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Signalling Mechanisms Used by the Orexin-1 Receptor

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HANNA PELTONEN

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ABSTRACT

The orexin-1 receptor (OX_1R) is a member of a superfamily of G-protein coupled receptors (GPCRs). Two hypothalamic neuropeptides, orexin-A/hypocretin-1 (Ox-A) and orexin-B/hypocretin-2, bind to and activate OX_1R and a related GPCR, the orexin-2 receptor. The physiological effects of the orexin system include regulation of feeding, energy metabolism, endocrine and autonomic systems, and sleep/wake cycle. At the cellular level, OX_1R stimulation leads to neuronal excitation, activation of the adenylyl cyclase pathway and several protein kinases, and an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). OX_1R couples to $G_{q/11}$-proteins and activates phospholipase Cβ (PLCβ) -pathway leading to release of Ca^{2+} from the intracellular Ca^{2+} stores and the subsequent capacitative Ca^{2+} entry (CCE) aiming to refill depleted stores. This is the main pathway utilized when high nanomolar Ox-A concentrations are evaluated, but at lower, and probably physiologically more relevant, concentrations there was a predominance of receptor-operated Ca^{2+} influx without any detectable release of stores. The aim of this study was to elucidate the poorly characterized signalling mechanisms used by the OX_1R leading to and regulating Ca^{2+} responses. The investigations with the patch clamp and Ca^{2+} imaging techniques in recombinant cell models demonstrated that even a low nanomolar Ox-A concentration was able to evoke a highly voltage-dependent polarization of the membrane, an increased ion current, and a robust elevation of $[Ca^{2+}]_i$. It was found that Ca^{2+} responses at low Ox-A concentrations were acutely dependent on voltage and extracellular Ca^{2+} concentration as well as being sensitive to blockers of receptor-operated Ca^{2+} entry. Protein kinase C (PKC) was found to be a crucial regulator of these responses. Based on the pharmacological profile of the responses and the experiments with dominant negative canonical transient receptor potential channel (TRPC) subtypes, the most probable ion channel involved in the Ca^{2+} influx is the diacylglycerol-activated and PKC-regulated TRPC3. At higher Ox-A concentrations, Ca^{2+} responses occurred also in the absence of extracellular Ca^{2+} and were sensitive to inhibitors of store release and CCE, pointing to involvement of the PLCβ-pathway. In addition to TRPC3 channel, phospholipase A_2 and protein kinase D1/3 were identified as novel targets of OX_1R mediated activation and as prominent modulators of Ca^{2+} oscillations induced by low nanomolar Ox-A concentrations. In conclusion, it was possible to confirm the present view of Ca^{2+} signalling of OX_1R and to identify novel players in the pathways activated by the receptor. It is hoped that these results will be useful in constructing a more comprehensive picture of OX_1R signalling and in developing efficient therapies for OX_1R -related disorders.

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TIIVISTELMÄ

Kaikki elävät olennot koostuvat soluista. Toimiakseen tarkoituksenmukaisesti solun on oltava jatkuvassa vuorovaikutuksessa ympäristönsä kanssa, mutta samalla suojattava itseään ympäristön mahdollisilta vaaroilta. Tämän vuoksi solua ympäröi solukalvo, joka läpäisee molekyylejä vain valikoidusti ja sisältää reseptoreja, jotka sitovat erilaisia viestiaineita ja välittävät viestejä solun sisään. Viesti etenee kemiallisina signalointipolkuina, kunnes solu kehittää tarkoituksenmukaisen vasteen. Oreksiini-1-reseptori (OX_1R), jonka aktivoimia signalointipolkuja tämä tutkimus tarkastelee, kuuluu laajaan G-proteiinivälitteisten reseptorien perheeseen yhdessä sisarreseptorinsa oreksiini-2-reseptorin kanssa. Hypotalamuksessa tuotetut neuropeptidit oreksiini-A (Ox-A) ja oreksiini-B sitoutuvat solukalvolla oreksiinireseptoreihin aiheuttaen niiden aktivaation. Kehon oreksiinisignaloinnilla on havaittu olevan moninaisia tehtäviä mm. syömisen, energiametabolian, hormonien erityksen, autonomisten toimintojen ja uni/valverytmin säätelyssä. Solutasolla OX_1R:n aktivaatio johtaa hermosolujen virittymiseen, adenylyylisyklaasipolun ja useiden proteiinikinaasien aktivoitumiseen sekä solunsisäisen kalsiumtason nousuun. Korkeilla nanomolaarisilla Ox-A-pitoisuuksilla stimuloitaessa OX_1R aktivoi fosfolipaasi Cβ-polun, joka johtaa solunsisäisten kalsiumvarastojen vapautumiseen ja sitä seuraavaan kalsiumin virtaukseen solun sisään. Sen sijaan matalammilla Ox-A-pitoisuuksilla reseptori näyttäisi aktivoivan solukalvon kalsiumkanavan ilman, että solunsisäiset kalsiumvarastot vapautuvat. Tämän tutkimuksen tarkoituksena oli valottaa ennestään huonosti tunnettuja kalsiumvasteisiin johtavia ja niitä säätteleviä OX_1R:n signalointimekanismeja. Tulokset tukivat edellä esitettyä näkemystä OX_1R:n kalsiumsignaloinnista. Proteiinikinaasi C:n havaittiin olevan merkittävässä roolissa Ox-A:n aiheuttamien kalsiumvasteiden säätelyssä. Tulosten perusteella diasyyliglyserolin aktivoima ja proteiinikinaasi C:n säätelemä TRPC3-kanava (canonical transient receptor potential channel 3) on todennäköisimmin vastuussa matalan Ox-A-pitoisuuden aiheuttamasta kalsiumvasteesta. Lisäksi fosfolipaasi A_2, proteiinikinaasi D1 ja proteiinikinaasi D3 tunnistettiin OX_1R:n aktivointikohteiksi ja matalan Ox-A-pitoisuuden aiheuttamien kalsiumoskillaatioiden huomattaviksi säätelijöiksi. Yhteenvetona tämä tutkimus valottaa aiemmin huonosti tunnettuja OX_1R:n signalointipolkuja ja tunnistaa niiden uusia jäseniä. Tulokset ovat hyödyllisiä luotaessa kattavaa kuvaa OX_1R:n signaloinnista ja kehitettäessä tehokkaita hoitomuotoja oreksiinisysteemin häiriöihin.

Yleinen suomalainen asiasanasto: reseptorit; solukalvot; solut - vuorovaikutus

To my beautiful daughters, Elli and "Kukka"

"Äiti, taasko sinä teet sitä värityskirjaa?"
- Elli 2,5 v -

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In Kuopio, April 2011

Hanna Peltonen

List of original publications

This dissertation is based on the following original publications referred to in the text by their Roman numerals I-IV.

I Larsson K P, Peltonen H M, Bart G, Louhivuori L M, Penttonen A, Antikainen M, Kukkonen J P and Åkerman K E O. Orexin-A-induced Ca^{2+} entry: evidence for involvement of TRPC channels and protein kinase C regulation. *Journal of Biological Chemistry 280: 1771-1781, 2005.*

II Näsman J, Bart G, Larsson K, Louhivuori L, Peltonen H and Åkerman K E O. The orexin OX_1 receptor regulates Ca^{2+} entry via diacylglycerol-activated channels in differentiated neuroblastoma cells. *Journal of Neuroscience 26: 10658-10666, 2006.*

III Peltonen H M, Magga J M, Bart G, Turunen P M, Antikainen M S H, Kukkonen J P and Åkerman K E O. Involvement of TRPC3 channels in calcium oscillations mediated by OX_1 orexin receptors. *Biochemical and Biophysical Research Communications 385: 408-412, 2009.*

IV Peltonen H M, Åkerman K E O and Bart G. A role for PKD1 and PKD3 activation in modulation of calcium oscillations induced by orexin receptor 1 stimulation. *Biochimica et Biophysica Acta 1803: 1206-1212, 2010.*

The publishers of the original publications have kindly granted permission to reprint the articles to this dissertation.

Contents

Abbreviations

2-APB	2-aminoethoxydiphenyl borate
AA	arachidonic acid
AC	adenylyl cyclase
ARC	arachidonic acid -regulated calcium entry
BrdU	5-bromo-2′-deoxyuridine
BSA	bovine serum albumin
BzATP	2′,3′-*O*-(4-benzoyl-benzoyl)-ATP
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
$[Ca^{2+}]_o$	extracellular calcium concentration
CAMKII	calcium/calmodulin-dependent protein kinase
cAMP	cyclic adenosine monophosphate
CCE	capacitative calcium entry
CHO-K1	Chinese hamster ovary cells
CICR	calcium induced calcium release
CMV	cytomegalovirus
CNS	central nervous system
CO_2	carbon dioxide
DAG	diacylglycerol
DAGKI	diacylglycerol kinase inhibitor R59022
Dex	dextromethorphan
DOG	dioctanoyl glycerol
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
EYFP	enhanced yellow fluorescent protein
FBS	fetal bovine serum
Fura-2AM	fura-2-acetoxymethyl ester
GABA	γ-aminobutyric acid
GDP	guanosine diphosphate
GFP	green fluorescent protein
GF-X	GF109203X
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate
H^+	hydrogen ion / proton
HBM	HEPES-buffered sodium ion based medium
HEK293	human embryonic kidney cells
IP_3	inositol 1,4,5-trisphosphate
IP_3R	inositol 1,4,5-trisphosphate receptor
K^+	potassium ion
KB-R	KB-R7943
MAFP	methyl arachidonyl fluorophosphonate
MAPK	mitogen activated protein kinase
Mg^{2+}	magnesium ion
Na^+	sodium ion

NHERF	Na^+/H^+ ion exchanger regulatory factor
Ni^{2+}	nickel ion
NMDA	N-methyl-D-aspartate
OX_1R	orexin-1 receptor
OX_2R	orexin-2 receptor
Ox-A	orexin-A
Ox-B	orexin-B
OxR	orexin receptor
PA	phosphatidic acid
PCR	polymerase chain reaction
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP -dependent protein kinase
PKC	protein kinase C
PKD	protein kinase D
PKD1kd	EGFP-tagged kinase-dead PKD1
PKD3kd	EGFP-tagged kinase-dead PKD3
PLA_2	phospholipase A_2
PLCβ	phospholipase Cβ
PLD	phospholipase D
PMCA	plasma membrane (Ca^{2+}+Mg^{2+})-ATPase
PPO	prepro-orexin
REM	rapid eye movement
RFP	red fluorescent protein
RGS	regulator of G-protein signalling
RIPA	radio-immunoprecipitation assay
ROC	receptor-operated calcium channel
RT-PCR	reverse transcriptase polymerase chain reaction
SERCA	sarcoplasmic reticulum and endoplasmic reticulum (Ca^{2+}+Mg^{2+})-ATPase
SKF	SKF-96365
SOC	store-operated calcium channel
SR	sarcoplasmic reticulum
TEA	tetraethylammonium
TM	transmembrane
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRP	transient receptor potential channel
TRPC	canonical transient receptor potential channel
Trpc1N	mtrpc1βN-EGFP-N3 (truncated TRPC1 construct with EGFP)
Trpc2N	mtrpc2N-EGFP-N1 (truncated TRPC2 construct with EGFP)
Trpc3N	EYFP-hstrpc3N-C1 (truncated TRPC3 construct with EYFP)
Trpc4N	EYFP-mtrpc4βdn-C1 (truncated TRPC4 construct with EYFP)
$TRPC6^{DN}$	a full-length, triple-mutated TRPC6 dominant-negative construct
Trpc7N	mtrpc7αdn-EGFP-N1 (truncated TRPC7 construct with EGFP)
TRPL	TRP-like channel
VOC	voltage-gated calcium channel
YFP	yellow fluorescent protein

1 Introduction

The superfamily of G-protein coupled receptors (GPCRs) is the largest group of receptors on the plasma membrane. They receive signals from the outside world and transmit them into the cell so that the cell is able to produce an appropriate response. Over 800 genes encode the members of this huge group of proteins (Fredriksson et al., 2003; reviewed in Oldham and Hamm, 2008). The importance of the GPCRs as drug targets is significant. In fact though the GPCR family represents the largest single fraction of the drug market, it has been speculated that only a very small number of the possible drugs targeting GPCRs have been developed so far (Fredriksson et al., 2003; Robas et al., 2003; Overington et al., 2006; Rosenbaum et al., 2009; Millar and Newton, 2010). One member of this remarkable receptor family is the orexin-1 receptor (OX_1R), on which this thesis is focused.

Orexins/hypocretins, orexin-A (Ox-A) and orexin-B (Ox-B), are neuropeptides and hormones produced in the brain by a rather small group of neurons in the lateral hypothalamus (Sakurai et al., 1998; de Lecea et al., 1998). Despite the small number of orexin neurons (Thannickal et al., 2000; Modirrousta et al., 2005), their projections spread virtually throughout the whole brain, indicating that the orexins play significant roles in multiple brain functions (de Lecea et al., 1998; Peyron et al., 1998). In addition to the central nervous system (CNS), orexins are expressed and able to act locally in the periphery (reviewed in Kirchgessner, 2002). The orexin system participates in the regulation of numerous physiological functions including feeding, energy metabolism, digestion, endocrine secretion, autonomic regulation, reproduction, sleep/wake cycle, and alertness (reviewed in Carter et al., 2009). Narcolepsy/cataplexy is a disease characterized by excessive daytime sleepiness, episodes of muscle weakness and abnormalities of rapid eye movement (REM) sleep. In dogs, mutations in the gene encoding the orexin-2 receptor (OX_2R) lead to a familial case of narcolepsy (Lin et al., 1999) and the link between orexin system and this disease is also rather firm in humans (Nishino et al., 2000; Peyron et al., 2000; Thannickal et al., 2000; Dalal et al., 2001; Ripley et al., 2001; Higuchi et al., 2002). Furthermore, the orexin system has been implicated in other disease states such as sleep apnea, Parkinson's disease, schizophrenia, depression and cancer (de Lecea and Sutcliffe, 2005; Spinazzi et al., 2006; Carter et al., 2009).

At the cellular level, orexins exert their effects by binding to and activating either of the two distinct GPCRs, OX_1R, and OX_2R (Sakurai et al., 1998). Probably the most prominent cellular response to stimulation of OX_1R is the increase in the intracellular calcium ion concentration ($[Ca^{2+}]_i$). Ca^{2+} is the most common second messenger within cells controlling all the aspects of cellular life: fertilization, proliferation, differentiation, development and cell death (Clapham, 1995; Berridge et al., 2000; Parkash and Asotra, 2010). It is almost miraculous how changes in the concentration of just one element can lead to such a great diversity of responses. Ca^{2+} fluxing into the cytosol can originate from two separate sources: from the extracellular space through specific Ca^{2+} permeable channels or from the intracellular Ca^{2+} stores (Clapham, 1995; Berridge et al., 2003). In addition to Ca^{2+} entry routes, also the spatial and temporal aspects of Ca^{2+} elevation diversify the possible signals. Transient increases of $[Ca^{2+}]_i$ lead to different responses than are seen with the repetitive Ca^{2+} signals known as Ca^{2+} oscillations which can carry

much more information in terms of amplitude, frequency, waveform or timing (Taylor and Thorn, 2001; Petersen et al., 2005; Parkash and Asotra, 2010).

Ca^{2+} signalling of OX_1R has been a topic of intense investigation. Activation of the phospholipase Cβ (PLCβ)-pathway leading to release of Ca^{2+} from the intracellular stores by Ox-A has been firmly established by several groups (Smart et al., 1999; Kukkonen and Akerman, 2001; Muroya et al., 2004; Johansson et al., 2007; Ekholm et al., 2007; Johansson et al., 2008). Furthermore, there are many lines of evidence indicating that the primary response to lower concentrations of Ox-A is a Ca^{2+} influx from the extracellular space (Lund et al., 2000; Kukkonen and Akerman, 2001; Ammoun et al., 2003; Magga et al., 2006; Ekholm et al., 2007; Johansson et al., 2007; 2008). Although many players activated by OX_1R stimulation and regulating the Ox-A induced Ca^{2+} responses have been identified, it is still not possible to discern the whole picture since important pieces of the puzzle are still missing. One special example is the still unidentified Ca^{2+} channels on the plasma membrane conducting Ca^{2+} into the cell after stimulation of OX_1R by a low concentration of Ox-A. A more detailed knowledge of orexin signalling inside the cells is crucial if one wishes to understand the cellular and systemic impacts of orexins and to develop methods to restore the balance of the orexin system disturbed in many disease states.

This study was conducted to elucidate the signalling mechanisms of OX_1R at a cellular level. These investigations in several recombinant cell models with Ca^{2+} imaging and immunological assays as the main methods have yielded novel findings in terms of the protein kinases and other signalling molecules activated by Ox-A, the characteristics and regulation of Ox-A induced Ca^{2+} signals and the Ca^{2+} channels responsible for the Ca^{2+} influx induced by low concentrations of Ox-A.

2 Review of the Literature

2.1 G-PROTEIN COUPLED RECEPTORS AND G-PROTEINS

2.1.1 The Large and Significant Family of G-protein Coupled Receptors

If one thinks about a single cell, it is far from an independent isolated unit but is in continuous contact with its environment and with other cells. The capability to sense the states and changes in the environment and to communicate with other cells represents a fundamental basis for cell survival and function. Since signalling molecules very rarely are able to pass through the plasma membrane protecting the cell from the possibly dangerous outside world, the cells have endowed their plasma membranes with specific receptors which can detect many different external signals and transmit their messages into the cells. The largest family of such membrane receptors is the family of GPCRs. It has been estimated that the human genome contains more than 800 genes encoding GPCR proteins (Fredriksson et al., 2003; reviewed in Oldham and Hamm, 2008). Thus about 3 - 4 % of the whole genome is dedicated to this task (Tuteja, 2009) which makes the GPCR superfamily not just the largest family of receptors, but also the largest of all protein families encoded in the genome (Lander et al., 2001; Venter et al., 2001; reviewed in Oldham and Hamm, 2008; Rosenbaum et al., 2009).

The importance of GPCR family and its members as drug targets cannot be overestimated. It represents the largest single fraction of the drug market i.e. at least one third of the currently marketed pharmaceutical agents target GPCRs, accounting for annual sales of several billion dollars (Robas et al., 2003; Overington et al., 2006; Millar and Newton, 2010). Furthermore, it has been speculated that only a very small number of the possible drugs targeting GPCRs have been developed so far and thus the potential for drug discovery is enormous (Fredriksson et al., 2003; Rosenbaum et al., 2009; Millar and Newton, 2010). The physiological importance of GPCRs has also been underlined by results obtained in knockout animal models which have revealed the pathological phenotypes involving a wide variety of systems, such as cardiovascular, nervous, endocrine and sensory systems (Rohrer and Kobilka, 1998; Karasinska et al., 2003). Furthermore, natural mutations within the genes encoding specific GPCRs have been linked to several hereditary diseases (Spiegel and Weinstein, 2004).

GPCRs have been named after their ability to couple to guanine nucleotide binding proteins (G-proteins), but they are also known as heptahelical receptors or 7-TM receptors based on their characteristic structure with 7 transmembrane (TM) domains. The TM regions are connected by three extracellular and three intracellular loops. The N-terminus of the receptor is located in the extracellular space while the C-terminus is intracellular. The conformation of the TM domains determines the state (active/inactive) of the receptor. The extracellular receptor surface is critical for ligand binding and the intracellular receptor surface is known to be involved in G-protein recognition and activation (Rosenbaum et al., 2009). The GPCR family is very diverse showing only a low degree of sequence conservation. In addition, the length and function of the intracellular and extracellular portions of GPCRs differ considerably. This means that a huge variety of ligands (e.g. photons of light, odorants and pheromones, sweet and bitter tastes, ions, hormones, and neurotransmitters) can bind to the extracellular proportion of their

specific receptors (Oldham and Hamm, 2008; Millar and Newton, 2010) and many different molecules participate in signal transduction or receptor regulation (Bockaert et al., 2004; Tilakaratne and Sexton, 2005). The diversity of GPCR signalling is also underlined by the broad spectrum of biological responses evoked after the stimulation of these types of receptors. These include sensory perception, changes in neuronal activity, cell growth, and hormonal regulation (reviewed in Rens-Domiano and Hamm, 1995; Tuteja, 2009).

2.1.2 Classification of G-protein Coupled Receptors

GPCRs can be divided into five families on the basis of their sequence and structural similarities: 1) rhodopsin, 2) secretin, 3) adhesion, 4) glutamate, and 5) frizzled/taste2 (Fredriksson et al., 2003; Oldham and Hamm, 2008; Rosenbaum et al., 2009). The rhodopsin family, which can be further subdivided into four groups: α, β, γ, and δ (Fredriksson et al., 2003), is the largest and most diverse, comprising of 672 members (Millar and Newton, 2010). Most of the family members share conserved sequence motifs such as the NsxxNPxxY motif in the seventh TM domain, the DRY motif at the border between the third TM domain and the second intracellular loop, the eighth α-helix in the C-terminus and the C-terminal palmitoylation site (Fredriksson et al., 2003; Oldham and Hamm, 2008). The rhodopsin family is also characterized by a shorter N-terminus compared with all the other GPCR families (Fredriksson et al., 2003; Oldham and Hamm, 2008). At least some of these common characteristics are believed to be important for protein stabilization and/or G-protein activation (Oldham and Hamm, 2008) and to result in shared structural features and activation mechanisms between the family members (Rosenbaum et al., 2009). The secretin family of GPCRs contains 15 members (Millar and Newton, 2010), binds rather large peptide ligands and has a long N-terminus with conserved cysteine residues participating in ligand binding (Fredriksson et al., 2003). Thirty-three members of the adhesion family have very extended N-termini, which are thought to be involved in cell adhesion (Fredriksson et al., 2003; Millar and Newton, 2010). The glutamate family with 22 members (Millar and Newton, 2010) is mainly composed of glutamate, γ-aminobutyric acid (GABA), and taste receptors (Fredriksson et al., 2003). The shared structural feature in the family is a so-called "Venus fly trap" for ligand binding in the N-terminus of the receptors (Fredriksson et al., 2003). The frizzled/taste2 family consists of 36 members (Millar and Newton, 2010) and includes two distinct clusters: the frizzled receptors mediating signals from secreted glycoproteins termed Wnt and the TAS2 receptors participating in bitter taste perception (Fredriksson et al., 2003).

2.1.3 Activation of G-proteins by G-protein Coupled Receptors

The extracellular signals detected by GPCRs are transferred into the cells by G-proteins which reside on the plasma membrane adjacent to the receptor and which bind to a binding pocket formed by the intracellular loops and the C-terminus of GPCRs upon receptor activation (Oldham and Hamm, 2008; Vilardaga, 2010; Ambrosio et al., 2011). The G-proteins belong to a larger family of GTPases, hydrolase enzymes, that can bind and hydrolyse guanosine triphosphate (GTP) to guanosine diphosphate (GDP). In addition to large heterotrimeric G-proteins, which are the conventional coupling partners of GPCRs, the family of GTPases includes small GTP-binding proteins as well as many factors involved in protein synthesis (reviewed in Rens-Domiano and Hamm, 1995). Heterotrimeric G-proteins are composed of three subunits. The α-subunit binds GTP and

possesses the intrinsic GTPase activity. The β-, and γ-subunits form a stable complex which is dissociable only by denaturation (Schmidt et al., 1992). The β-subunit requires the presence of the γ subunit to allow it to fold properly (Schmidt and Neer, 1991; Higgins and Casey, 1994).

Figure 1 represents the signalling cycle of heterotrimeric G-proteins, which has also been extensively described in several reviews (Neer, 1995; Rens-Domiano and Hamm, 1995; Cabrera-Vera et al., 2003; Tuteja, 2009). In the inactive state, α-, β- and γ-subunits form a heterotrimer and the α-subunit is associated with GDP. When a specific ligand binds to its receptor and activates it, a conformational rearrangement takes place in the receptor's TM domains (particularly TM3 and TM6) which allows an interaction to occur between the receptor and the G-protein (Oldham and Hamm, 2008; Vilardaga, 2010; Ambrosio et al., 2011). Two models of interaction have been proposed. In the "collision model", free lateral diffusion of molecules within the plasma membrane results in an interaction between the activated receptor and the G-protein. The opposing "precoupling model" postulates that there is an interaction between the receptor and the G-protein already prior to receptor activation (Oldham and Hamm, 2008; Vilardaga, 2010). Both α- and βγ-subunits have been shown to bind to the activated receptor (Oldham and Hamm, 2008) and this binding lowers the affinity of α-subunit for GDP. Once GDP is released, a stable high-affinity tetrameric complex is formed between the activated receptor and the heterotrimeric G-protein (Oldham and Hamm, 2008). This complex is however transient, since GTP, the cytosolic concentration of which is several-fold higher than that of GDP, rapidly binds to the α- subunit (Oldham and Hamm, 2008) and destabilizes the complex. Both α- and βγ-subunits are released and able to activate effector systems. It is possible that the subunits are not completely dissociated but conformational rearrangements allow them to interact with downstream effectors (Vilardaga, 2010). The signalling through the G-protein is terminated, when GTP is hydrolysed to GDP and the βγ-complex binds again to the α-subunit. This step is probably catalysed by a specific group of proteins termed regulators of G-protein signalling (RGS) (Oldham and Hamm, 2008; Tuteja, 2009). The ligand is released from the receptor and the cycle is ready to start all over again.

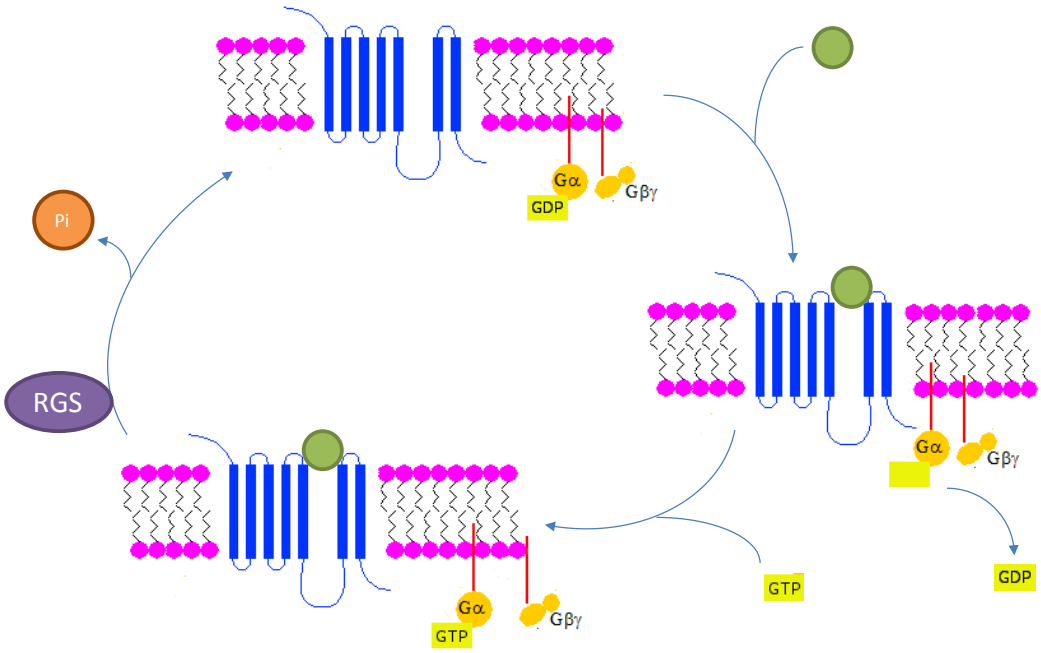

Figure 1. Receptor-mediated G-protein activation (Graphics by J. Peltonen). Binding of a specific ligand to its receptor induces a conformational change in the receptor structure. The activated receptor couples with the intracellular heterotrimeric G-protein composed of α- and βγ-subunits. This coupling promotes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α-subunit and the dissociation of the subunits from the receptor. Both α- and βγ-subunits are free to activate various effectors inside the cell. When the intrinsic GTPase activity of the α-subunit hydrolyses GTP to GDP and Pi, the βγ-subunit reassociates with the α-subunit and the cycle is complete. A regulator of G-protein signalling (RGS) can accelerate the intrinsic GTPase activity of α-subunit (Cabrera-Vera et al., 2003; Oldham and Hamm, 2008; Tuteja, 2009).

2.1.4 Effectors Activated by G-proteins

G-proteins play an important role not only in transferring the signals but also in determining the specificity and temporal characteristics of the cellular responses induced by extracellular signals (reviewed in Rens-Domiano and Hamm, 1995). Activated G-proteins modulate the activity of various enzymes, ion channels and transcription factors, which are called effectors because changes in their activity lead to the changes in ionic composition, in second messenger levels or in transcription processes, that ultimately are reflected to cellular responses and in systemic functions such as embryonic development, gonadal development, learning and memory, and organismal homeostasis (reviewed Neer, 1995; Neves et al., 2002).

There are 16 genes (Downes and Gautam, 1999) with over 20 gene products including the splice variants encoding for Gα-subunits in the mammalian genome (Oldham and Hamm, 2008). Based on their activation profiles and sequence similarities, α-subunits can be divided into four families: $G\alpha_{i/o}$, $G\alpha_{s}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Oldham and Hamm, 2008). Each Gα-family activates a distinct profile of effectors. The members of Gα-families and their known effectors are listed in Table 1. Sometimes the coupling between GPCR and G-protein is very selective and the receptor is able to discriminate even between related

G-proteins within the same family. However, in many cases, a single receptor can activate more than one G-protein and thereby modulate multiple intracellular signalling pathways (Oldham and Hamm, 2008). Furthermore, a single G-protein may be capable of engaging multiple signalling pathways.

Table 1. Classification of Gα-subunits and their effectors (adapted from Cabrera-Vera et al., 2003)

Family	Member	Effectors
$G\alpha_{i/o}$	$G\alpha_{i1}$	Adenylyl cyclase
	$G\alpha_{i2}$	Rap1 GTPase activating protein
	$G\alpha_{i3}$	GRIN1 and 2
	$G\alpha_{oA}$	GTPase of tubulin
	$G\alpha_{oB}$	src
	$G\alpha_{z}$	Ca^{2+} and K^{+} channels
	$G\alpha_{t1}$	cGMP-PDE
	$G\alpha_{t2}$	DAG kinase
	$G\alpha_{g}$	Rho
		PI4-kinase
$G\alpha_{s}$	$G\alpha_{s(S)}$	Adenylyl cyclase
	$G\alpha_{s(L)}$	GTPase of tubulin and Src
	$G\alpha_{olf}$	Adenylyl cyclase
$G\alpha_{q/11}$	$G\alpha_{q}$	Phospholipase Cβs and Bruton's tyrosine kinase
	$G\alpha_{11}$	
	$G\alpha_{14}$	
	$G\alpha_{15}$	
	$G\alpha_{16}$	
$G\alpha_{12/13}$	$G\alpha_{12}$	Na^{+}/H^{+} exchanger 1 and phospholipase D
	$G\alpha_{13}$	p115RhoGEF and inducible nitric oxide synthase

Abbreviations used: cGMP-PDE = cyclic guanosine monophosphate - phosphodiesterase E, DAG = diacylglycerol, GEF = guanine nucleotide exchange factor, GRIN = G-protein-regulated inducer of neurite outgrowth, GTP = guanosine triphosphate, GTPase = an enzyme hydrolyzing GTP, PI4 = phosphatidylinositol 4, Rap1 = Ras-related protein 1, Rho = Rho GTPase.

Five and 12 mammalian genes (Downes and Gautam, 1999) encode for 6 Gβ- and 12 Gγ-subunits, respectively (Oldham and Hamm, 2008). The association between these subunits is not a random process (Pronin and Gautam, 1992; Schmidt et al., 1992; Iniguez-Lluhi et al., 1992), but different β- and γ-subunits have varying affinities for one another (Yan et al., 1996). For a long time, the βγ-complex was seen as a rather passive player in G-protein signalling. It was thought simply to assist the α-subunit to interact with the receptor (Fung, 1983; Hekman et al., 1987; Florio and Sternweis, 1989) and to limit the signalling period by forming inactive heterotrimers with the α-subunits (Neer, 1995). Subsequently, it was found that also the βγ-complex can interact with and regulate a wide variety of second messengers in the cells (reviewed in Cabrera-Vera et al., 2003; Tuteja, 2009) including various isozymes of the PLCβ family (Blank et al., 1992; Camps et al., 1992; Park et al., 1993; Smrcka and Sternweis, 1993), certain G-protein responsive cation channels (Clapham and Neer, 1997; Schneider et al., 1997; Jan and Jan, 1997), and a

number of kinases (Pumiglia et al., 1995; Langhans-Rajasekaran et al., 1995; Tang and Downes, 1997). Thus, it is evident that βγ- and α-subunits interact with a number of common effectors and additionally the βγ-complex can interact with many effectors not regulated by α-subunits.

2.1.5 Regulation of G-protein Coupled Receptor Signalling

The receptors do not only couple to the G-proteins, but also interact with many other proteins with signalling and regulatory functions (reviewed in Bockaert et al., 2004; Tilakaratne and Sexton, 2005; Millar and Newton, 2010). These proteins regulate all aspects of GPCR signalling, from the ligand binding to the receptor localization and from the G-protein selectivity to the receptor desensitization. The nature of the interacting proteins regulates the signalling specificity of a GPCR in concert with the nature of the heterotrimeric G proteins to which it is coupled. The availability of these proteins can profoundly affect the activity of the receptors. As mentioned above, one important class of proteins regulating GPCR signalling is the family of RGSs which act to stimulate the GTPase activity of G proteins (particularly of $G_{i/o}$ and $G_{q/11}$) and thereby to attenuate the signalling process (Dohlman and Thorner, 1997; Arshavsky and Pugh, 1998; Kristiansen, 2004) or in some cases also to speed up the activation of signalling (Zerangue and Jan, 1998; Kristiansen, 2004).

Typically, activation of a GPCR has five major consequences: 1) activation and inhibition of specific signalling pathways in the cells via G-proteins and/or G-protein-independent signalling, 2) short-term desensitization of the receptor, 3) endocytosis of the receptor, 4) recycling of the receptor back to the plasma membrane, or 5) degradation of the receptor in lysosomes (Kristiansen, 2004). Short-term desensitization is usually accomplished via phosphorylation of GPCRs by G-protein coupled receptor kinases (GRKs) followed by β-arrestin binding to the receptor. This uncouples the receptor from the G-protein at the plasma membrane. The complex formed by β-arrestin and the phosphorylated receptor is then subjected to endocytosis via clathrin-coated pits. In endocytotic vesicle, the receptor is dephosphorylated by protein phosphatases and either guided back to the plasma membrane (resensitization) or shunted to lysosomes for degradation. Lysosomal degradation of receptors and/or the down-stream components leads to long-term desensitization. Other ways to down-regulate the signalling for longer periods of time are decreased synthesis of receptor proteins and/or downstream proteins and enhanced mRNA degradation.

2.1.6 Complexity of G-protein Coupled Receptor Signalling

The signalling of GPCRs is far more complex than originally was anticipated. Individual receptors may couple to more than one G-protein subtype and an individual G-protein can engage multiple signalling pathways. Furthermore, GPCRs are known to activate signalling pathways also independently of G-proteins (Rosenbaum et al. 2009; Tuteja, 2009; Millar and Newton, 2010). It has been found that different ligands acting on the same receptor induce different sets of cellular responses. The ON/OFF model of receptor conformations cannot explain this phenomenon, but the receptors presumably display distinguishable active conformational states, which are induced by different agonists and correlate to specific signalling outputs (Rosenbaum et al., 2009; Millar and Newton, 2010; Vilardaga, 2010; Ambrosio et al., 2011). Dimerization/oligomerization of GPCRs further complicates the issue. Many GPCRs assemble with identical (homodimers) or distinct (heterodimer) receptors creating receptor dimers with pharmacological and functional

properties distinct from their monomeric units (Bai, 2004; Milligan, 2004; Oldham and Hamm, 2008). Given that an individual organ or a small region of a tissue may express up to 100 distinct GPCRs (Hakak et al., 2003), the significance of heterodimerization is obvious and this has been further highlighted by several studies (Lee et al., 2002; Liang et al., 2003). In addition to other receptors, GPCRs can also modulate the function of ion channels by directly interacting with them (Liu F. et al., 2000; Kitano et al., 2003). The diversity of GPCR signalling seems enormous and thus much work will be still needed to reveal individual characteristics of specific receptors as well as common themes shared by multiple GPCRs.

2.2 CELLULAR CALCIUM METABOLISM

Ionized calcium (Ca^{2+}) is the most common second messenger in cells, ranging from bacteria to the specialized neurons in the human body. It controls almost everything that we do and impacts on nearly every aspect of cellular life: fertilization, differentiation, development, proliferation and ultimately cell death (Clapham, 1995; Berridge et al., 2000; Parkash and Asotra, 2010). Ca^{2+} gradients within cells have been proposed to be involved, even to initiate cell migration, exocytosis, lymphocyte killer cell activation, acid secretion, transcellular ion transport, neurotransmitter release, gap junction regulation, and numerous other functions (reviewed in Tsien and Tsien, 1990; Clapham, 1995). Gene transcription extends the impact of Ca^{2+} signalling into long-term changes in the life of a cell (reviewed in Clapham, 2007; Parkash and Asotra, 2010).

A molecule can act as a signalling agent only if its concentration can be tightly regulated. Unlike other common second messengers, Ca^{2+} cannot be metabolized, but the cells have evolved numerous proteins to keep $[Ca^{2+}]_i$ low (Clapham, 2007). Normal $[Ca^{2+}]_i$ is approximately 100 nM while Ca^{2+} concentration in the extracellular space is 20 000-fold higher reaching mM range (Clapham, 2007; Parkash and Asotra, 2010). Transporter proteins pump Ca^{2+} out of the cells or into the intracellular Ca^{2+} stores located within cell organelles such as endoplasmic reticulum (ER) and mitochondria. These transporters include the sarcoplasmic reticulum (SR) and ER (Ca^{2+}+Mg^{2+})-ATPases (SERCAs), which transport Ca^{2+} into SR and ER, plasma membrane (Ca^{2+}+Mg^{2+})-ATPases (PMCAs) which pump Ca^{2+} out of the cells across the plasma membrane and mitochondrial Ca^{2+} transporters and channels (Berridge et al., 2000; 2003; Clapham, 2007; Parkash and Asotra, 2010) (Figure 2). Furthermore, several buffer molecules form complexes with free Ca^{2+} thereby limiting its concentration (Berridge et al., 2000; Clapham, 2007; Parkash and Asotra, 2010). Ca^{2+} signals are conveyed through the magnitude, location and duration of the changes in $[Ca^{2+}]_i$ (Barritt, 1999; Petersen et al., 2005; Parkash and Asotra, 2010). These are usually initiated by the binding of an extracellular signalling molecule to its plasma membrane receptor (Barritt, 1999; Petersen et al., 2005). Upon receptor stimulation, the local $[Ca^{2+}]_i$ can rise to concentrations as high as 1 μM (Berridge et al., 2000; Clapham, 2007). Ca^{2+} can originate from two sources: 1) from the extracellular space through plasma membrane Ca^{2+} channels or 2) from intracellular Ca^{2+} stores in cell organelles (Berridge et al., 2003).

Ca^{2+} channels on the plasma membrane (Figure 2) can be classified based on the mechanisms regulating their activity. Voltage-gated Ca^{2+} channels (VOCs) are expressed mostly in excitable cells but also in many nonexcitable cell types and they are activated by changes in membrane potential. The channels are selective to Ca^{2+} and modulated by neurotransmitters, G-proteins, and diffusible messengers (Tsien and Tsien, 1990; Berridge

et al., 2003; Clapham, 2007; Parkash and Asotra, 2010). There are four subtypes of VOCs: L-, T-, N-, and P-type. For example, activity of VOCs is responsible for example for contraction in muscles, pacemaking in the heart and neurotransmitter release in brain (Catterall, 2000). Receptor-operated Ca^{2+} channels (ROCs) are activated following the stimulation of a receptor by its specific agonist. There are two subtypes of ROCs: 1) the ligand-gated Ca^{2+} channels (for example N-methyl-D-aspartate (NMDA) receptor), which are directly activated by a ligand without the intervention of a diffusible cytosolic messenger, and 2) second messenger-operated Ca^{2+} channels, which are activated by a second messenger produced as a result of receptor activation by its ligand (Tsien and Tsien, 1990; Berridge et al., 2000; 2003). Second messengers proposed to activate Ca^{2+} channels include inositol 1,4,5-trisphosphate (IP_3) (Kuno and Gardner, 1987; Mozhayeva et al., 1990; Vaca and Kunze, 1995; Kiselyov et al., 1997), diacylglycerol (DAG) (Hofmann et al., 1999; Okada et al., 1999), inositol 1,3,4,5-tetrakisphosphate (Irvine and Moor, 1986), Ca^{2+} itself (von Tscharner et al., 1986; Loirand et al., 1991; Braun and Schulman, 1995; Lidofsky et al., 1997; Congar et al., 1997; Leech and Habener, 1997), cyclic guanosine monophosphate (Finn et al., 1996), cyclic adenosine monophosphate (cAMP) (Finn et al., 1996; Lenz and Kleineke, 1997), and arachidonic acid (AA) (Peppelenbosch et al., 1992; Shuttleworth, 1996; Munaron et al., 1997; Shuttleworth and Thompson, 1998). Some channels can also be directly activated by G-proteins (von zur Muhlen et al., 1991; Krautwurst et al., 1992; Berven et al., 1994; Iwasawa et al., 1997). In addition, store-operated Ca^{2+} channels (SOCs) are sometimes classified under the superfamily of ROCs (Barritt, 1999), but here they will be located as a separate group in order to distinguish between ROCs, which are considered to be independent of the intracellular Ca^{2+} stores and SOCs, that sense the state of the stores and open when the stores are emptied (Barritt, 1999; Putney, 2005; Clapham, 2007). The signalling pathway leading to opening of SOCs is described below (see chapter 2.2.1 The Phospholipase Cβ Pathway). It has been proposed that the opening of SOC does not increase the Ca^{2+} concentration in the cytosol but that Ca^{2+} enters directly into the empty store through the channel (Chakrabarti and Chakrabarti, 2006). The prolonged search for the molecular entity responsible for capacitative Ca^{2+} entry (CCE) led to the first real breakthrough, when a plasma membrane protein called Orai1 was identified as a channel forming subunit required for CCE (Feske et al., 2006; Zhang et al., 2006; Prakriya et al., 2006; Yeromin et al., 2006). The most probable signal mediator between the emptied intracellular Ca^{2+} stores and Orai1 is stromal interaction molecule 1 (STIM1), a single pass transmembrane protein primarily residing in the ER (Lewis, 2007). The exact signal transduction mechanism between these two proteins is currently unknown. In addition to the channel types mentioned above, Ca^{2+} can enter into the cells via mechanically operated Ca^{2+} channels, tonically active Ca^{2+} channels, or via gap junctions between neighbouring cells (Tsien and Tsien, 1990).

Intracellular Ca^{2+} stores can be released in response to stimulation of either of two types of channels on ER: IP_3 receptors (IP_3Rs) and ryanodine receptors (Berridge et al., 2000; 2003; Clapham, 2007; Parkash and Asotra, 2010) (Figure 2). The signalling pathway leading to activation of IP_3Rs and discharge of Ca^{2+} from the intracellular stores will be introduced in the next chapter (2.2.1 The Phospholipase Cβ Pathway). Both IP_3Rs and ryanodine receptors can be activated by Ca^{2+} and thus give rise to a phenomenon called Ca^{2+} induced Ca^{2+} release (CIRC) (Berridge et al., 2000; 2003; Parkash and Asotra, 2010).

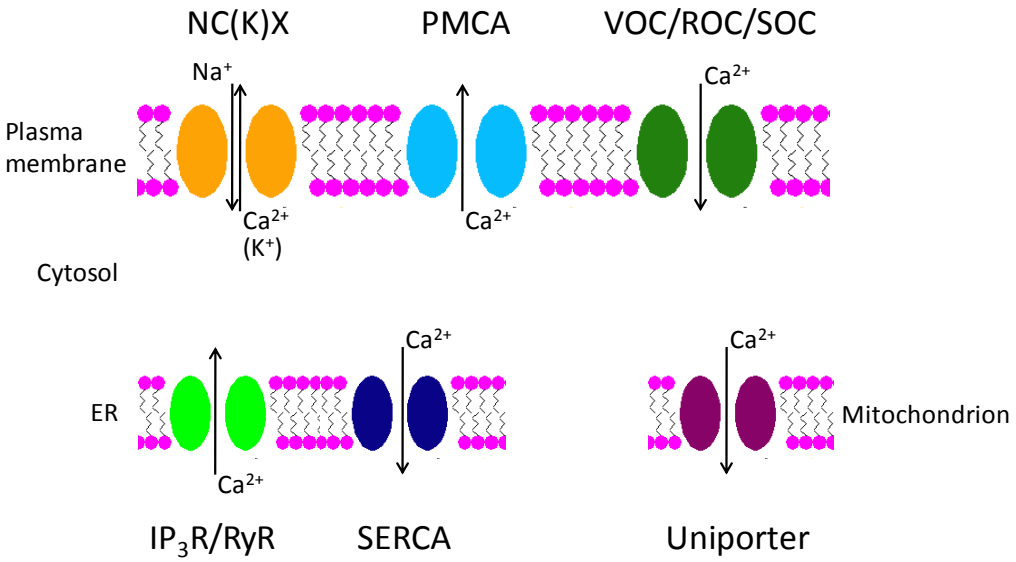

Figure 2. Various ion transporters and channels participating in the regulation of Ca^{2+} homeostasis and signalling (Graphics by J. Peltonen). Mechanisms extruding Ca^{2+} from the cytosol include plasma membrane (Ca^{2+}+Mg^{2+})-ATPases (PMCAs) pumping Ca^{2+} out of the cell through the plasma membrane, sarcoplasmic reticulum and endoplasmic reticulum (ER) (Ca^{2+}+Mg^{2+})-ATPases (SERCAs) storing Ca^{2+} in ER and the mitochondrial uniporter transferring Ca^{2+} to mitochondrial stores. Additionally Na^+/Ca^{2+} exchangers (NCX) and Na^+/Ca^{2+}-K^+ exchangers (NCKX) on the plasma membrane exchange one Ca^{2+} ion for three Na^+ ions (NCX) or co-transport one K^+ ion with one Ca^{2+} ion in exchange for four Na^+ ions (NCKX) lowering the intracellular Ca^{2+} concentration. Specific Ca^{2+} signals are generated when voltage-gated Ca^{2+} channels (VOCs), receptor-operated Ca^{2+} channels (ROCs) or store-operated Ca^{2+} channels (SOCs) allow Ca^{2+} to flow into the cell or when Ca^{2+} is released from the ER stores via inositol 1,4,5-trisphosphate receptors (IP_3Rs) or ryanodine receptors (RyRs) (Clapham, 2007; Parkash and Asotra, 2010).

2.2.1 The Phospholipase Cβ Pathway

PLCs comprise a family of phosphodiesterase enzymes that cleave the polar head groups from inositol lipids (reviewed in Rebecchi and Pentyala, 2000). The enzymes within this family have been divided into four groups (β, γ, δ, and ε) based on their structural differences. The PLCβ-subfamily consists of 4 isozymes (β1 - β4) and additional splice variants (reviewed in Rhee, 2001) and is characterized by an extended C-terminal sequence (reviewed in Exton, 1996; Morris and Scarlata, 1997). Of these isozymes, PLCβ1 and PLCβ3 are the most widely expressed in mammalian tissues (reviewed in Rebecchi and Pentyala, 2000; Rhee, 2001). The main function of PLCs is to hydrolyze the highly phosphorylated lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), and to generate the ubiquitous second messengers IP_3 and DAG in response to plasma membrane receptor stimulation (reviewed in Rebecchi and Pentyala, 2000). The PLCβ isozymes of PLC family are activated by the stimulation of receptors coupled to $G_{q/11}$- (Smrcka et al., 1991; Taylor et al., 1991; Lee et al., 1992; Wu et al., 1992; reviewed in Exton, 1996; Yoshida and Imai, 1997; Rebecchi and Pentyala, 2000) or $G_{i/o}$-subtypes (Moriarty et al., 1990; reviewed in Yoshida and Imai, 1997) of G-proteins. Both α- and βγ-subunits of G-proteins have been shown to activate PLCβ (Lee et al., 1992; Wu et al., 1992; Blank et al., 1992; Camps et al.,

1992; Park et al., 1993; Smrcka and Sternweis, 1993; reviewed in Morris and Scarlata, 1997; Yoshida and Imai, 1997; Rebecchi and Pentyala, 2000) by interacting with separate regions of the enzyme (reviewed in Yoshida and Imai, 1997; Rhee, 2001). Since activation via the βγ-subunit seems to be weaker, it has been suggested that only abundant G-protein heterotrimers of $G_{o/i}$-family can produce sufficient amounts of activated βγ-subunits for PLCβ activation (Exton, 1996; Morris and Scarlata, 1997). Other PLC subfamilies (γ, δ, and ε) do not seem to be regulated by G-proteins (Lopez et al., 2001, reviewed in Rhee and Choi, 1992, Morris and Scarlata, 1997). The activity of PLCβ is also regulated by phosphorylation induced by cAMP-dependent protein kinase (PKA) and/or by protein kinase C (PKC) (Rebecchi and Pentyala, 2000) and the activity of all isozymes in PLC superfamily is dependent on Ca^{2+} (reviewed in Exton, 1996; Rhee, 2001). One interesting feature of PLCβs is that they function as GTPase activating proteins for their $G\alpha_{q/11}$ activator thereby providing a feedback mechanism to limit their own activity (Biddlecome et al., 1996; reviewed in Morris and Scarlata, 1997; Rhee, 2001).

IP_3 released by action of PLCβ (or other PLC) is a soluble molecule, which can diffuse from the plasma membrane to the cytosol. There it binds to IP_3R in the ER. Three isoforms of IP_3Rs derived from different genes have been identified to date (reviewed in Yoshida and Imai, 1997; Foskett et al., 2007) and they are expressed in a tissue- and cell type-specific manner while type 1 IP_3R is the most prevalent (Nakagawa et al., 1991; Sudhof et al., 1991; Ross et al., 1992; De Smedt et al., 1994). IP_3Rs belong to a superfamily of ion channels with six TM domains but they are unique in their localization near to intracellular Ca^{2+} stores in the ER. Four monomeric subunits of IP_3Rs come together to form a massive homotetrameric nonselective cation channel (Taylor and Laude, 2002; Clapham, 2007; Parkash and Asotra, 2010).

The activity of IP_3Rs is biphasically regulated by Ca^{2+} (Taylor and Laude, 2002; Parkash and Asotra, 2010). Low levels of Ca^{2+} are stimulatory while higher concentrations become inhibitory (Parker and Ivorra, 1990; Bezprozvanny et al., 1991; Parys et al., 1992; reviewed by Berridge et al., 2000; Clapham, 2007). This might represent an important feedback mechanism to protect the cell from the toxic effect of high $[Ca^{2+}]_i$. It seems likely that Ca^{2+} binds to two distinct sites on the channel complex and the role of IP_3 is to promote channel opening by controlling whether Ca^{2+} binds to the stimulatory or inhibitory sites. Working in conjunction, IP_3 and Ca^{2+} activate the IP_3R complex and allow release of Ca^{2+} from the intracellular Ca^{2+} stores (Taylor and Laude, 2002). The release triggers Ca^{2+} influx from the extracellular space through SOCs in order to refill the emptied stores (Clapham, 2007). The conventional view of PLCβ-pathway starting from the activated receptor and leading to the Ca^{2+} influx from the extracellular space is depicted in Figure 3.

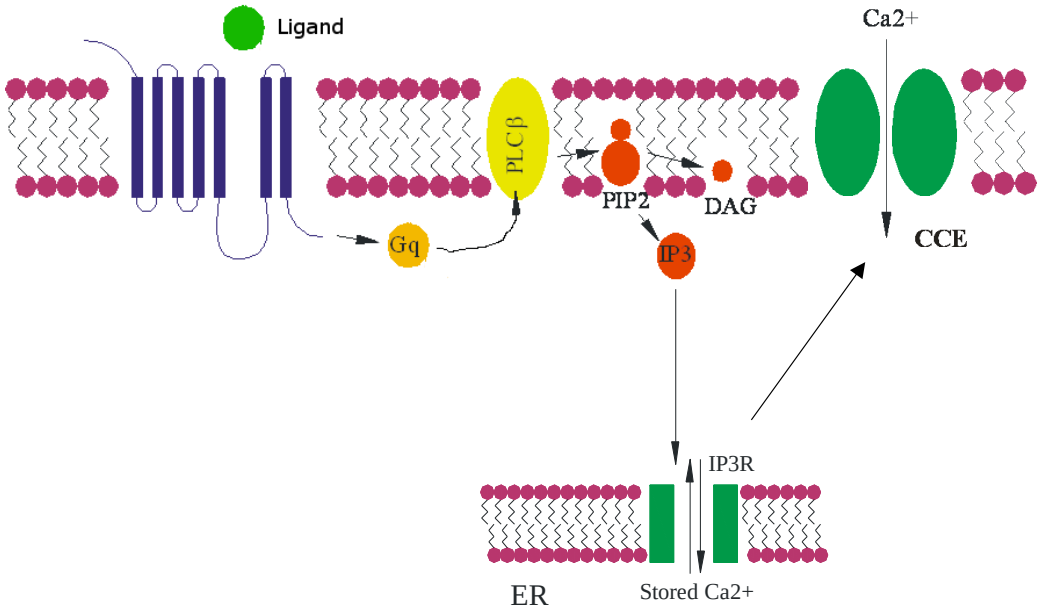

Figure 3. Conventional view of the phospholipase Cβ (PLCβ)-pathway (Graphics by J. Peltonen). Binding of a specific ligand to its receptor induces activation of the heterotrimeric G_q-protein and dissociation of the α- and βγ-subunits. α-subunit activates PLCβ on the plasma membrane. PLCβ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) and generates diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 diffuses to the cytosol and binds to IP_3Rs on the endoplasmic reticulum (ER). This leads to the discharge of the Ca^{2+} stores on the ER. Release of Ca^{2+} from the intracellular sources induces capacitative Ca^{2+} entry (CCE) from the extracellular space which aims to refill the emptied stores.

Several protein kinases including PKA, PKC and Ca^{2+}/calmodulin-dependent protein kinase (CAMKII) have been shown to regulate the activity of IP_3Rs via phosphorylation (Supattapone et al., 1988; Volpe and Alderson-Lang, 1990; Ferris et al., 1991; Quinton and Dean, 1992; Joseph and Ryan, 1993). The signalling of IP_3 is terminated when IP_3 is further converted by the action of several distinct kinases and phosphatases to a variety of inositol phosphates, some of which have also been implicated in intracellular signalling (reviewed in Rhee, 2001).

Release of stored Ca^{2+} and Ca^{2+} influx through SOCs are not the only signalling events following the activation of PLCβ, but DAG produced along with IP_3 also plays important signalling roles while staying bound to the plasma membrane. DAGs are glycerol derivatives in which two hydroxyl groups are substituted by fatty acids through ester bond formation (Brose and Rosenmund, 2002). The mammalian cell contains at least 50 structurally distinct species of the physiological relevant isomer of DAG, 1,2-diacyl-*sn*-glycerol, whose fatty acyl groups can be polyunsaturated, di-unsaturated, mono-unsaturated, or saturated (Hodgkin et al., 1998; Wakelam, 1998). Polyunsaturated forms of DAG produced by hydrolysis of PIP_2 by PLCs seem to be the most effective moieties in signalling (Hodgkin et al., 1998; Wakelam, 1998). DAGs can also be produced by other means, especially via the action of phospholipase D (PLD) on phosphatidylcholine to

form phosphatidic acid (PA), which is in turn converted to DAG in a reaction catalyzed by phosphatidic acid phosphatase. Usually the same receptors activating PLCs also activate PLD. However, DAG species produced via the PLD pathway are mono-unsaturated or saturated and they are unlikely to play a role in cellular signalling (Hodgkin et al., 1998; Wakelam, 1998).

The main target of DAG is PKC. The large PKC family of serine/threonine kinases comprises 10 members which can be classified based on their divergent N-terminal regulatory domains. Conventional PKC isoforms (α, βI, βII, and γ) contain binding domains for DAG, Ca^{2+} and lipids (phosphatidyl-serine, PIP_2). Novel PKCs (δ, θ, ε, and η/L) are activated by DAG and bind lipids but unlike conventional isoforms, these enzymes are insensitive to Ca^{2+}. Atypical PKC enzymes (ζ, ι/λ) are not regulated by either DAG or by Ca^{2+} while still possessing binding domain for lipids (Newton, 2001; van Rossum and Patterson, 2009). DAG binds to the cysteine rich region (C1 domain) of conventional and novel PKC isoforms (Muramatsu et al., 1989; Kaibuchi et al., 1989; Burns and Bell, 1991) leading to the activation and translocation of these enzymes to the plasma membrane (reviewed in Bell and Burns, 1991; Hug and Sarre, 1993). After activation, PKCs are thought to regulate a multitude of intracellular processes, ranging from cell proliferation to apoptosis and from regulation of transcription to neurotransmitter/hormone release (reviewed in Nishizuka, 1995; Dempsey et al., 2000). Regulation of cell proliferation and apoptosis by PKC can be mediated via its effects on protein kinases of the mitogen activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathway (reviewed in Rozengurt, 2007).

In addition to the PKC isoforms, DAG is able to bind to the C1 domains of a large number of proteins with diverse functions. The known C1-domain-containing proteins activated by DAG are chimaerins (Ahmed et al., 1990; 1993; Areces et al., 1994; Caloca et al., 1997; 2001), protein kinase Ds (PKDs) (Valverde et al., 1994; Hayashi et al., 1999; Sturany et al., 2001), Ras guanine nucleotide releasing protein (Ebinu et al., 1998; Lorenzo et al., 2000), Munc13s (Betz et al., 1998) and DAG kinases (Shindo et al., 2001; Shindo et al., 2003; reviewed in Brose and Rosenmund, 2002). The family of PKDs consists of PKCμ/PKD1 (Johannes et al., 1994; Valverde et al., 1994), PKD2 (Sturany et al., 2001) and PKCν/PKD3 (Hayashi et al., 1999). PKDs are serine/threonine kinases with distinct characteristics from the PKC kinase family (Valverde et al., 1994; Van Lint et al., 1995). They are independent of Ca^{2+} (Van Lint et al., 1995) but their activation usually requires phosphorylation of specific residues by novel PKC isoforms (Zugaza et al., 1996; 1997; Matthews et al., 1997; Waldron et al., 1999; 2001). PKDs have been shown to play essential roles in many cellular functions, including Golgi organization and function, immune response, proliferation and apoptosis (reviewed in Rykx et al., 2003). The termination of signalling of polyunsaturated DAG molecules occurs via actions of DAG kinases and DAG lipases which convert DAG into PA and AA, respectively (reviewed in Wakelam, 1998; Hodgkin et al., 1998).

2.2.2 Calcium Oscillations

As described earlier, Ca^{2+} is a ubiquitous signalling agent, necessary for life. However, a prolonged increase in $[Ca^{2+}]_i$ can be deleterious and lead to cell death. Therefore, Ca^{2+} signals are usually delivered as brief transients instead of as a sustained elevation of $[Ca^{2+}]_i$ (Berridge et al., 2000; Clapham, 2007; Parkash and Asotra, 2010). Single peaks in $[Ca^{2+}]_i$ can activate certain cellular processes, such as secretion of cellular material in membrane-bound vesicles, or they can trigger muscle contraction (Berridge et al., 2000).

However, more information can be encoded into repetitive transients known as Ca^{2+} oscillations in terms of amplitude, frequency, waveform or timing (Berridge et al., 2000; Taylor and Thorn, 2001; Parkash and Asotra, 2010). For example, the pattern and frequency of Ca^{2+} oscillations encodes for differential regulation of cellular functions, including gene expression (Dolmetsch et al., 1998; Li et al., 1998) and cell metabolism (Kasai and Augustine, 1990; Tse et al., 1993; Pralong et al., 1994; Hajnoczky et al., 1995) while the cell can also intepret modest changes in the amplitude of Ca^{2+} spikes, for example by activating different genes in response to the changes in the amplitudes (Dolmetsch et al., 1997). The widely accepted current view is that Ca^{2+} oscillations are a nearly universal phenomenon occurring under physiological conditions in response to a wide range of signals in both excitable and non-excitable cells (Tsien and Tsien, 1990; Berridge, 1995). Several studies have demonstrated Ca^{2+} oscillations in response to stimulation of GPCRs with physiological concentrations of agonists (which are often lower than the concentrations generally used in signalling studies, i.e. concentrations known to elicit a sustained elevation in $[Ca^{2+}]_i$) (Fu et al., 1991; Akagi et al., 1997; Sergeeva et al., 2000; Luo et al., 2001; Wu X. et al., 2002; Rey et al., 2006b).

In electrically excitable cells, the oscillations are generated by VOCs driven by membrane potential fluctuations (reviewed in Tsien and Tsien, 1990). Most models of Ca^{2+} oscillations induced by GPCR stimulation in non-excitable cells predict the involvement of periodic release and the reuptake of Ca^{2+} from the intracellular Ca^{2+} stores. Additionally, Ca^{2+} influx from the extracellular space has an essential role in refilling the stores and maintaining the oscillation pattern (reviewed in Tsien and Tsien, 1990; Taylor and Thorn, 2001; Berridge, 2005). Sometimes, both initial Ca^{2+} release from IP_3-sensitive stores and subsequent CIRC from ryanodine-sensitive stores are believed to be involved (reviewed in Tsien and Tsien, 1990; Mikoshiba et al., 1993; Bootman et al., 1996). However, there is growing body of evidence indicating that also in non-excitable cells, the oscillations can be derived solely from the function of the plasma membrane Ca^{2+} channels independently of Ca^{2+} stores (Fu et al., 1991; Grimaldi et al., 2003; Shlykov and Sanborn, 2004; Rey et al., 2006b). Despite these seemingly contradictory results and models, one can draw the conclusion that Ca^{2+} influx plays a major role in generation and maintenance of oscillatory Ca^{2+} signals.

There is no consensus concerning the Ca^{2+} influx pathway involved in Ca^{2+} oscillations but at least CCE via SOCs (Bootman et al., 1996; Sergeeva et al., 2000; Bird and Putney, 2005) and a novel non-capacitative Ca^{2+} entry activated by AA (Akagi et al., 1997; Shuttleworth and Thompson, 1999; Wu X. et al., 2002) have been proposed as candidates. Neither of these Ca^{2+} entry pathways has been suggested to be entirely responsible for the Ca^{2+} oscillations but to function in cooperation with the intracellular Ca^{2+} stores. The studies implicating the exclusive contribution of Ca^{2+} entry in oscillatory Ca^{2+} signals mostly identify canonical transient receptor potential channels (TRPC) as the channels responsible for the Ca^{2+} entry and oscillations (Grimaldi et al., 2003; Shlykov and Sanborn, 2004; Rey et al., 2006b). This large family of cation channels are more closely described in the next chapter (2.2.3 Canonical Transient Receptor Potential Channels). Now an attempt will be made to clarify the possible role of AA in triggering the Ca^{2+} influx involved in Ca^{2+} oscillations.

AA can be produced via multiple pathways in the cells. It is present in the phospholipids of plasma membranes of the cells and can potentially be released by a number of phospholipases (reviewed in Dennis et al., 1991). Activation of PLC or PLD generates AA indirectly via DAG, which can be converted to AA by diglyceride lipase.

On the other hand, phospholipase A_2 (PLA_2) generates AA as a direct product after phospholipid hydrolysis (reviewed in Diaz and Arm, 2003; Hirabayashi et al., 2004). PLA_2 can be activated by various extracellular stimuli including agonists of GPCRs. Thus GPCRs seem to trigger multiple either independent or interconnected pathways (see for example Lin et al., 1992; Winitz et al., 1994; Xing and Insel, 1996; Handlogten et al., 2001; Kurrasch-Orbaugh et al., 2003 or review Rozengurt, 2007). AA has been shown to induce Ca^{2+} influx in various cell types (Shuttleworth, 1996; Munaron et al., 1997; Shuttleworth and Thompson, 1998) and several reports have demonstrated its significant role in Ca^{2+} oscillations (Shuttleworth, 1996; Akagi et al., 1997; Shuttleworth and Thompson, 1998; 1999; Wu X. et al., 2002). Shuttleworth et al. (2004) proposed a model of Ca^{2+} oscillations in which stimulation of GPCR with a low concentration of agonist leads to a generation of AA and activation of a novel non-capacitative Ca^{2+} influx pathway, called AA-regulated Ca^{2+} entry (ARC). Ca^{2+} flowing to the cytosol increases the likelihood that already a low concentration of IP_3 will trigger repetitive Ca^{2+} release. Thus Ca^{2+} influx through ARC is essential for the generation of Ca^{2+} oscillations and in the modulation of the frequency although Ca^{2+} is originated from the intracellular IP_3-sensitive Ca^{2+} stores. At higher concentrations of agonist, the amount of generated IP_3 is sufficient to discharge the stores alone and to activate CCE which inhibits ARC. This leads to a sustained Ca^{2+} elevation instead of oscillations. Furthermore, they argued that the channels responsible for ARC and TRP channels are different molecular entities. However, involvement of TRP channels in Ca^{2+} oscillations have been demonstrated in several studies (Wu X. et al., 2002; Grimaldi et al., 2003; Launay et al., 2004; Shlykov and Sanborn, 2004; Bird and Putney, 2005; Rey et al., 2006b; Wedel et al., 2007) and therefore multiple Ca^{2+} entry pathways may well be involved in Ca^{2+} oscillations depending on the specific GPCR and cell model.

2.2.3 Canonical Transient Receptor Potential Channels

TRP ion channels were first identified in *Drosophila* visual system. It was found that a spontaneously occurring *Drosophila* mutant lacking a functional copy of the trp gene responded to continuous light with a transient receptor potential instead of a sustained response (reviewed in Minke and Cook, 2002; Flockerzi, 2007). The channel encoded by the gene was named after the abnormal response as the TRP channel. To date, at least 28 mammalian homologs of this channel encoded by distinct genes have been identified (Clapham et al., 2003; Flockerzi, 2007; Rowell et al., 2010). All the members have similar overall structure with six TM domains, both N- and C-terminus in the cytosol and a pore-forming region between the fifth and the sixth TM domain (Benham et al., 2002; Chakrabarti and Chakrabarti, 2006; Rowell et al., 2010) (Figure 4). The members of TRP superfamily show a high degree of structural diversity and the most conserved region is the one forming the pore, which shares structural homology with the *Drosophila* TRP (Minke and Cook, 2002; Rowell et al., 2010). A functional channel is formed when four similar or different TRP subunits assemble together to form homo- or heterotetramer (Benham et al., 2002; Clapham, 2003, Chakrabarti and Chakrabarti, 2006; Flockerzi, 2007). Often the channels are reported to be nonselective and permeable to Ca^{2+}, other divalent cations and Na^+ (reviewed in Clapham, 2003; Vazquez et al., 2004; Pedersen et al., 2005; Chakrabarti and Chakrabarti, 2006; Parkash and Asotra, 2010; Rowell et al., 2010). TRP channels have a central role in Ca^{2+} homeostasis in virtually all cells (Benham et al., 2002). Many members of the superfamily are utilized in mediating sensory signals, such as light, smells, pheromones, bitter or sweet tastes and changes in temperature (Clapham, 2003; Flockerzi, 2007).

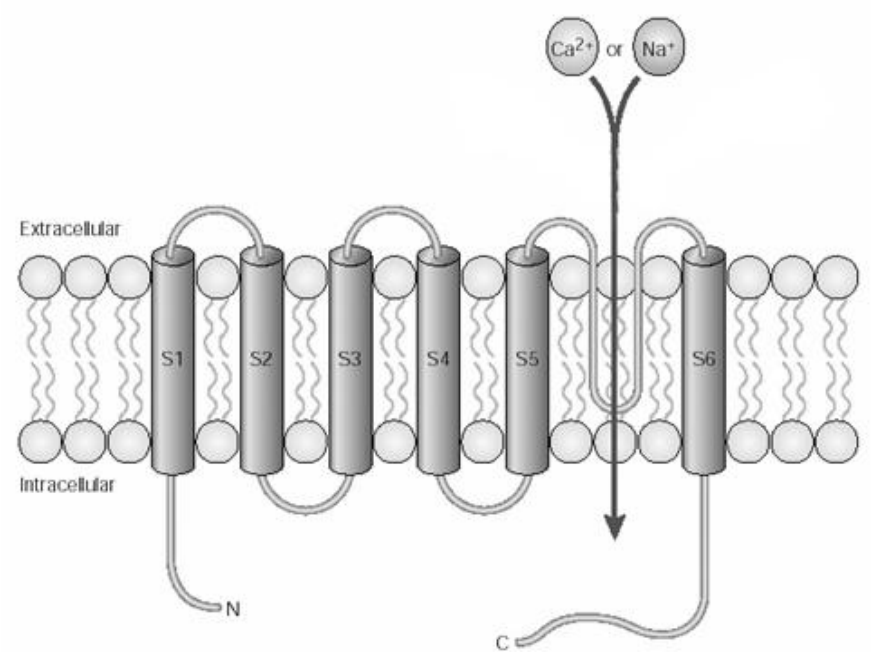

Figure 4. Architecture of transient receptor potential (TRP) channels (adapted from Clapham et al. 2001). TRP channels contain six transmembrane (TM) domain (S1 – S6). Both N- and C-terminus are in the intracellular site of the membrane. Channels are nonselective and permeable to both calcium ions (Ca^{2+}) (and other divalent cations) and sodium ions (Na^{+}).

The superfamily of mammalian TRP channels can be divided into six sub-families (Minke and Cook, 2002; Clapham, 2003; Flockerzi, 2007). The canonical TRP (TRPC) channel subfamily is one of them and consists of 7 members, TRPC1-7 (Benham et al., 2002; Clapham, 2003; Clapham et al., 2003; Flockerzi, 2007). This subfamily is further divided into three groups based on sequence homology and functional similarities (Clapham, 2003). The first group includes TRPC1, TRPC4 and TRPC5. The first identified human homolog of *Drosophila* TRP channel was TRPC1 channel (Wes et al., 1995; Zhu et al., 1995; Zitt et al., 1996). It has a widespread expression and ability to coassemble with all of the other TRPCs (Xu et al., 1997; Lintschinger et al., 2000; Strubing et al., 2001). Recombinant expression of TRPC1 and either TRPC4 or TRPC5 in cells results in a novel nonselective heterotetrameric channel with distinct properties from homotetrameric channels (Strubing et al., 2001). Murine TRPC4 and TRPC5 can form homotetrameric cation channels (Okada et al., 1998; Schaefer et al., 2000). TRPC2 alone forms the second group of TRPC channels. It is expressed in tissues of mouse, rat and bovine, but it is a pseudogene in humans (Wes et al., 1995; Wissenbach et al., 1998; Liman et al., 1999; Vannier et al., 1999; Hofmann et al., 2000). TRPC2 has been postulated to play a role in pheromone signalling (Liman et al., 1999; Stowers et al., 2002; Leypold et al., 2002). The third group of TRPC channels encompasses TRPC3, TRPC6 and TRPC7 channels, which are characterized by their low selectivity for Ca^{2+} over Na^{+} and their sensitivity to $[Ca^{2+}]_i$ (Zitt et al., 1997; Boulay et al., 1997; Okada et al., 1999; Jung et al., 2002; Estacion et al., 2006 reviewed in Dietrich et al., 2005; Eder et al., 2005). TRPC3 has been reported to be

constitutively active (Hurst et al., 1998; Albert et al., 2006). Most mammalian cells express more than one type of TRPC channels (reviewed in Chakrabarti and Chakrabarti, 2006).

The activation mechanisms of TRPC channels have been under an intense investigation. They have been acknowledged to be good candidates for both SOCs and ROCs activated by stimulation of GPCRs and receptor tyrosine kinases which activate the PLC signalling pathway (Clapham, 2003; 2007; Parkash and Asotra, 2010; Rowell et al., 2010). There is abundant evidence both for and against these functions. The first view is supported by the findings that TRPC channels are often activated after Ca^{2+} release from the intracellular Ca^{2+} stores and that there seems to be a physical contact between the channels and IP_3Rs. TRPC1/4/5 are generally thought to be the primary channels mediating CCE (Chakrabarti and Chakrabarti, 2006; Ambudkar et al., 2007). The physical interaction between IP_3R and TRPCs has been directly demonstrated in the cases of TRPC1 (Lockwich et al., 2000; Singh et al., 2000; Mehta et al., 2003) and TRPC3 (Kiselyov et al., 1998; Boulay et al., 1999; Ma et al., 2000; Zhang et al., 2001). All members of TRPC family possess a similar binding site for IP_3R in their C-terminus and are therefore capable of functioning as units of SOCs. A ubiquitous Ca^{2+} binding protein, calmodulin, competitively binds to the same region of the TRPC sequence as IP_3R and inhibits the channel activity (Tang et al., 2001; Zhang et al., 2001). The function of TRPC channels can therefore be indirectly modulated by $[Ca^{2+}]_i$. In addition to TRPC1 (Zitt et al., 1996; Zhu et al., 1996; Sinkins et al., 1998; Liu X. et al., 2000; Xu and Beech, 2001; Brough et al., 2001) and TRPC3 (Groschner et al., 1998; Li et al., 1999), also TRPC2 (Vannier et al., 1999; Gailly and Colson-Van Schoor, 2001; Jungnickel et al., 2001), TRPC4 (Philipp et al., 2000; Freichel et al., 2001), TRPC5 (Kanki et al., 2001), and TRPC7 (Riccio et al., 2002) seem to be activated after store depletion. TRPC channels have been shown to associate with STIM1 and Orai1 proteins, recently characterized essential components for CCE (Ambudkar et al., 2007; Liao et al., 2009; Rowell et al., 2010). However, nearly every report showing a store-operated activation of a specific TRPC channel seems to be accompanied by another study pointing to another direction. PLCβ and other components of the phosphatidylinositol pathway are important in the activation of *Drosophila* TRP channels (Harteneck et al., 2000; Clapham et al., 2001; Montell et al., 2002; Hardie, 2003). In accordance with this hypothesis, many TRPC channels have been demonstrated to be activated by the downstream products of PLC hydrolysis, most notably by DAG but also by AA or some other metabolite (reviewed in Benham et al., 2002; Montell et al., 2002). DAG potentiation of TRPC function seems to be a direct effect and not mediated via activation of PKC by DAG (Zitt et al., 1997). The principal targets for DAG activation seem to be TRPC3/6/7 (Hofmann et al., 1999; Ma et al., 2000; Venkatachalam et al., 2001; Chakrabarti and Chakrabarti, 2006; Rowell et al., 2010). Although often described as a SOC, TRPC1 can also function as a DAG activated channel in a recombinant model system in the presence of low extracellular Ca^{2+} (Lintschinger et al., 2000; reviewed in Pedersen et al., 2005). There is recent evidence to suggest that STIM1 and Orai1 proteins are not needed for TRPC1 function (DeHaven et al., 2009; Rowell et al., 2010) and that the complexes formed by TRPC channels and Orai1 proteins can also behave as ROCs (Liao et al., 2009). TRPC4 and TRPC5 are not activated by DAG, but probably by some other product downstream of PLC activation (Philipp et al., 1998; Okada et al., 1998; Hofmann et al., 1999; Schaefer et al., 2000). One candidate is AA (Broad et al., 1999; Mignen and Shuttleworth, 2000), which has been shown to activate *Drosophila* TRP-like (TRPL) channels (Chyb et al., 1999). One potential mediator between GPCR and TRPC is also PIP_2 (reviewed in Clapham, 2003). Taking these various results and models into

consideration, it can be concluded that TRPC channels likely participate in the formation of several different types of ion channels (Chakrabarti and Chakrabarti, 2006). Flockerzi (2007) concluded that most TRP channels do not fulfil the characteristics typical of SOCs but it still may turn out that one or more of TRP channels participate in CCE.

The important role of TRPC channels in the generation of Ca^{2+} oscillations in non-excitable cells has already been mentioned above. DAG analogs have been shown to promote Ca^{2+} oscillation in some cell models and the effect has been postulated to be mediated via DAG-activated TRPC3 (or TRPC6/7) (Grimaldi et al., 2003; Shlykov and Sanborn, 2004). In the model system used by Bird and Putney (2005), recombinantly expressed TRPC3 was able to support the Ca^{2+} oscillation induced by metacholine (an agonist of one GPCR, the cholinergic muscarinic receptor) when SOCs were inhibited. Similarly, Wedel et al. (2007) observed that Ca^{2+} oscillations induced by metacholine could be supported by non-store-operated channel mechanisms. They observed Ca^{2+} entry in response to AA in human embryonic kidney cells (HEK293) and Ca^{2+} oscillations in TRPC3-expressing cells even when SOCs were blocked. There is also evidence that TRPC4 or TRPC1 may be involved in Ca^{2+} oscillations elicited by stimulation of GPCRs (Wu X. et al., 2002; Rey et al., 2006b). The study of Wu X. et al. (2002) linked Ca^{2+} oscillations induced by carbachol (an agonist of the muscarinic receptor) together with AA and TRPC4. They characterized Ca^{2+} oscillations that are independent of intracellular Ca^{2+} stores, but maintained by AA regulated Ca^{2+} entry, probably through TRPC4. Rey et al. (2006b) characterized Ca^{2+} oscillations induced by stimulation of the Ca^{2+} sensing receptor by aromatic amino acids in HEK293 cells and reported independence of PLCβ-pathway and crucial role of TRPC1. They proposed a model where the receptor forms a multiprotein complex through its cytoplasmic tail with GTPase Rho, filamin-A (a potential scaffolding protein) and TRPC1. This interaction leads to TRPC1 channel opening and Ca^{2+} entry. Calmodulin which is activated by the increase in $[Ca^{2+}]_i$ participates in the generation of oscillations by inhibiting TRPC1 channel and initiating the downward phase of the Ca^{2+} spike.

The multiprotein signalling complexes seem to be common among GPCRs and especially signalling molecules of PLCβ-pathway have been shown to form assemblies. One example comes from the visual system of *Drosophila* where a scaffolding protein, G-protein, PLC, TRP and TRPL channels, PKC and calmodulin exist as a united complex (reviewed in Barritt, 1999; Bockaert et al., 2004). Scaffolding proteins are generally required to bring the participants together and the complexes are located in specific regions at the plasma membrane, such as caveolaes. Co-immunoprecipitation studies have revealed multiple interaction partners of mammalian TRPC channels in addition to IP_3R mentioned already earlier. TRPC1 seems to form a multiprotein complex with SERCA, $G\alpha_{q/11}$ and PLCβ (Lockwich et al., 2000). It can directly interact with one GPCR, the metabotropic glutamate receptor mGluR1 (Kim et al., 2003) and co-immunoprecipitates with an scaffolding protein, ezrin and an abundant protein in caveolaes, caveolin-1 (Lockwich et al., 2000; Singh et al., 2000; Brazer et al., 2003). TRPC4 forms a multiprotein complex with PLC, the scaffolding protein Na^+/H^+ exchanger regulatory factor and the actin cytoskeleton (Tang et al., 2000) and the complex is located in caveolae (Torihashi et al., 2002).

2.3 THE OREXIN SYSTEM

2.3.1 Discovery of Orexin System

The orexin system consisting of two hypothalamic neuropeptides orexin-A/hypocretin-1 (Ox-A) and orexin-B/hypocretin-2 (Ox-B) and the corresponding GPCRs orexin-1 receptor (OX_1R) and orexin-2 receptor (OX_2R) was found by two independent research groups in 1998 using two different approaches (de Lecea et al., 1998; Sakurai et al., 1998). First de Lecea et al. (1998) as a result of their search for unique mRNA transcripts in the hypothalamus described two hypothalamic peptides, hypocretin-1 and -2 (hypothalamic member of the incretin family), derived from the same precursor (preprohypocretin) and sharing substantial amino acid identities with each other and with the gut hormone, secretin. Only few weeks later a report with a careful characterization of two hypothalamic peptides named as orexin-A and orexin-B based on their orexinergic effects when administered centrally to rats was published by Sakurai et al. (1998). The aim of the study of Sakurai et al. was to screen extracts from rat and bovine brains for possible ligands of orphan GPCRs. Consequently, in the same report they were able to describe OX_1R (orphan receptor HFGAN72) and OX_2R as specific receptors for orexin peptides. Soon it became clear that hypocretins and orexins were two different names for the same peptides. No consensus on the nomenclature has been reached and both are still in use today, but here the name orexin is used consistently for clarity's sake.

2.3.2 General Aspects of Orexins and Their Receptors

Ox-A and Ox-B are derived from the same 131-amino acid precursor peptide prepro-orexin (PPO) by proteolytic processing (de Lecea et al., 1998; Sakurai et al., 1998). In the human genome, the gene encoding PPO is located in chromosome 17q21 and consists of two exons and one intron (de Lecea et al., 1998; Sakurai et al., 1998; 1999). Ox-A is a 33-amino acid peptide of 3562 Da with two sets of intrachain disulfide bonds formed by four cysteine residues (Sakurai et al., 1998). Ox-B is a linear 28-amino acid peptide of 2937 Da consisting of two α-helices (Lee et al., 1999). The sequences of orexin peptides are 46 % identical with each other (Sakurai et al., 1998) (Figure 5). Both peptides are C-terminally amidated and the N-terminus of Ox-A is additionally protected by pyroglutamyl residue (Sakurai et al., 1998; Lee et al., 1999). The functional significance of the structural differences of the peptides can be the higher stability of Ox-A (Kastin and Akerstrom, 1999). Since both termini of Ox-A are blocked by posttranslational modifications and it has two intrachain disulfide bonds, this peptide may be more resistant to inactivating peptidases. Sakurai et al. (1998) found no significant structural similarities to known families of regulatory peptides while de Lecea et al. (1998) reported a substantial identity with secretin. Both peptides, Ox-A and Ox-B, are highly conserved among the species from the frog, *Xenopus laevis* (Shibahara et al., 1999) to mammals (Sakurai et al., 1998; Dyer et al., 1999).

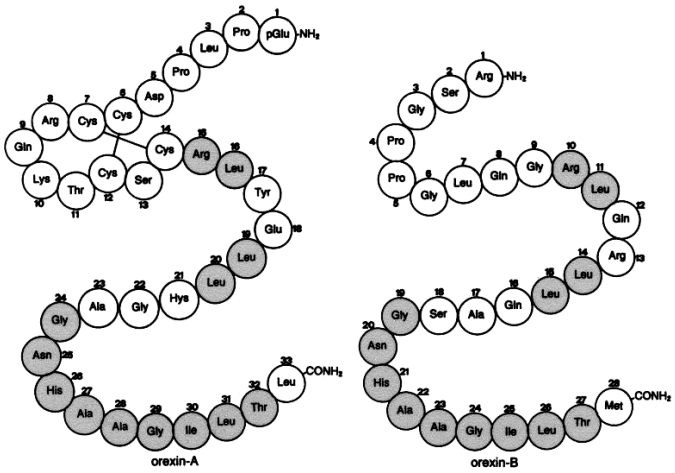

Figure 5. Amino acid sequence of human orexin-A (Ox-A) and orexin-B (Ox-B) (adapted from Spinazzi et al., 2006). Both peptides are C-terminally amidated. Additionally Ox-A possesses an N-terminal pyroglutamyl residue and two intramolecular disulfide bridges between cysteine residues. Identical amino acids between the two peptides are shaded.

De Lecea et al. (1998) did not identify receptors for the novel peptides but proposed that they acted through dedicated GPCRs that activate adenylyl cyclase (AC). They also predicted the existence of two receptor subtypes based on the differences in amino acid sequences of the two orexin peptides. OX_1R and OX_2R were identified by Sakurai et al. (1998). The receptors greatly resemble each other, sharing an amino acid identity of 64 % (Sakurai et al., 1998). They have a classical structure of GPCRs with seven TM domains, an extracellular N-terminus and a cytosolic C-terminus (Figure 6). They belong to the β group of the rhodopsin family of GPCRs (Fredriksson et al., 2003). OX_1R comprises 425 amino acids and OX_2R 444 amino acids (reviewed in Kukkonen et al., 2002). Both receptor genes are highly conserved between the species (Sakurai et al., 1998; reviewed in Kukkonen et al., 2002). OX_1R have been reported to exclusively couple to the $G_{q/11}$ subgroup of G-proteins while OX_2R interacts also with $G_{i/o}$ and G_s (Sakurai et al., 1998; van den Pol et al., 1998; Karteris et al., 2001; Randeva et al., 2001; Zhu et al., 2003). As a result, OX_1R signalling is mainly excitatory while OX_2R can mediate both excitatory and inhibitory signals depending on the postsynaptic neurons (reviewed in Willie et al., 2001). Later, an interaction between OX_1R and G_s or G_i has also been suggested (Holmqvist et al., 2005; Magga et al., 2006). OX_2R seems to bind both Ox-A and Ox-B in a non-selective manner while OX_1R is substantially more selective to Ox-A (Sakurai et al., 1998). Both receptors are highly specific for orexin peptides and do not bind other neuropeptides (Holmqvist et al., 2001; Smart et al., 2001). In addition to the orexin peptides, neither full nor partial agonists of the receptors have been discovered or developed. However, several antagonists with distinct selectivities (e.g. SB-334867-A, SB-674042, TCS-OX2-29, JNJ-10397049 and ACT-078573) have been synthetized (Smart et al., 2001; Hirose et al., 2003; Langmead et al., 2004; McAtee et al., 2004; Brisbare-Roch et al., 2007; for review see Boss et al., 2009) and at least one of them (SB-334867) seems to exert a partial agonist action (Bengtsson et al., 2007).

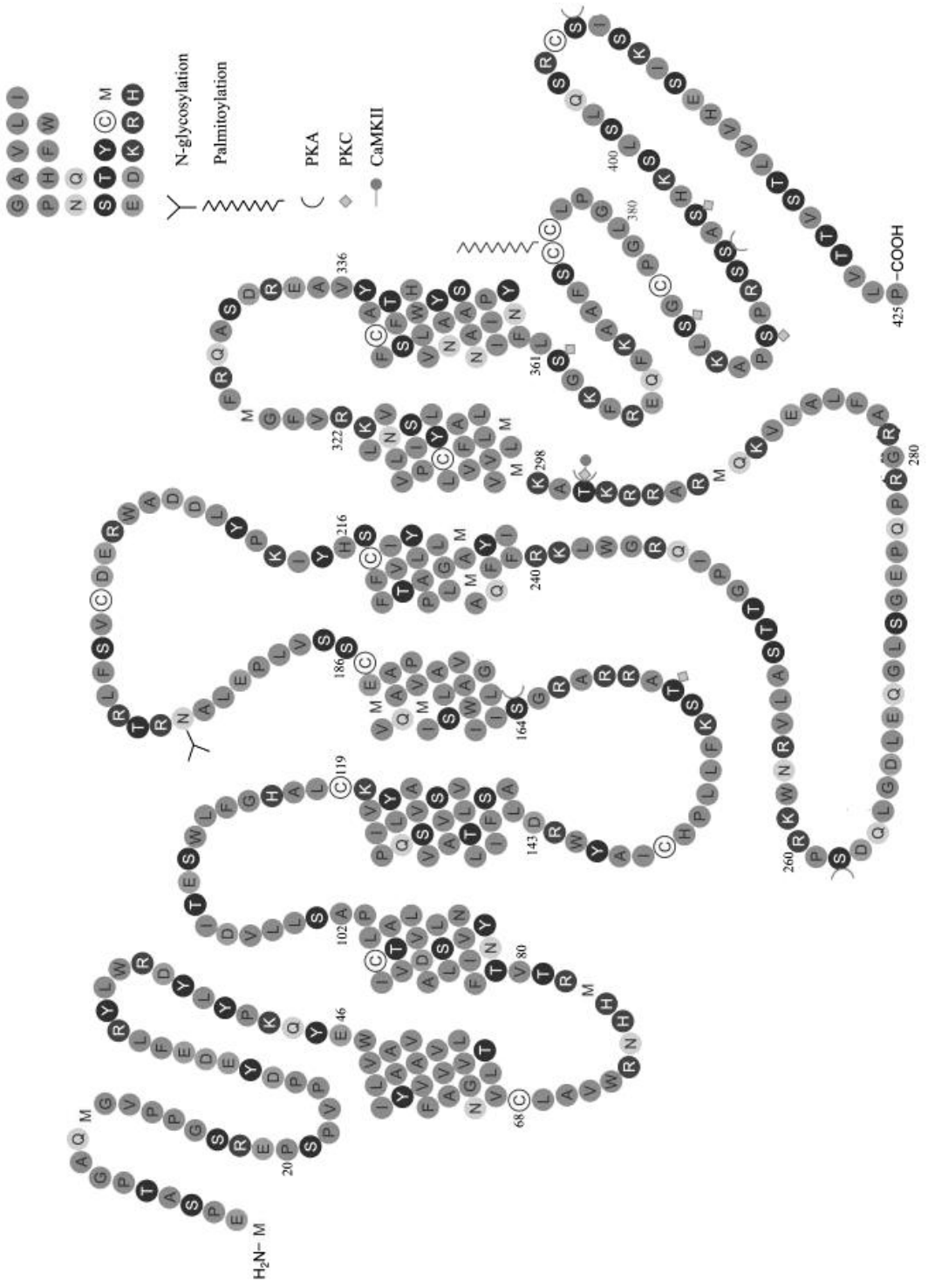
G A V L I
P H F W
N Q
S T Y C M
E D K R H
N-glycosylation
Palmitoylation
PKA
PKC
CaMKII
H2N– M
425 P –COOH

Figure 6. Amino acid sequences of human orexin-1 receptor (OX_1R) (adapted from Kukkonen et al. 2002). Putative N-glycolysation sites, putative phosphorylation sites for cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) and possible palmitoylated cysteines are shown. Grayscale coloring of the amino acids represents various amino acid families (nonpolar, polar with amide or hydroxyl group, acidic and basic). Additionally, cysteines (C) and methionines (M) are individually colored.

2.3.3 Orexin System in the Central Nervous System and the Periphery

Originally orexin peptides were detected almost exclusively in the brain except, for a small amount found in the testis (de Lecea et al., 1998; Sakurai et al., 1998). mRNAs of orexin and PPO were found from the cells of the lateral hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). An exhaustive immunohistochemical study of PPO containing neurons and fibers in the rat brain conducted by Peyron et al. (1998) confirmed the localization of PPO immunoreactive neurons in the perifornical nucleus, the dorsomedial hypothalamic nucleus, and in the dorsal and lateral hypothalamic areas. Date et al. (1999) used specific antibodies for Ox-A and Ox-B and found that the same neurons express both peptides. The orexin neurons are organized bilaterally and symmetrically (Sakurai et al., 1998; Peyron et al., 1998). Some neurons in posterior hypothalamic areas, in the subthalamus (the zona incerta, subincertal, and subthalamic nuclei) and in dorsomedial hypothalamic nucleus also contain orexins (Sakurai et al., 1998; Peyron et al., 1998; Nambu et al., 1999; Date et al., 1999). The presence of orexin neurons has been confirmed in the brain of numerous species including rat, bovine, monkey, mouse, human, frog, and hamster (de Lecea et al., 1998; Sakurai et al., 1998; Elias et al., 1998; Broberger et al., 1998; Horvath et al., 1999a; 1999b; van den Pol, 1999; Galas et al., 2001; Mintz et al., 2001). Female rats seem to have a larger content of Ox-A peptide and OX_1R mRNA in their hypothalamus than males (Taheri et al., 1999; Johren et al., 2001). The total number of orexin neurons has been estimated to be 6 700 in rat brain (Modirrousta et al., 2005) and 70 000 in human brain (Thannickal et al., 2000).

Orexin neurons have a relatively depolarized resting potential and they are spontaneously active regularly generating Na^+-dependent action potentials (Li et al., 2002; Eggermann et al., 2003; Yamanaka et al., 2003b). The depolarized and active state of these cells is dependent on a constitutively active non-selective cation channel, most probably involving TRPC5 (Cvetkovic-Lopes et al., 2010). The activity of the neurons is modulated by neural inputs from diverse brain centres and by substances that enter the brain from the bloodstream (Li et al., 2002; Yamanaka et al., 2003a; 2003b; Sakurai T. et al., 2005; Yoshida et al., 2006). Orexin neurons express receptors for many neurotransmitters and neuromodulators (de Lecea and Sutcliffe, 2005) and some of these confer orexin neurons with a capability to detect hormonal signals related to the metabolic state of the organism. The same trend continues in the sensitivity of the orexin neurons to glucose (Muroya et al., 2001; Yamanaka et al., 2003a; Burdakov et al., 2005), triglycerides (Wortley et al., 2003; Chang et al., 2004), carbon dioxide and pH (Williams et al., 2007). Neuronal co-expression of certain neurotransmitters/hormones is considered as evidence for their involvement in the systemic effects for these substances. The orexin neurons have been suggested to co-express dynorphin and galanin (feeding-regulating neuropeptides) (Hakansson et al., 1999; Chou et al., 2001), glutamate (a most common excitatory neurotransmitter) (Abrahamson et al., 2001; Collin et al., 2003; Rosin et al., 2003; Torrealba et al., 2003), and prolactin (a hormone associated with reproduction and lactation) (Risold et al., 1999).

Although orexins are produced by only a small group of neurons, these neurons project to almost all brain areas (de Lecea et al., 1998; Peyron et al., 1998) as well as all levels of the spinal cord (van den Pol, 1999). The brain structures receiving dense projections from orexin neurons include the hypothalamus, olfactory bulb, cerebral cortex, thalamus, and brainstem (Peyron et al., 1998; Date et al., 1999; Horvath et al., 1999b; van den Pol, 1999; Mondal et al., 1999a; 1999b; Shibata et al., 2008). It seems that the orexin neurons do not represent a homogenous cell population but the expression patterns and thereby the sensitivities to various substances vary among the cells (Hakansson et al., 1999; Muroya et al., 2001; Niimi et al., 2001). The heterogeneity of the orexin neurons is further emphasized by the distinct projection patterns of the various subgroups of neurons (Winsky-Sommerer et al., 2004; Espana et al., 2005). In all mammalian species examined, the distributions of orexin neurons and projections are very similar (Horvath et al., 1999b; Moore et al., 2001; Nixon and Smale, 2007)

Furthermore, the receptors OX_1R and OX_2R were first detected exclusively in the brain (Sakurai et al., 1998). They are expressed in a pattern consistent with orexin nerve fibers (Trivedi et al., 1998; Lu et al., 2000; Hervieu et al., 2001; Marcus et al., 2001) in the hypothalamus, cerebral cortex, hippocampus, amygdala, and several nuclei of the subthalamus, thalamus, and brain stem. The distribution patterns of OX_1R and OX_2R are distinct, but partially overlapping (Trivedi et al., 1998; Marcus et al., 2001). This suggests different physiological roles for each receptor subtype. Hervieu et al. (2001) detected OX_1R mRNA and protein also in the spinal cord and the dorsal root ganglia. Orexin neurons express OX_2R and are directly activated by Ox-B as a positive-feedback signal (Yamanaka et al., 2010).

The extensive study by Kirchgessner and Liu (1999) provided the first clue that orexins and their receptors might be expressed also in the peripheral tissues. Currently the presence of the peptides and/or receptors is firmly established at least in plasma, vagal afferent neurons, gastrointestinal tract and enteric nervous system directly controlling it, pancreas, adrenal gland, kidney, testis and ovaries (reviewed in Kirchgessner, 2002; Heinonen et al., 2008). The species tested include human, rat, mouse and guinea pig. There are also some indications that orexins and/or their receptors may be present in lung, heart, adipose tissue, placenta, thyroid gland, penis and epididymis (reviewed in Heinonen et al., 2008).

2.3.4 Systemic Effects of Orexins

The hypothalamus plays a key role in maintaining energy homeostasis and an appropriate state of arousal. It is a relay center of various metabolic, autonomic, endocrine and environmental factors and coordinates the behavioral, metabolic and neuroendocrine responses (Bernardis and Bellinger, 1993; 1996). The lateral hypothalamus, a region where there is extensive orexin expression, is especially important in food intake and behavioral arousal. Nevertheless, the diffuse nature of orexin projections suggests that there are multiple physiological roles for orexin peptides in addition to the initially suggested roles in feeding and sleeping (de Lecea et al., 1998; Sakurai et al., 1998) and furthermore, the detection of orexins and their receptors in the peripheral tissues extends the possible functions of these peptides. Table 2 illustrates the diversity of systemic functions proposed to be modified by orexins.

Table 2. Proposed systemic effects of orexins (reviewed in Burdakov, 2007; Heinonen et al. 2008; Kukkonen et al., 2002; Carter et al., 2009; Kuwaki, 2008; Beuckmann and Yanagisawa, 2002; Willie et al., 2001; Sutcliffe and de Lecea, 2002).

Systemic function	Proposed effect of orexins	Site of action
Feeding	Food intake ↑	CNS
Drinking	Water intake ↑	CNS
Metabolism	Metabolic rate ↑	CNS
	Proliferation of adipocytes ↑	Adipose tissue
	PPAR-γ-2 ↑	Adipose tissue
	Lipolysis ↓	Adipose tissue
Digestion	Gastric acid secretion in stomach ↑	CNS
	Bicarbonate secretion in duodenum ↑	Intestine
	Intestinal motility ↑↓	Intestine
	Pancreas juice volume ↑	Pancreas
Sleeping and alertness	Wakefulness ↑	CNS
	Slow-wave and REM sleep ↓	CNS
	Attention ↑	CNS
	Learning and memory ↑	CNS
Stress response	Locomotor activity ↑	CNS
	Distressed behaviour ↑	CNS
Addiction	Reward seeking ↑	CNS
	Drug addiction ↑	CNS
Pain	Pain ↓	CNS
Autonomic functions	Heart rate ↑	CNS
	Blood pressure ↑	CNS
	Breathing ↑	CNS
	Oxygen consumption ↑	CNS
	Chemosensitivity to CO_2 ↑	CNS
	Body temperature ↑	CNS
Reproduction	Male sexual arousal ↑	CNS
	Copulation ↑ [1]	CNS
	Spermatogenesis ↑↓	Testis
	Reproduction ↑↓	Ovary
	Nursing ↑↓ [3]	CNS
Hormone secretion	Arginine-vasopressin ↑	CNS
	Cholecystokinin ↑	Intestine
	Somatostatin ↑	CNS
	CRF ↑	CNS
	ACTH ↑	CNS and adrenal gland
	ACTH ↓	Pituitary gland
	Corticosterone ↑	CNS and adrenal gland
	Epinephrine and norepinephrine ↑	CNS and adrenal gland
	Prolactin ↓	CNS
	LHRH ↑	CNS
	LH ↑	Pituitary gland
	Neurotensin ↑	CNS
	Vasoactive intestinal polypeptide ↑	CNS
	Growth hormone ↓	CNS
	Insulin ↑↓	Pancreas
	Glucagon ↓ [4]	Pancreas
	Melatonin ↓ [5]	Pineal gland
	Testosterone ↑ [2]	Testis

Abbreviations used: ACTH = Adrenocorticotropin, CNS = Central nervous system, CO_2 = carbon dioxide, CRF = Adrenocorticotropin-releasing factor, LH = Luteinizing hormone, LHRH = Luteinizing hormone-releasing hormone, PPAR-γ-2 = Peroxisome proliferator-activated receptor γ-2, REM sleep = Rapid eye movement sleep. [1](Muschamp et al., 2007; Hoskins et al., 2008; Bai, 2009). [2](Barreiro et al., 2004). [3](Garcia et al., 2003; Espana et al., 2004; Wang et al., 2003; D'Anna and Gammie, 2006). [4](Goncz et al., 2008). [5](Mikkelsen et al., 2001).

The first evidence for a physiological role of orexins in feeding behaviour came already from Sakurai et al. (1998), who showed that the central administration of Ox-A and Ox-B stimulated food consumption in the rats in a dose-dependent manner. Further studies from several research groups have verified the important role of Ox-A in the regulation of feeding behaviour. However, the results obtained with Ox-B are more variable and harder to interpret. Investigations with the selective OX_1R antagonist (SB-334867-A) and with an anti-orexin antibody confirm that Ox-A acts endogenously to control natural eating behaviour (Haynes et al., 2000; Yamada et al., 2000; Rodgers et al., 2001). However the regulation seems to be complex and subject to diurnal variations (Haynes et al., 1999; Yamanaka et al., 1999). The total food intake during the chronic treatment with Ox-A does not change significantly and/or the body weight remains the same (Ida et al., 1999; Haynes et al., 1999; Yamanaka et al., 1999). Thus, the most important task of Ox-A in this context seems to be the initiation of food intake, but long-term energy homeostasis may be predominantly controlled by other factors than Ox-A. Some of the orexigenic effects might be mediated via other physiological responses evoked by orexins such as increased alertness and prolonged wakefulness or increased metabolic rate (Lubkin and Stricker-Krongrad, 1998; Willie et al., 2001; Hara et al., 2001). Hypoglycemia has been shown to activate orexin neurons and this has been postulated to result in increased alertness and intensified search for food (Moriguchi et al., 1999; Cai et al., 2001; Yamanaka et al., 2003a; Burdakov et al., 2005; Burdakov and Alexopoulos, 2005).

Central administration of orexins has been shown to increase wakefulness, decrease both slow-wave and REM sleep and disrupt sleep patterns (Hagan et al., 1999; Piper et al., 2000; Bourgin et al., 2000; Methippara et al., 2000; Espana et al., 2001; Xi et al., 2001; Huang et al., 2001; Yamanaka et al., 2002). Adamantidis et al. (2007) developed an exceptional novel technique to artificially excite orexin neurons and showed that excitation produced an increase in the probability of transition from sleep to wakefulness during both slow-wave and REM sleep. Electrophysiological studies suggest that the effects of orexins on wakefulness and vigilance are induced by exciting histaminergic neurons in the tuberomammillary nucleus (Eriksson et al., 2001; Bayer et al., 2001; Yamanaka et al., 2002) and monoaminergic/cholinergic neurons in the brain stem nuclei (Hagan et al., 1999; Brown et al., 2001; Burlet et al., 2002). The neuronal activity of orexin neurons has been increased by administration of a wake-promoting therapeutic agent, modafinil (Chemelli et al., 1999; Scammell et al., 2000) or by short-term sleep deprivation (Estabrooke et al., 2001). The increased arousal induced by orexins is also apparent from the observations of behavioral studies showing that Ox-A and Ox-B increases locomotor activity especially in terms of the time spent grooming and searching, respectively (Hagan et al., 1999; Ida et al., 1999; 2000; Duxon et al., 2001). Orexin antagonists have been shown to promote sleep in animals and several compounds are currently being tested as potential drugs for treating insomnia (Scammell and Winrow, 2011). Collectively these results highlight the crucial role of orexins in triggering and maintaining alertness and wakefulness. In addition, the tight linkage of the orexin system to the circadian rhythm and the fact that, in rats, the orexin system is most active during the active dark phase (Date et al., 1999; Taheri et al., 2000; Abrahamson et al., 2001; Hervieu et al., 2001; Mikkelsen et al., 2001; Fujiki et al., 2001; Yoshida et al., 2001; Estabrooke et al., 2001) supports this conclusion.

In the periphery, cells expressing orexins and/or their receptors are found primarily in the organs involved in feeding and energy metabolism (Kirchgessner, 2002; Heinonen et al., 2008) and the plasma levels of orexins are regulated by the nutritional status (Komaki

et al., 2001; Adam et al., 2002; Ehrstrom et al., 2005; Bronsky et al., 2007). Thus orexins are believed to have peripheral effects in controlling digestion and metabolism. Ox-A stimulates gastric acid secretion in stomach via the vagal pathway (Takahashi et al., 1999; Okumura et al., 2001; Ehrstom et al., 2005) and bicarbonate secretion in duodenum independently of vagal control (Flemstrom et al., 2003; Bengtsson et al., 2007). Orexins also seem to have an effect on intestinal motility but whether the effect is stimulatory or inhibitory is currently under debate due to the variability in the results (Heinonen et al., 2008). The endocrine glands are another prominent group of peripheral tissues expressing orexins and/or their receptors (Kirchgessner, 2002; Heinonen et al., 2008). Orexins have been shown to regulate the secretion of numerous hormones related to energy metabolism, autonomic functions, stress responses, and reproduction both in the hypothalamus and in the periphery and consequently stimulatory and/or inhibitory effects of orexins on these systemic functions have been demonstrated (Willie et al., 2001; Sutcliffe and de Lecea, 2002; Kukkonen et al., 2002; Burdakov, 2007; Heinonen et al., 2008; Kuwaki, 2008; Carter et al., 2009).

2.3.5 Diseases Linked to the Orexin System

Narcolepsy/cataplexy is a disease characterized by excessive daytime sleepiness, episodes of muscle weakness and abnormalities of REM sleep. In large breed dogs, Doberman pinschers and Labrador retrievers, there have been reports of a familial disease caused by several mutations in the gene encoding for OX_2R (Lin et al., 1999). Although the narcolepsy is usually considered as a non-genetic disease in humans, a firm link to the orexin system has been established by observations of dramatically reduced levels of Ox-A in cerebrospinal fluid and a slightly decreased Ox-A concentration in the plasma of narcoleptic individuals (Nishino et al., 2000; Dalal et al., 2001; Ripley et al., 2001; Higuchi et al., 2002; Knudsen et al., 2010) and absence of orexin neurons in the lateral hypothalamus of post-mortem human narcoleptic brains (Peyron et al., 2000; Thannickal et al., 2000). The degeneration of hypothalamic orexin neurons detected in post-mortem studies (Peyron et al., 2000; Thannickal et al., 2000) and the strong association of a certain Human Leukocyte Antigen haplotype with narcolepsy (Honda et al., 1986b) indicates that narcolepsy might be an autoimmune disorder. A single case of human narcolepsy has been reported to be associated with a mutation in PPO, which probably impairs correct trafficking and processing of the peptide (Peyron et al., 2000). The involvement of the orexin system in the regulation of energy metabolism is supported by findings of a significantly higher average body mass index (Schuld et al., 2000; Nishino et al., 2001) and an elevated risk to develop type 2 diabetes (Honda et al., 1986a) of narcoleptic patients than age-matched control subjects, despite lower daily caloric intake. Interestingly, narcolepsy is also associated with olfactory dysfunction (Bayard et al., 2010). In addition to narcolepsy, the orexin system has been proposed to be involved in other disease states including obstructive sleep apnea syndrome, Parkinson's disease, schizophrenia, depression and some types of cancer (de Lecea and Sutcliffe, 2005; Spinazzi et al., 2006; Carter et al., 2009).

Chemelli et al. (1999) generated a PPO knock-out mouse model with a phenotype strongly resembling human and canine narcolepsy. These mice exhibit reduced activity during the dark phase, behavioural arrests and disruption of REM sleep regulation. In addition to regulation of sleep/wake cycle and vigilance, the phenotype of PPO knock-out mice also provided support for some of the other putative systemic functions of orexins. PPO knock-out mice display a diminished behavioural response to stressful

stimuli (Georgescu et al., 2003; Kayaba et al., 2003), have a lower basal blood pressure (Kayaba et al., 2003) and a decreased chemosensitivity to carbon dioxide (CO_2) during wakefulness (Deng et al., 2007) than their wild-type littermates. Transgenic animals eat less than their wild-type counterparts but still maintain the same body weight, implying that there is a difference in energy homeostasis and metabolic rate (Willie et al., 2001).

Single receptor knock-out mouse models have been generated in order to unravel the possible distinct involvement of orexin receptor subtypes in the systemic effects induced by the orexins. However, the appearance of compensatory systems may well complicate the interpretation of the results. OX_1R knock-out mice exhibit no overt behavioural changes except for more rapid cycling between vigilance states whereas OX_2R knock-out mice suffer from a mild narcoleptic disorder (Willie et al., 2001). Double receptor knock-out mice and PPO knock-out mice seem to have the exactly same phenotype (Willie et al., 2001). Thus, it seems that both receptor subtypes participate in regulation of sleep/wake cycle and severe narcolepsy develops only with the disruption of both subtypes.

Hara et al. (2001) created an interesting mouse model for following the possible progression of human narcolepsy. In these mice, the orexin neurons are specifically and progressively ablated. Consequently, the mice display behavioral arrests, disrupted REM sleep and a poorly consolidated sleep pattern. There are several findings indicating that there have been changes in the basal metabolism and the energy homeostasis of these animals. The transgenic mice suffer from late-onset obesity and have an elevated risk for type 2 diabetes, despite eating less than the control animals (Hara et al., 2001). Additionally they fail to respond with normal vigilance and locomotor activity to fasting (Yamanaka et al., 2003a). The abnormalities in the emotional state-dependent adjustment of the central autonomic regulation on circulation and respiration observed in these mice point to an essential role for the orexin system in controlling autonomic functions and emotional behaviours (Kuwaki, 2011). Central administration of Ox-A can reduce cataplexy and prolong the wake time in these animals (Mieda et al., 2004). Similarly, an improvement in the condition of narcoleptic dogs after systemic administration of Ox-A has been reported (John et al., 2000), although another research group was unable to confirm this finding (Fujiki et al., 2003).

2.3.6 Cellular Effects of Orexins

The basis of all the systemic effects of orexins listed above is the cellular responses induced by orexin peptides binding to either of the two orexin receptors. Activation of orexin receptors have been shown to activate a wide variety of intracellular signalling pathways.

Neuronal Excitation

Orexin peptides reside in the synaptic vesicles in axon terminals of orexin neurons and are released in response to excitation of the cells (de Lecea et al., 1998; Peyron et al., 1998; Horvath et al., 1999a). They have been reported to have actions on presynaptic neurons and also at postsynaptic sites (van den Pol et al., 1998; Burlet et al., 2002; Acune-Goycolea and van den Pol, 2009). Generally, orexin peptides are believed to be excitatory and to increase the neuronal electrical activity of the receptive neuron. Already in the first report describing orexins, a prominent excitatory effect was observed i.e. an increase in the frequency of postsynaptic currents in 75 % of the synaptically coupled rat hypothalamic neurons (de Lecea et al., 1998). Later, orexins have been shown to excite several types of target neurons in various brain regions including many areas and nuclei of

hypothalamus (Liu et al., 2001; Bayer et al., 2001; Eriksson et al., 2001; Yamanaka et al., 2002; Samson et al., 2002; Acuna-Goycolea and van den Pol, 2009; Klisch et al., 2009), thalamus (Govindaiah and Cox, 2006; Huang et al., 2006), brain stem (Hagan et al., 1999; Bourgin et al., 2000; Hwang et al., 2001; Burlet et al., 2002; Takahashi et al., 2002; Liu et al., 2002; Brown et al., 2002; Korotkova et al., 2002; Yang and Ferguson, 2002; 2003; Yang et al., 2003; Grabauskas and Moises, 2003; Soffin et al., 2002; 2004; Takahashi et al., 2005; Muschamp et al., 2007), forebrain (Eggermann et al., 2001; Wu M. et al., 2002; 2004; Kolaj et al., 2008; Arrigoni et al., 2010), and cortex (Song et al., 2005; Xia et al., 2005a), as well as in spinal cord (Antunes et al., 2001; van den Top et al., 2003) and in peripheral tissues (Kirchgessner and Liu, 1999). This extensive collection of electrophysiological data provides support for the roles of orexins in the regulation of various physiological functions such as feeding, cardiovascular functions, sexual behavior, wakefulness, attention, gastric acid secretion, gut motility, and endocrine secretion.

Excitation of the target neurons via postsynaptic effects is evident from membrane depolarization, increased firing rate, prolonged firing, and/or enhanced inward current. Very often the primary mechanism behind excitation has been postulated to be a closure of potassium channels (van den Top et al., 2003; Grabauskas and Moises, 2003; Wu et al., 2004; Xia et al., 2005a; Govindaiah and Cox, 2006; Huang et al., 2006; Kolaj et al., 2008), but there may also be involvement of an activation of Na^+/Ca^{2+} exchanger (Eriksson et al., 2001; Wu M. et al., 2002; 2004; Acuna-Goycolea and van den Pol, 2009) or nonselective cation channel (Hwang et al., 2001; Liu et al., 2002; Brown et al., 2002; Yang and Ferguson, 2002; 2003; Huang et al., 2006; Kohlmeier et al., 2008). Probably the excitation results from more than one cellular mechanism. The intracellular second messengers: $G_{i/o}$-proteins (van den Top et al., 2003), PLC (Yang et al., 2003; Xia et al., 2005a; Song et al., 2005), PKC (Yang et al., 2003; Xia et al., 2005a; Song et al., 2005), and/or PKA (Korotkova et al., 2002; van den Top et al., 2003) have been proposed to lie along the signalling pathways leading to excitation. Presynaptic excitation caused by stimulation of orexin receptors induces increased release of neurotransmitters: glutamate (van den Pol et al., 1998; Kodama and Kimura, 2002; John et al., 2003; Borgland et al., 2008), GABA (van den Pol et al., 1998; Wu M. et al., 2002; Viggiano et al., 2004), noradrenaline (Hirota et al., 2001, Walling et al., 2004), histamine (Huang et al., 2001; Ishizuka et al., 2002; 2006; Hong et al., 2005), serotonin (Tao et al., 2006), and acetylcholine (Bernard et al., 2003; 2006; Fadel et al., 2005). The mechanisms accounting for the presynaptic actions of orexins remain largely unknown, but may involve the influx of extracellular Ca^{2+} (Hirota et al., 2001; John et al., 2003) and PKC (Borgland et al., 2008).

Some inhibitory effects of orexins on neuronal activity have also been reported. Ox-A significantly suppresses glucoreceptor neurons in the ventromedial hypothalamic nucleus, a brain area which is believed to mediate the cessation of eating (Shiraishi et al., 2000). This is probably one way through which Ox-A can stimulate feeding. Orexins seem to have both inhibitory and excitatory effects on neurons of the hypothalamic suprachiasmatic nucleus, which is the main circadian pacemaker in the brain (Brown et al., 2008; Klisch et al., 2009). Orlando et al. (2001) demonstrated an inhibitory presynaptic effect of orexins on the depolarization-stimulated release of serotonin.

Increase in Intracellular Calcium Concentration

Another well characterized cellular response evoked by stimulation of orexin receptors is the dose-dependent transient increase in $[Ca^{2+}]_i$, which was already observed by Sakurai et al. (1998) in Chinese hamster ovary cells (CHO-K1) and HEK293 cells stably transfected

with OX_1R. Both orexin peptides acting on either of the two receptors are able to elicit a Ca^{2+} response in recombinant cell models (Sakurai et al., 1998; Smart et al., 1999; Lund et al., 2000; Okumura et al., 2001; Holmqvist et al., 2002; Ammoun et al., 2003) as well as in native systems, such as cultured neurons from the rat lateral and medial hypothalamus (van den Pol et al., 1998), the rat tuberomammillary nucleus (Eriksson et al., 2001), the rat arcuate nucleus (Muroya et al., 2004), the murine midbrain (Narita et al., 2007), the rat spinal cord (van den Pol, 1999), the rat dorsal root ganglion (Ozcan et al., 2010), and the ovine pituitary gland (Xu et al., 2002; 2003). Furthermore, Ox-A has been shown to induce an increase in $[Ca^{2+}]_i$ in duodenal enterocytes isolated from the rat duodenal mucosa or in biopsy specimens of human duodenum (Bengtsson et al., 2009), in brain slices from the murine laterodorsal tegmentum and dorsal raphe (Kohlmeier et al., 2004; 2008), in dopamine neurons isolated from the rat ventral tegmental area (Nakamura et al., 2000; Uramura et al., 2001), in primary cultured neurons from the rat spinal cord (Xia et al., 2005b) and in cell lines of intestinal neuroendocrine cells (STC-1) (Larsson et al., 2003) as well as in pancreatic tumor cells (AR42J) (Harris et al., 2002). Consequently, there are clear indications for a physiological relevance of the Ca^{2+} response induced by orexins in endogenous systems.

Coupling of orexin receptors to the $G_{q/11}$ subgroup of G-proteins (Sakurai et al., 1998; van den Pol et al., 1998; Randeva et al., 2001; Zhu et al., 2003) indicates that the stimulation of the receptor activates PLCβ-pathway leading to Ca^{2+} release from the intracellular stores and subsequent CCE from the extracellular space. Several studies have confirmed the activation of this signalling pathway (Smart et al., 1999; Kukkonen and Akerman, 2001; Muroya et al., 2004; Ekholm et al., 2007; Johansson et al., 2007; 2008). However, when lower, and probably a more physiological concentration of Ox-A or Ox-B has been used, the Ca^{2+} response is acutely dependent on the extracellular Ca^{2+} concentration pointing to the involvement of Ca^{2+} influx instead of release of the cation from its storage sites (Lund et al., 2000; Ammoun et al., 2003; Magga et al., 2006). The importance of Ca^{2+} influx as a primary response is also evident from the results demonstrating that membrane depolarization which decreases the driving force of Ca^{2+} influx, is able to abolish the Ca^{2+} response (Lund et al., 2000) and that attenuation of IP_3 elevation and Ca^{2+} release from the intracellular stores leaves the Ca^{2+} influx untouched (Ekholm et al., 2007). The ROCs and SOCs activated after OX_1R stimulation have different pharmacological profiles implicating the involvement of distinct molecular entities (Kukkonen and Akerman, 2001) and OX_1R seems to activate ROCs through a signalling pathway independent of $G_{q/11}$-protein activation (Magga et al., 2006). Based on these results, a signalling model has been proposed in which the receptor-operated Ca^{2+} influx is the primary response and IP_3 production, Ca^{2+} release and CCE are subsequent to this response after potentiation of PLC activity by Ca^{2+} influx (Lund et al., 2000; Kukkonen and Akerman, 2001). Johansson et al. (2007) provided further proof for the model by demonstrating the central role of the receptor-activated Ca^{2+} influx in amplifying the OX_1R induced PLC response. Thus at a low concentration of Ox-A, Ca^{2+} influx is required to sufficiently activate PLC and produce IP_3. On the other hand, if OX_1R is prevented from coupling to the receptor-activated Ca^{2+} influx pathway, the receptor can utilize other Ca^{2+} influx pathways to activate Ca^{2+}-dependent downstream processes (Ammoun et al., 2006a). Also CCE is capable to amplify PLC response (Johansson et al., 2007). Later, it has been shown that OX_1R is capable of activating various phospholipases depending on the Ox-A concentration stimulating the receptor (Johansson et al., 2008). At the lowest concentration of Ox-A capable of inducing a Ca^{2+} response, PLD is activated and the the

second messengers most likely produced are DAG and PA. At 10-100-fold higher concentration, an activation of PLC can be also detected, but the production of IP_3 is still absent. Finally, still higher concentrations of Ox-A probably activate a different type of PLC, which hydrolyses PIP_2 to produce DAG and IP_3 (Johansson et al., 2008). Thus, the present model completely excludes the involvement of PLCβ-pathway at low concentrations of Ox-A with the response being solely derived from the Ca^{2+} influx. The differential coupling of OX_1R to phospholipases clearly explains the differences in Ca^{2+} signalling observed at low and high concentrations of Ox-A. At high concentration of Ox-A, OX_1R behaves like a classical $G_{q/11}$-coupled receptor while at low concentrations, an activated Ca^{2+} channels is sufficient to elicit cellular responses.

Ca^{2+} influx, which is dependent on the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and mediated via a similar non-voltage-gated Ca^{2+} channels as the one characterized in stably transfected nonexcitable CHO-K1 and HEK293 cells also seems to be the primary response to OX_1R stimulation in recombinant neuron-like cell systems (Neuro-2A murine neuroblastoma cells and PC12 human pheochromocytoma cells) (Holmqvist et al., 2002). In native systems, orexins induce increased Ca^{2+} current which is often considered a response indendent of the store release (van den Pol et al., 1998; Eriksson et al., 2001; Xu et al., 2002; 2003; Kohlmeier et al., 2004; 2008; Xia et al., 2005b). Kohlmeier et al. (2004) observed Ca^{2+} oscillations among other response types after Ox-A stimulation of young mouse brain slices taken from the laterodorsal tegmentum and the dorsal raphe. The Ca^{2+} responses were derived from the PKC-dependent Ca^{2+} influx via L-type VOCs and not dependent on Ca^{2+} release from the intracellular store. Also other reports have proposed the involvement of L-type channels (Uramura et al., 2001; Xu et al., 2002; 2003; Larsson et al., 2003) and the importance of PKC (van den Pol et al., 1998; Uramura et al., 2001; Xu et al., 2002; 2003; Kohlmeier et al., 2008; Ozcan et al., 2010) in the generation of orexin induced Ca^{2+} responses in neuronal systems. Thus the signalling sequence leading to Ca^{2+} elevation in neurons seems to consist of activation of the orexin receptor and PLC, the production of DAG, the activation of PKC and finally phosphorylation of the L-type channel by PKC, leading to channel opening. Nonexcitable cells, which have been widely used as model systems to study signalling of orexin receptor do not express VOCs, and therefore the molecular entity responsible for the receptor-activated Ca^{2+} influx has to be different.

Although in most systems orexins have been reported to increase the $[Ca^{2+}]_i$, there are also exceptions. Ox-A and Ox-B have attenuated Ca^{2+} oscillations and decreased $[Ca^{2+}]_i$ levels in isolated propiomelanocortin-containing neurons from the rat arcuate nucleus. The effect is probably mediated via OX_2R and $G_{i/o}$-proteins (Muroya et al., 2004). Orexins also decrease $[Ca^{2+}]_i$ levels of glucose-responsive neurons in the rat ventromedial hypothalamus (Muroya et al., 2004). It is possible that orexins do not elevate Ca^{2+} in all cell types or that other signals generated by the receptor activation counteract Ca^{2+} elevation.

Adenylyl Cyclase Pathway

In both recombinant cell lines and native cells, orexin receptor activation has been shown to lead to the activation of AC and production of cAMP, which in turn can activate PKA (Malendowicz et al., 1999; Mazzochi et al., 2001b; Randeva et al., 2001; Nanmoku et al., 2002; Holmqvist et al., 2005; Karteris et al., 2005; Ammoun et al., 2006a; Gorojankina et al., 2007; Tang et al., 2008). The orexin-evoked activation of the AC pathway is significant in the adrenal gland, where orexins stimulate secretion of glucocorticoids from

adrenocortical cells through cAMP-dependent process (Malendowicz et al., 1999; Mazzochi2001b; Ziolkowska et al., 2005). On the other hand, many studies using different models and methods have failed to detect the orexin induced activation of AC or the elevation of cAMP or to link the AC pathway to the cellular responses induced by orexins (van den Pol et al., 1998; Samson and Taylor, 2001; Mazzochi et al., 2001a; Harris et al., 2002; Xu et al., 2002; 2003; Larsson et al., 2003; Yang et al., 2003; Chen and Randeva, 2004; Kohlmeier et al., 2004; Magga et al., 2006). Thus this response seems to be rarer and more cell type/tissue specific than for example, the Ca^{2+} response. This becomes more conceivable when bearing in mind the possible tissue specific expression of different AC isoforms and G-protein subtypes. Interestingly, Mazzochi et al. (2001a) demonstrated that orexins utilize different signalling pathways in pheochromocytoma to stimulate catecholamine release and in normal adrenal tissue to increase glucocorticoid secretion. The PLCβ-pathway is predominant in the former response while AC-pathway is used in the latter case.

Orexins can also have other than stimulatory roles in AC signalling. Zhu et al. (2003) presented the inhibitory effect of activated OX_2R on forskolin stimulated AC activation. Steidl et al. (2004) and Goncz et al. (2008) demonstrated the decrease of cAMP level after Ox-A treatment in hamster glucagon-secreting α-cells and primary human hematopoietic stem/progenitor cells, respectively. Although the orexins seem to increase AC activity in adrenal cortical cells (Malendowicz et al., 1999; Mazzochi et al., 2001b; Nanmoku et al., 2002), the effect is opposite in adrenal medulla cells where Ox-A decreases the cAMP level (Nanmoku et al., 2002). Thus it seems that the regulation of the activity of AC by OX_1R occurs via multiple pathways. The receptor can couple to $G_{i/o}$-protein to inhibit cAMP generation, to G_s-protein to stimulate cAMP generation and to $G_{q/11}$-protein to activate PLCβ-pathway leading to activation of PKC, which in turn can stimulate cAMP generation (Holmqvist et al., 2005).

Activation of Protein Kinases

Orexins have been demonstrated to activate several types of protein kinases including PKC, PKA, MAPKs, adenylate-cyclase-kinase and phosphoinositide-dependent kinase 1 (Kukkonen et al., 2002; Holmqvist et al., 2005; Milasta et al., 2005; Ammoun et al., 2006a, 2006b, Ekholm et al., 2007; Tang et al., 2008; Ramanjaneya et al., 2009; Goncz et al., 2008). As described above, the activation of PKA and PKC has been proposed based on the indirect data showing the activation of PLCβ- and AC-pathways by orexins and demonstrating the effects of specific inhibitors on orexin induced responses. The exact subtypes activated by orexins have not yet been identified, but Holmqvist et al. (2005) were able to suggest the activation of PKCδ based on their experiments with pharmacological inhibitors.

Several studies have examined the specific activation of MAPKs: ERK1, ERK2 and p38, but not ERK5 and c-Jun N-terminal kinase, by both Ox-A and Ox-B (Kukkonen et al., 2002; Milasta et al., 2005; Ammoun et al., 2006a; 2006b; Ekholm et al., 2007; Tang et al., 2008; Ramanjaneya et al., 2009). The response might be mainly transmitted through OX_1R, but also a role for OX_2R in activation of ERK1/2 and p38 has been suspected (Tang et al., 2008; Ramanjaneya et al., 2009). The studies on activation mechanisms point to the involvement of multiple G-proteins and signalling pathways. The main pathways leading to activation of MAPKs seem to be G_q/PLC/PKC and G_s/AC/cAMP/PKA, but also the involvement of G_i-protein has been implicated (Tang et al., 2008; Ramanjaneya et al., 2009). The predominance of a signalling pathway depends on the peptide (Ox-A or Ox-B)

stimulating the receptor, the receptor subtype (OX_1R or OX_2R) which is activated and the cellular environment. In addition to the above pathways, also small GTP-binding protein Ras, phosphoinositide-3-kinase and protein kinase Src are thought to participate in signalling leading to activation of ERK1/2 (Ammoun et al., 2006a). Ca^{2+} influx seems to have a central role, because inhibition of influx pathways can fully attenuate ERK phosphorylation (Ammoun et al., 2006a). Inhibition of IP_3 elevation and consequent Ca^{2+} release from the intracellular stores do not affect the ERK response indicating that the effect is not solely due to the increase in $[Ca^{2+}]_i$ but the influx of the cation is required (Ekholm et al., 2007).

MAPKs can regulate both cell proliferation and apoptosis and orexins seem to play a dual role in the regulation of cell survival. In a recombinant cell system, Ammoun et al. (2006b) demonstrated that Ox-A caused a delayed cell death independently of the elevation of $[Ca^{2+}]_i$ and signalling molecules generally associated to apoptosis, p53 and caspase. Activity of p38 was essential for induction of apoptosis, while the ERK pathway was protective. Spinazzi et al. (2005) investigated the role of Ox-A in the proliferation of cultured adrenocortical cells endogenously expressing orexin receptors. They found that Ox-A stimulates cell proliferation via OX_1R and MAPK p42/p44, while Ox-B inhibits it via OX_2R and p38.

3 Aims of the Study

The physiologically prominent role of orexin signalling in the human body is widely acknowledged. Many cellular effects of orexins like elevation of $[Ca^{2+}]_i$ and activation of protein kinases have been repeatedly reported, but the signalling pathways leading to these responses have not been fully identified or characterized. However, the detailed knowledge of the signalling inside the cell is critical if one wishes to understand the effects of orexins at the systemic level and to interfere with the orexin system in order to treat the orexin-related disorders. This study was carried out to elucidate the signalling pathways activated by the stimulation of OX_1R. The specific aims of the present study were:

1. To recognize various cellular responses induced by stimulation of OX_1R by Ox-A
2. To characterize Ca^{2+} responses induced by different concentrations of Ox-A in several recombinant cell models
3. To identify signalling pathways participating on the regulation of Ox-A induced Ca^{2+} responses
4. To elucidate which Ca^{2+} channels are responsible for the Ca^{2+} influx induced by low concentration of Ox-A

4 Materials and Methods

4.1 CELL CULTURE (I-IV)

The cell lines used in this study were based on Chinese hamster ovarian CHO-K1 cells (I), human embryonic kidney HEK293 cells (III, IV) and human neuroblastoma IMR32 cells (II). All the cell lines were obtained from American Type Culture Collection. CHO-K1 cells are a widely used recombinant cell model to explore signalling pathways activated by GPCRs. However, this cell line has been derived from a rodent species and in order to acquire results which would be more relevant in human tissues, the HEK293 cell line is often used. Both CHO-K1 and HEK293 cells are non-excitable. As a model of excitable cells, the neuroblastoma cell line IMR32 was used. It is an adrenergic human cell line that can be differentiated in vitro to extend long axon-like processes with numerous growth cones (Clementi et al., 1986; Carbone et al., 1990) and thus provides a model of mature neurons in human.

The CHO-K1 and HEK293 cell lines were transfected to stably express the human OX_1R. The generation of CHO-hOX_1-C1 cells (I) has been described by Lund et al. (2000). CHO-hOX_1-C1 cells were cultured in Ham's F-12 cell culture medium supplemented with 100 units/ml penicillin G, 80 units/ml streptomycin, 400 μg/ml geneticin, and 10 % fetal bovine serum (FBS). HEK293OX_1R (III, IV) cells were prepared essentially the same way as HEK-OX_1-FLAG as described by Magga et al. (2006). HEK293OX_1R cells were cultured in Dulbecco's Modified Eagle's cell culture medium supplemented with 100 units/ml penicillin-streptomycin, 0.05 mg/ml hygromycin, and 10 % FBS. Successful transfections of CHO-hOX_1-C1 and HEK293OX_1R cells were confirmed by functional studies. Low nanomolar concentrations of Ox-A were sufficient to induce robust Ca^{2+} responses in these cell lines. In single cell Ca^{2+} imaging recordings, all stably transfected cells responded to Ox-A, while no responses even at high concentrations of Ox-A (1 μM) were detected in non-transfected cell lines. IMR32 cells (II) were grown in standard Minimum Essential cell culture medium with 100 units/ml penicillin-streptomycin, and 10 % FBS. The cell cultures were grown in 260 ml culture flasks (Nunc A/S) in an air-ventilated humified incubator (37 °C, 5 % CO_2). All the cell lines were cultured continuously and when the cultures reached 80 – 100 % confluency, the cells were detached by using phosphate-buffered saline containing 0.2 g/l ethylenediaminetetraacetic acid and transferred to new cell culture bottles with fresh medium.

4.2 POLYMERASE CHAIN REACTION ASSAYS

4.2.1 mRNA of Transient Receptor Potential Channels in CHO-hOX_1-C1 cells (I)

Total RNA (0.5 μg) was extracted from the cells using TRIzol (Invitrogen), reverse-transcribed to make cDNA using the SuperscriptII cDNA synthesis kit (Invitrogen) and amplified by using general trpc-specific degenerate primers 5'-nggvmchytgcagathtc-3' and 5'-nckhgcaaayttccaytc-3'. The polymerase chain reaction (PCR) was as follows: 95 °C for 5 min, 50 °C for 30 s, 72 °C for 30 s, and 94 °C for 30 s, 30 cycles. Amplified DNA was gel-purified and inserted into a PgemTeasy plasmid (Promega) and sequenced. PCR

product identification was done using BLAST (basic local alignment search tool) program (Altschul et al., 1997).

For expression analysis, specific primers for each trpc mRNA subtype were designed and tested. Nucleotide sequences, retrieved from the GenBankTM data base, were aligned with MacMolly Tetra (version 3.10, align ppc program, Soft Gene GmbH). The primers are presented in Table 3.

PCR conditions were optimized. The optimal annealing temperature for trpc1, trpc3, trpc6 and trpc7 was 55 °C and for trpc2, trpc4 and trpc5 59.5 °C. One or 0.5 μl (trpc1/trpc2) of the 20 μl cDNA reaction was amplified with channel-specific primers using optimized conditions. Identical amounts of PCRs were run on a 1.5 % agarose Tris-Borate-EDTA gel, stained with SYBRgreenI (Molecular Probes) according to the manufacturer's instructions, and scanned on a Storm 860 imaging system (Amersham Biosciences). Quantification of signal was done using ImageQuant program.

Table 3. Primer pairs for detection of trpc channels in CHO-hOX_1-C1 cells

Channel subtype	**Primer sequence**
TRPC1-5'	CTTGTTCTGTTTTCCTTCAC
TRPC1-3'	AAGCAGGTGCCAATGAACGA
TRPC2-5'	TCATCCTGACTGCCTTCC
TRPC2-3'	CCAGGAACTGAGGCATGT
TRPC3-5'	ACTACCTTGGGGCCAAAG
TRPC3-3'	CTACATCACTGTCATCCTC
TRPC4-5'	GTGGAGAAGGGGGACTATGC
TRPC4-3'	CCACGGCTCCAACCACCT
TRPC5-5'	TCCCTCTACCTGGCAACT
TRPC5-3'	AAAGAGCGTGGAGAAGGC
TRPC6-5'	CTCTGAAGGTCTTTATGC
TRPC6-3'	TCATCCTCAATTTCCTGG
TRPC7-5'	GCTGAAATACGACCACAA
TRPC7-3'	ATGAGGCACATCTTGATTC

4.2.2 mRNA of Transient Receptor Potential Channels in differentiated IMR32 cells (II)
IMR32 cells were differentiated for 6, 8, and 10 days. Total RNA (5 μg) was extracted using TRIzol (Invitrogen) and reverse-transcribed to make cDNA using SuperscriptII and oligo-dT (Invitrogen). An aliquot of the first-strand cDNA template (approximately the equivalent of 250 ng total RNA) was amplified with an annealing temperature of 55 °C for 30 cycles with Dynazyme II (Finnzymes, Espoo, Finland) using the specific primers presented in Table 4.

The primers were designed according to sequences available from the European Biology Laboratory database and at least one intron region was included to avoid amplification of genomic DNA and unprocessed RNA. The PCR reactions were electrophoretically analyzed on 2 % agarose gels and stained with ethidium bromide, and images were collected using a GelDoc imaging system (Bio-Rad). Amplified DNA fragments were gel purified and ligated into pGemTeasy (Promega) and sequenced. Sequences were identified using the BLAST program (Altschul et al., 1997).

Table 4. Primer pairs for detection of trpc channels in IMR32 cells

Channel subtype	Primer sequence
TRPC1–5′	GGGTCCATTACAGATTTCAA
TRPC1–3′	AAGCAGGTGCCAATGAACGA
TRPC3–5′	GTATGTGGACAGTTACGTC
TRPC3–3′	CTACATCACTGTCATCCTC
TRPC4 –5′	TGGGATGGCGGACTTCAG
TRPC4 –3′	ATGCCTTTGCAGGTTAACCC
TRPC5–5′	GTGGAGAAGGGGGACTATGC
TRPC5–3′	CCTCACTTGATAAGGCAATG
TRPC6 –5′	CTCTGAAGGTCTTTATGC
TRPC6 –3′	TCATCCTCAATTTCCTGG
TRPC7–5′	AACCCAGCGTTTACAACG
TRPC7–3′	ATGAGGCACATCTTGATTC

4.2.3 mRNA of Protein Kinase Ds in HEK293OX$_1$R cells (IV)

Total RNA (5 μg) was extracted from the cells using TRIzol (Invitrogen), reverse-transcribed to make cDNA using Revertaid (Fermentas) and amplified using Dynazymes (Finnzymes) and specific primers: PKD1 5′-GCCAGCTTCGTAATGAGG-3′/5′-CCTGCCCTTTTCACTTGA-3′, PKD2 5′-CGCTCTTCCAGAACAACACG-3′/5′-ACGAAGTAGGTGGCATTGG-3′, and PKD3 5′-CATGCCTGTTACTCCTCAAGC-3′/5′-AACTGGCCTGAACCAAGC-3′. The primers were designed using eprimer3 (EMBOSS). PCR conditions were as follows: 95 °C for 5 min, 94 °C for 20 s, 72 °C for 20 s, and 55 °C for 20 s, 30 cycles. PCR reactions were purified and sequenced using the ABI-prism system. Sequences were identified using the BLAST program (Altschul et al., 1997).

4.3 MEASUREMENTS OF ARACHIDONIC ACID RELEASE (III)

HEK293OX$_1$R cells were cultured on poly-L-ornithine-coated 24-well plates to 50 % confluence. 0.1 μCi [^{3}H]-AA ([5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid, New England Nuclear Corp. GesmbH) was added in each well and the cells were cultured for another 20 h. The incubation medium was removed and the cells were washed twice with the culture medium without serum but supplemented with 2 mg/ml bovine serum albumin. The stimulations with Ox-A were performed at 37 °C in 250 μl/well of this same medium. After 7 min of stimulation, 200 μl of the medium from each well was transferred to an Eppendorf tube on ice. These samples were spun down for 1 min at 4 °C and 150 μl of the medium was transferred to a scintillation tube and the scintillation cocktail (HiSafe3, Wallac-PerkinElmer) was added. Cell remnants on the 24 well plates were dissolved in 0.1 M NaOH and the scintillation cocktail was added. The radioactivity was counted in a scintillation counter after allowing the samples to set for 24 h.

4.4 TRANSFECTIONS AND TRANSDUCTIONS

4.4.1 DNA Constructs (I, III, IV)

Truncated forms of five trpc channels (trpc1, trpc2, trpc3, trpc4 and trpc7) exerting dominant negative effects as well as functional trpc3 channel were constructed by a standard protocol by subcloning a restriction fragment of desired size from a parent

vector into digested target vector (Table 5, Figure 7). Verification that all constructs were correct and inframe with enhanced green/yellow fluorescent protein (EGFP/EYFP) was done by automated sequencing.

Table 5. Construction of truncated forms of trpc1, trpc2, trpc3, trpc4 and trpc7 and functional form of trpc3

Construct	**Parent vector (restriction)**	**Fragment size (bp)**	**Target vector (restriction)**
mtrpc1βN-EGFP-N3 (trpc1N)	pcDNAtrpc1βFLAG[1] (NsiI-BamHI)	1480	pEGFP-N3[2] (BglII-PstI)
mtrpc2N-EGFP-N1 (trpc2N)	pcDNA-mtrpc2 clone 14[3] (BamHI-PstI)	2552	pEGFP-N1[2] (BglI-PstI)
EYFP-hstrpc3N-C1 (trpc3N)	human trpc3 cDNA[4] (BamHI-StuI, partial digest)	1620	pEYFP-C1[2] (BglII-SmaI)
functional trpc3 channel[5] (TRPC3FLAG)	human trpc3 cDNA[4] (BamHI-SpHI)		pIRES-hrGFP1a[6]
EYFP-mtrpc4βdn-C1 (trpc4N)	mtrpc4β-stop-EYFP (SalI-EcoRV)[7]	1520	pEYFP-C1[2] (SalI-SmaI)
mtrpc7αdn-EGFP-N1 (trpc7N)	PCIneomtrpc7α[8] (NheI-SacII)	1485	pEGFP-N1[2] (BglI-PstI)

[1]Gift from J. Frey (Engelke et al., 2002), [2](BD Biosciences), [3]gift from L. Birnbaumer (Vannier et al., 1999), [4]gift from C. Harteneck (Hofmann et al., 1999), [5]the last three residues are replaced by a triple FLAG epitope tag, [6](Stratagene), [7]gift from M. Nowycky (Obukhov and Nowycky, 2002), [8]gift from T. Okada (Okada et al., 1999).

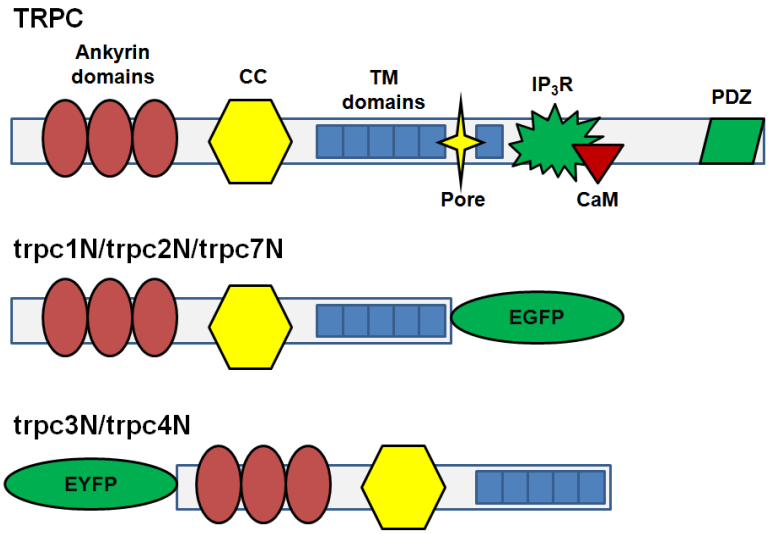

Figure 7. Schematic representation of truncated trpc channel constructs (Graphics by J. Peltonen). N-terminus of TRPC channels contains repeated ankyrin domains and a coiled-coil domain (CC). The channels have 6 transmembrane (TM) domains and the pore region is located between the fifth and sixth TM domain. C-terminus may display domains binding to inositol 1,4,5-trisphosphate receptor (IP_3R), calmodulin (CaM) and PDZ-domains (Clapham, 2003). In truncated channel constructs, the N-terminus of the channel is intact, but the pore region and the C-terminus have been deleted. Constructs are tagged with enhanced green/yellow fluorescent protein (EGFP/EYFP).

Trpc6^{DN} exerting a dominant negative effect was a gift from T. Gudermann (Hofmann et al., 2002). Kinase dead PKD constructs, PKD1K612W and PKD3K605A, used in this study were gifts from A. Hausser (Hausser et al., 2002) and O. Rey (Rey et al., 2006a), respectively. These constructs are called kinase dead, because the catalytic activity associated with wild-type PKD1 and PKD3 proteins were absent in these constructs as a result of point mutations of selected amino acids known to be essential for kinase activity.

4.4.2 Baculovirus (II)

In order to tranduct human neuroblastoma IMR32 cells, we designed baculovirus constructs to drive the expression of EGFP, OX$_1$R, and truncated forms of trp channels (trpc3N and trpc6^{DN}). All these recombinant baculoviruses were obtained using the Bac-to-Bac expression system (Invitrogen).

EGFP

An *Ase*I (blunted)–*Not*I fragment from pEGFP–N1 (BD Biosciences Clontech), including the cytomegalovirus (CMV) promoter and the gene for GFP, was subcloned into a *Sna*BI–*Not*I gap in pFastBac1 (Invitrogen), removing the polyhedrin promoter. The resultant vector was called pFastBac–CMV–GFP.

OX$_1$R

The OX$_1$R cDNA (in pcDNA3, gift from M. Detheux) was processed by PCR to remove the stop codon and subsequently subcloned into pEGFP–N3 (BD Biosciences Clontech). The OX$_1$R cDNA fused to the cDNA for GFP was then transferred to pFastBac–CMV–GFP as an *Eco*RI–*Not*I fragment. An untagged OX$_1$R construct was generated by subcloning the whole coding sequence of OX$_1$R cDNA into pFastBac–CMV–GFP, the GFP from vector being cut out. For plasma membrane localization of the red fluorescent protein (RFP) Discosoma red (DsRed)-Monomer, a CAAX motif from K-ras, KKKKKSKTKCVIM, was added to the *Eco*RI–*Bam*HI gap of pDsRed-Monomer-C1 (BD Biosciences Clontech) by ligation of two complementary oligonucleotides, and subsequently the RFP fused to CAAX was transferred to pFastBac–CMV–GFP (GFP cut out) with *Bsh*TI and *Sph*I.

Trpc3N

Trpc3N was constructed as described above and transferred to pFastBac-CMV–GFP, and the GFP cDNA was replaced by complementary oligonucleotides encoding a V5 epitope, followed by a STOP codon.

Trpc6^{DN}

The htrpc6^{DN} fused to YFP in pcDNA3 (gift from T. Gudermann) (Hofmann et al., 2002) was subcloned into pFastBac–CMV–GFP with *Bam*HI and *Xba*I (GFP removed).

4.4.3 Transfection Procedure (I, III, IV)

CHO-hOX$_1$-C1 and HEK293OX$_1$R cells were transfected by using FuGENE 6 (Roche Applied Science) according to the manufacturer's recommendations 18 – 24 hours after plating the cells on Petri dishes (35 mm inner diameter). One μg of DNA and 3 μl or 6 μl of FuGENE 6 were used for CHO-hOX$_1$-C1 and HEK293OX$_1$R cells, respectively. The cells were used in experiments after 24 – 48 h of transfection. Stable transfection of HEK293OX$_1$R cells by EGFP-PKD3 was achieved by transfecting the cells by the conventional procedure and by selecting the transfected cells with 400 μg/ml geneticin.

4.4.4 Transduction Procedure (II)

IMR32 cells were transduced by using recombinant baculoviruses. 0.5 ml of a high titer virus stock (10^7 pfu/ml), originating from *Spodoptera frugiperda* (Sf9) cell (Massotte, 2003; Aloia et al., 2009) infection, was spun down in a microcentrifuge at 12 000 rpm for 30 min. The pelleted viruses were resuspended in IMR32 cell culture medium, added to Petri dishes (35 mm inner diameter) with cells, and incubated until experimental use.

4.5 CALCIUM IMAGING AND PATCH CLAMP RECORDINGS

4.5.1 Media (I-IV)

The HEPES-buffered Na^+ based medium (HBM) consisted of 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose, 1 mM p-[dipropylsulfamoyl]benzoic acid (probenecid, Sigma-Aldrich), 20 mM HEPES, and 1.2 $MgCl_2$. The pH was adjusted to 7.4 with NaOH. For experiments with IMR32 cells (II) and for electrophysiology (I), probenecid and $MgCl_2$ were usually omitted, respectively. Ca^{2+} free medium was obtained by omitting $CaCl_2$. The tetraethylammonium (TEA) and K^+ based media were prepared by replacing Na^+ with TEA or K^+, respectively and by mixing this medium with the conventional one in order to achieve the desired TEA/K^+ concentration.

In electrophysiological experiments (I), the intracellular electrode solution used in the whole-cell voltage clamp recordings consisted of 136 mM Cs^+ aspartate, 30 mM HEPES, 10 mM NaCl, 4 mM ATP (Mg^{2+} salt, Sigma-Aldrich), and 0.6 mM GTP (Na^+ salt, Sigma-Aldrich). In current clamp recordings, Cs^+ aspartate was replaced by K^+ aspartate. The Ca^{2+} concentration was measured by using fura-2, pentapotassium salt (Molecular Probes) and adjusted to approximately 140 nM by adding 50 μM ethylene glycol tetraacetic acid (EGTA, Sigma-Aldrich) and 25 μM fura-2-acetoxymethyl ester (fura-2AM, Molecular Probes/Invitrogen). The effect of a high intracellular Ca^{2+} buffer capacity was tested in some 4 and 1 mM, respectively, or to 10 and 2.8 mM, respectively. The pH was adjusted to experiments by increasing the concentrations of EGTA and Ca^{2+} to 7.25 with CsOH or KOH.

4.5.2 Preparation of the Cells

For the Ca^{2+} imaging measurements in cell suspension, the cells (CHO-hOX_1-C1 (I) and HEK293OX_1R (III, IV)) were grown in 800 ml cell culture flasks in order to obtain a large quantity of cells. Near confluent cultures were grown, the cells were detached by standard protocol and spun down (1000 rpm, 3 min). The cell pellet was resuspended to pre-warmed HBM and loaded with fluorescent Ca^{2+} indicator fura-2AM (4 μM) (Grynkiewicz et al., 1985) for 20 – 30 min at 37 °C. The cells were washed once with Ca^{2+}-free HBM and divided into aliquots. CHO-hOX_1-C1 cells were stored on ice as pellets without medium and HEK293OX_1R at room temperature as cell suspensions.

For single cell Ca^{2+} imaging experiments (I-IV), the cells were plated on Petri dishes (35 mm inner diameter) containing circular glass coverslips (25 mm inner diameter) in 2 ml of medium. The cell densities varied according to cell line and the particular experiment protocol which was being used. The density of 125 000 cells per plate was used for CHO-hOX_1-C1 (I). 250 000 HEK293OX_1R cells (III, IV) were added per plate in other than oscillation experiments, in which the density of 400 000 cells per plate was used in order to obtain confluent monolayer of the cells, which is required for Ox-A induced Ca^{2+} oscillations to occur. In the case of IMR32 cells (II), confluent monolayer of cells from a

260 ml culture bottle were divided 1:3 or 1:4 and plated on dishes. In order to trigger differentiation in IMR32 cells, 5 μM 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) was added on the day after seeding. Thereafter, the medium was exchanged to fresh one containing BrdU every second or third day. Before the measurements of any above mentioned cell lines, the medium was exchanged to HBM and the cells were loaded with 4 μM fura-2AM for 20 - 30 min at 37 °C.

In the electrophysiological studies (I), the cells were harvested from 260 ml cell culture bottles and plated on Petri dishes (35 mm inner diameter) containing square glass coverslips (22 x 22 mm) to a confluency of approximately 50 – 70 % on the day of use. After 3 h, the medium was exchanged to HBM and the cells were loaded with 2 μM fura-2AM for 20 min at 37 °C.

4.5.3 Calcium Measurements in Cell Suspension

One pellet of CHO-hOX$_1$-C1 (I) or HEK293OX$_1$R cells (III, IV) was resuspended in HBM at 37 °C and placed in a stirred quartz microcuvette in a thermostated cell-holder within a PTI QuantaMaster™ fluorescence spectrophotometer. Fluorescence was monitored at the wavelengths 340/380 (excitation) and 505 nm (emission). The experiments were calibrated by using 60 μg/ml digitonin, which gives the maximum value of fluorescence, and 10 mM EGTA, which gives the minimum value of fluorescence.

4.5.4 Single Cell Calcium Imaging Experiments

The Ca^{2+} imaging experiments (I-IV) were performed and the data were analyzed by using the intracellular imaging InCyt2™ fluorescence imaging system (Intracellular Imaging). The cells were perfused with HBM at 37 °C and excited by alternating wavelengths of 340 and 380 nm by using narrow band excitation filters. The emitted fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera. One ratio image was acquired per second. Fluorescence from 340 and 380 nm exposures were imported into Microcal OriginTM 6.0, and the ratios (340/380) were calculated. The cells over-expressing EGFP-tagged constructs were identified by EGFP fluorescence with 450 – 480 nm ultraviolet light.

CHO-K1 cells have been shown previously to respond to 2′,3′-*O*-(4-benzoyl-benzoyl)-ATP (BzATP) via activation of purinergic P2X (P2$_Z$/P2X$_7$) receptors (Michel et al., 1998). In our setup, the response of BzATP was not affected by transfection. Therefore, CHO-hOX$_1$-C1 cells were stimulated with 100 μM BzATP (Sigma-Aldrich) at the end of each experiment and used the response as a control to cancel day to day variance (I). The responses evoked by Ox-A in individual cells were normalized with the response of BzATP. Oxotremorine (Research Biochemicals International), an agonist of muscarinic receptors, was used in IMR32 cells (II) to study the effect of TRPC6^{DN} on Ca^{2+} response induced by GPCR other than OX$_1$R. In HEK293OX$_1$R cells (III, IV), which did not respond to BzATP, the responses evoked by 1 nM Ox-A concentration were normalized against those obtained with 100 nM Ox-A, which was unaffected by transfection.

4.5.5 Patch Clamp Recordings (I)

Ca^{2+} currents were measured in voltage clamp mode at 28 °C by using the standard whole-cell configuration (Hamill et al., 1981) while at the same time monitoring the $[Ca^{2+}]_i$ by fura-2AM technique. The $[Ca^{2+}]_i$ in cells in the vicinity of the patched cell was monitored as controls. The coverslip with the cells was attached to the bottom of an RC-24 fast exchange chamber (Warner Instruments Inc.) and positioned on top of the

microscope. Cells were perfused with HBM by a gravity-controlled drug delivery system. The perfusates were converging in a perfusion manifold and funneled through an SH-27B in-line heater (Warner Instruments Inc.) located just before the chamber inlet to obtain the desired temperature. Patch pipettes (model PG150T, Harvard Apparatus) were prepared with a PC-10 puller and flame-polished in a microforge MF-900 (Narishige) to a resistance of 3.6 – 3.8 megaohms measured in the bath solution. The patch clamp amplifier Axopatch 200A was connected to a computer via the AD/DA Digidata 1320E SCSI interface (Axon Instruments). Voltage protocols and data acquisition were controlled with pClamp 8.1 (Axon Instruments). Cells were compensated for the pipette capacitance, whereas following whole-cell access, the series resistance was analogically compensated to 60 – 70 %. Liquid junction potential was calculated using pClamp8.1 and subtracted from the recordings giving a more accurate clamping potential of approximately 60 mV. In general, voltage ramps (-80 to +80 mV; 320 ms) were applied every 5 or 7.5 s. Data were digitally sampled at 3.8 kHz and filtered at 2 kHz by using the low pass Bessel filter on the recording amplifier. In current clamp experiments, data were digitally sampled at 5 kHz and filtered at 2 kHz. Combined fluorescence recordings were obtained with a second computer running the TILLvisION Multi-Color Ratio ImagingSystem (TILL Photonics GmbH), and saved for later analysis. The system consisted of a polychrome IV and a 12-bit IMAGO CCD camera under the control of an external control unit. An inverted microscope (Nikon) was used to visualize the fluorescence. Ultraviolet light was guided through an epifluorescence condenser, and cells were excited through a dichroic mirror (DM430, Nikon). The emission was measured through a 510 nm cut-off filter (Nikon). The imaging protocol was designed to acquire images at 340 and 380 nm every 1–3 s. A transistor-transistor logic (TTL) trigger pulse synchronized the patch clamp and imaging recordings; the TTL pulse was controlled by TILLvisION 4.0 to trigger the voltage clamp data acquisition by using the "digitizer start input" option in the pClamp 8.1. After ending the recordings, fluorescence from 340 and 380 nm of selected regions of interest was analyzed and converted into $[Ca^{2+}]_i$ as described previously (Lund et al., 2000). Voltage clamp and image data were then combined in Microcal OriginTM 6.0 for visualization and final analysis.

4.5.6 Test Reagents (I-IV)

Various test reagents used in Ca^{2+} imaging and patch clamp recordings and their most probable modes of action are listed in Table 6.

Table 6. Test reagents used in Ca^{2+} imaging and Patch clamp recordings

Reagent, abbreviation	**Manufacturer**	**Mode of action**
2-Aminoethoxydiphenyl borate, 2-APB (I, III)	Calbiochem	Antagonist of IP_3Rs and inhibitor of CCE
Arachidonic acid, AA (III)	Sigma-Aldrich	Product of PLA_2 action
2',3'-*O*-(4-benzoyl-benzoyl)-ATP, BzATP (I)	Sigma-Aldrich	Agonist of purinergic P2X ($P2_Z$/$P2X_7$) receptors
R59022, DAGKI (I)	Calbiochem	Inhibitor of DAG kinase
Dextromethorphan, Dex (I, II)	Sigma-Aldrich	Blocker of NMDA receptor channels and VOCs
Digitonin (I, III, IV)	Merck (I), Sigma-Aldrich (III, IV)	Solubilizer of plasma membranes
Dioctanoyl glycerol, DOG (I, II)	Sigma-Aldrich	Analog of DAG, activator of PKC
Ethylene glycol tetraacetic acid, EGTA (I, III, IV)	Sigma-Aldrich	Chelator of Ca^{2+}
GF109203X, GF-X (I, II)	Calbiochem	Inhibitor of PKC
Potassium chloride, KCl (I, II)	Sigma-Aldrich	Depolarizor of plasma membrane
KB-R7943, KB-R (II)	Tocris Cookson	Blocker of the reverse operation mode of NCX
Methyl arachidonyl fluorophosphate, MAFP (III)	Cayman Chemical	Inhibitor of PLA_2
Magnesium chloride, $MgCl_2$ (I-III)	Sigma-Aldrich	Blocker of Ca^{2+} channels
Nickel chloride, $NiCl_2$ (I)	Sigma-Aldrich	Blocker of Ca^{2+} channels
ω-conotoxin (II)	Tocris Cookson	Blocker of N-type VOCs
Orexin-A, Ox-A (I-IV)	Bachem	Agonist of OX_1R
Oxotremorine (II)	RBI	Agonist of muscarinic receptors
SKF-96365, SKF (I)	Calbiochem	Inhibitor of CCE
Tetraethylammonium, TEA (I)	Sigma-Aldrich	Blocker of K^+ channels
Thapsigargin (I, II)	RBI	Inhibitor of ER Ca^{2+}-ATPase
12-*O*-tetradecanoylphorbol-13-acetate, TPA (I, II)	Sigma-Aldrich	Activator of PKC

Abbreviations used: CCE = capacitative Ca^{2+} entry, DAG = diacylglycerol, ER = endoplasmic reticulum, IP_3R = inositol 1,4,5-trisphosphate receptor, NCX = Na^+/Ca^{2+} exchanger, NMDA = N-methyl-D-aspartate, OX_1R = orexin-1 receptor, PKC = protein kinase C, PLA_2 = phospholipase A_2, RBI = Research Biochemicals International, VOC = voltage-gated Ca^{2+} channel.

4.5.7 Statistics (I-IV)

Ratios of fluorescence from 340 nm and 380 nm exposures (ratio 340/380) were calculated with Microcal Origin 6.0. Absolute levels of $[Ca^{2+}]_i$ and changes in Ca^{2+} levels ($\Delta[Ca^{2+}]_i$) were obtained utilizing standard curve determined with known Ca^{2+} concentrations. The differences between two groups were evaluated by the unpaired Student's t-test. One-way ANOVA test followed by Scheffe's test was used to determine the differences between more than two groups. p-values < 0.05 were considered as statistically significant.

4.6 SUBCELLULAR FRACTIONATION (IV)

HEK293OX_1R cells or HEK293OX_1R-EGFP-PKD3 cells were grown to near confluency on Petri dishes (10 mm inner diameter) in 8 ml of medium and treated with 1 nM or 50 nM Ox-A in HBM for 5 min. Cytosolic and membrane fractions were isolated according to Brott et al. (1998).

4.7 IMMUNOLOGICAL ASSAYS (IV)

4.7.1 Immunoblotting

In order to detect protein kinases activated by Ox-A, specific antibodies were used: anti-active ERK1/2 (Promega), anti-PKD1S916p (Cell Signalling), anti-PKCδT505p (Cell Signalling), anti-PKCα/βT638/641p (Cell Signalling). Anti-actin (Sigma-Aldrich) was used as a loading control. HEK293OX_1R cells were grown to near confluency on Petri dishes (35 mm inner diameter) in 2 ml of medium. The medium was exchanged to HBM and the cells were treated with 5 nM Ox-A at 37°C. HBM was removed, dishes were transferred on ice and cells were lyzed in a Radio-Immunoprecipitation Assay (RIPA) buffer (50 mM Tris, 150 mM NaCl, 1 % Triton X-100, 10 nM sodium fluoride (NaF), 100 μM orthovanadate). The lysates were used in western blotting. 5 - 10 μg of protein were run on 7.5 or 10 % acrylamide gel, transferred to polyvinylidene fluoride membrane and probed with antibodies according to the manufacturer's instructions. Positive bands were detected with ECL+ (Enhanced Chemiluminescence) Western Blotting Detection Reagents (GE Healthcare) and scanned on STORM™ imaging system (GE Healthcare). The same western blot protocol was used to detect active PKD1 and PKD3 after subcellular fractionation described in section 4.6. Anti-EGFP (Clontech) followed by anti-active PKD (744/748) (Cell Signalling) was used in the case of PKD3 and anti-PKD1S916p (Cell Signalling) in the case of PKD1. Anti-OX_1R (Alpha Diagnostics) was used as a loading control.

4.7.2 Immunoprecipitation of Protein Kinase D3

HEK293OX_1R-EGFP-PKD3 cells were seeded on Petri dishes (60 mm inner diameter). When the cultures were nearly confluent medium was exchanged to HBM and the cells were treated with 1 and 50 nM Ox-A for 5 min. Cells were lyzed in RIPA buffer and the lysate was precleared with Dynabeads® Protein G (Invitrogen) for 1 h at 4 °C. The beads used in preclearing were replaced with the beads cross-linked to anti-EGFP polyclonal antibody (Clontech). The mixture was incubated at 4 °C over night with constant turning. The beads were then captured, washed three times with phosphate buffered saline (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7,2) and eluted in sample

buffer (62,5 mM Tris-HCl (pH 6,8), 25 % glycerol, 2 % sodium dodecyl sulphate, 0,01 % bromophenol blue, 5 % β-mercaptoethanol). The eluates were used in western blotting as described above in chapter 4.7.1 (8 % acrylamide gel). Anti-active PKD (744/748) (Cell Signalling) was used to detect active PKD3.

4.8 EPIFLUORESCENCE MICROSCOPY OF HEK293OX$_1$R-EGFP-PKD3 CELLS (IV)

HEKOX1R-EGFP-PKD3 cells were plated on 12-well plates containing circular glass coverslips (0.9 mm inner diameter) in 1 ml of medium. At near confluency, medium was replaced with HBM with 1 or 50 nM Ox-A. At selected times (5 and 30 min), cells were fixed in formalin and fluorescent cells were observed with Olympus IX71 epi-fluorescence microscope, Olympus IX71. Images were captured using Olympus DP controller software.

5 Results

5.1 CELLULAR RESPONSES TO OREXIN-A

5.1.1 Membrane Depolarisation

Orexin peptides are known to be excitatory and the excitation of the target neurons can often be observed via membrane depolarization (rewieved in Kukkonen et al., 2002; Ferguson and Samson, 2003). The present whole-cell current clamp recordings with an intracellular K^+-based solution revealed that 0.3 nM Ox-A evoked a highly voltage-dependent depolarization of CHO-hOX1-C1 cell membrane (I, Fig. 5C and 5D).

5.1.2 Elevation of Intracellular Calcium Concentration

Dose-dependent transient increase in $[Ca^{2+}]_i$ is a well characterized cellular response to stimulation of orexin receptors in both recombinant cell models and native systems (reviewed in Kukkonen et al., 2002). Ox-A induced Ca^{2+} responses were observed and characterized in all 3 recombinant cell models used in this study: CHO-hOX_1-C1 cells, IMR32 cells transiently transduced by OX_1R and HEK293OX_1R cells. In these cell lines, 3 nM or higher concentrations of Ox-A induced a two-phase Ca^{2+} response, consisting of a sharp peak and a delayed stable phase (see for example I, Fig. 1B, 2D and 2E, II, Fig. 1E and III, Fig. 1B, gray trace). Lower concentrations of Ox-A induced different Ca^{2+} responses in different cell lines: 1) In CHO-hOX_1-C1 cells, a transient elevation of $[Ca^{2+}]_i$ was observed, 2) in IMR32 cells, 1 nM Ox-A resulted in a stable elevation of $[Ca^{2+}]_i$ (II, Fig. 2A), which lasted for over 10 min and 3) in HEK293OX_1R cells, prolonged stimulation with a low concentration of Ox-A resulted in repetitive spike patterns of $[Ca^{2+}]_i$ known as Ca^{2+} oscillations, the frequencies of which were about 7 mHz (III, Fig. 1A). A similar oscillation pattern was also seen when HEK293OX_1R cells were stimulated with a high concentration (100 nM) of Ox-A in the absence of extracellular Ca^{2+} (III, Fig. 1B).

5.1.3 Increase of the Ion Current across the Membrane

In CHO-hOX_1-C1 cells, combined whole-cell voltage clamp and Ca^{2+} imaging recordings were undertaken in order to dissect Ox-A activated membrane current. A large proportion (about 45 %) of the patched cells responded to Ox-A stimulation with an increase in inward current and Ca^{2+} elevation (I, Fig. 4A), when the intracellular Ca^{2+} was buffered to a resting level similar to that in the intact cells.

5.1.4 Activation of Protein Kinases

Previously orexins have been demonstrated to activate several types of protein kinases (Kukkonen et al., 2002; Milasta et al., 2005; Ammoun et al., 2006a; 2006b; Ekholm et al., 2007; Tang et al., 2008; Goncz et al., 2008; Ramanjaneya et al., 2009). In order to identify Ox-A activated protein kinases in HEK293OX_1R cells, it was decided to treat near-confluent cells with 5 nM Ox-A and lyse them at different time points in RIPA buffer. Total protein lysates were tested by western blotting being achieved with antibodies against phosphorylated forms of various protein kinases. It was found that the levels of phosphorylated forms of ERK1/2, PKCδ and PKD1 were increased in a time-dependent manner in Ox-A treated samples compared to untreated controls (IV, Fig. 1A and B). This

study was the first one to identify PKD1 as a downstream target of OX_1R. This finding led us to explore if other types of PKDs were expressed and activated by Ox-A in this cell model. Expression of all three subtypes of PKDs in HEK293OX_1R cells was confirmed by reverse transcriptase PCR (RT-PCR) with sub-type specific primers (confirmed by sequencing). There is no subtype specific antibody for PKD3 but phosphorylation of all three PKD subtypes can be detected using an antibody against phosphorylated serine residue 744/748 in the active loop (Rykx et al., 2003). To test if PKD3 was specifically activated by Ox-A, HEK293OX_1R cells were stably transfected with an EGFP-tagged PKD3 construct (Rey et al., 2006a). Immunoprecipitation with anti-GFP antibody of control and Ox-A treated cells and subsequent western blot detection with anti-active PKD(744/748) revealed increased phosphorylation of PKD3 after stimulating HEK293OX_1R cells with 1 nM Ox-A for 5 min (IV, Fig. 4A).

5.2 CHARACTERIZATION OF OREXIN-A INDUCED ELEVATION OF INTRACELLULAR CALCIUM CONCENTRATION AND MEMBRANE CURRENT

5.2.1 Dependence on Extracellular Calcium

In order to distinguish if Ca^{2+} was derived from the intracellular stores or from the extracellular space, the dependence of Ox-A induced Ca^{2+} responses on $[Ca^{2+}]_o$ was tested. In the absence of extracellular Ca^{2+}, Ca^{2+} influx from the extracellular space is prevented and only the release of Ca^{2+} from the intracellular stores can be seen. For example when CHO-hOX_1-C1 cells were treated with 100 nM thapsigargin, an inhibitor of ER Ca^{2+}-ATPase causing discharge of the intracellular Ca^{2+} stores (Thastrup et al., 1990), only a peak response was observed (I, Fig. 1C). In the presence of extracellular Ca^{2+}, this was followed by a delayed stable phase corresponding to CCE activated by store depletion (I, Fig. 1C). In all tested cell lines, the Ca^{2+} response to 3 nM or higher concentration of Ox-A had the same characteristics as that obtained with thapsigargin. In the presence of extracellular Ca^{2+}, a two-phase response was observed while in the absence of extracellular Ca^{2+}, only the peak response remained (see for example I, Fig. 1B, 2D and 2E, II, Fig. 1E and III, Fig. 1B, gray trace). However, Ca^{2+} responses induced by lower ($\leq$ 3 nM) concentrations of Ox-A were acutely dependent on $[Ca^{2+}]_o$. No response was detected when the cells were stimulated in the absence of extracellular Ca^{2+} (see for example II, Fig. 1D and F and III, Fig. 2B). In IMR32 cells transiently expressing OX_1R, the dependence on extracellular Ca^{2+} was also evident if extracellular Ca^{2+} was removed during the stable elevation of $[Ca^{2+}]_i$. Under these conditions, the response was readily and reversibly blocked (II, Fig. 2b). In addition, the Ca^{2+} oscillations induced by 1 nM Ox-A in HEK293OX_1R cells were reversibly blocked by removal of Ca^{2+} from the perfusion solution (III, Fig. 1A). Figure 8 summarizes the effect of $[Ca^{2+}]_o$ on Ca^{2+} responses induced by Ox-A.

Similarly as the Ca^{2+} response, also the membrane current evoked by 0.3 nM Ox-A was dependent on extracellular Ca^{2+} (I, Fig. 4A). Interestingly, the concentration-response relations of Ox-A induced membrane current and Ca^{2+} elevation differed significantly. The measured membrane current increased steeply from 0.1 to 0.3 nM Ox-A, after which no further increase in the magnitude of current could be evoked, even if the Ca^{2+} elevation continued to rise with increasing Ox-A concentrations (I, Fig. 4B).

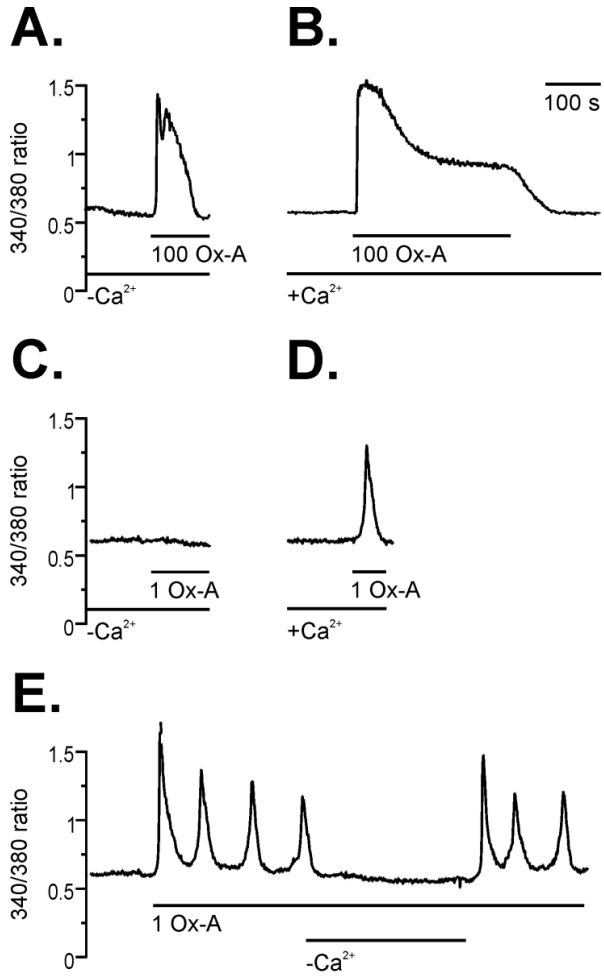

Figure 8. Representative single cell calcium (Ca^{2+}) imaging recordings showing the effect of extracellular Ca^{2+} on Ca^{2+} responses elicited by high (100 nM) and low (1 nM) concentrations of orexin-A (Ox-A). Periods of stimulation are indicated by horizontal bars. When a cell expressing orexin-1 receptor is stimulated by 100 nM Ox-A in the absence of extracellular Ca^{2+}, only a transient elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is observed (A). In the presence of extracellular Ca^{2+}, 100 nM Ox-A induced a biphasic Ca^{2+} response (B). No response is detected when 1 nM Ox-A is used in the absence of extracellular Ca^{2+} (C), but in the presence of extracellular Ca^{2+}, a short pulse of 1 nM Ox-A induces transient elevation of $[Ca^{2+}]_i$ (D) and a longer stimulation results in a repetitive spike pattern known as Ca^{2+} oscillations (E). The oscillations cease if Ca^{2+} is removed from the perfusion solution.

5.2.2 Sensitivity to Pharmacological Methods to Block Calcium Entry Pathways

To confirm the necessity of Ca^{2+} entry from the extracellular space for the Ca^{2+} responses induced by low concentrations of Ox-A, a panel of pharmacological inhibitors known to block Ca^{2+} entry pathways were examined. The effects seen with the inhibitors were not due to changes in resting membrane potential as these compounds caused no or only marginal changes in our current clamp recordings in CHO-hOX$_1$-C1 cells. Next the effects

of individual test reagents are described. The main findings are then summarized in Table 7.

Magnesium (Mg^{2+})

Mg^{2+} (5 mM), a nonselective Ca^{2+} channel blocker, dramatically reduced (by about 70 %) the Ca^{2+} elevation (I, Fig. 1A and 1E) and membrane current (I, Fig. 4C) induced by 0.3 nM Ox-A in CHO-hOX_1-C1 cells. When IMR32 cells transiently expressing OX_1R were stimulated with 1 nM Ox-A in the presence of 10 mM Mg^{2+}, only a small and transient Ca^{2+} response was observed instead of a prolonged stable elevation of $[Ca^{2+}]_i$ (II, Fig. 2b). Furthermore, 10 mM Mg^{2+} reversibly blocked the sustained Ca^{2+} elevation when applied during the stimulation with 1 nM Ox-A (II, Fig. 2B and 2C). The same inhibitory effect of a high concentration of Mg^{2+} was seen also in HEK293OX_1R cells: 10 mM Mg^{2+} reversibly blocked the Ca^{2+} oscillations induced by 1 nM Ox-A, decreasing the oscillation frequency by 87 % (III, Fig. 3A and 3E). Mg^{2+} treatment did not have any visible effect on the Ca^{2+} responses (peak responses or Ca^{2+} oscillations seen in the absence of extracellular Ca^{2+}) induced by higher concentrations of Ox-A (≥ 3 nM) or by thapsigargin in any of the tested cell lines (I, Fig. 1C and 1E, II, Fig. 4A, III, Fig. 3B and 3E).

Tetraethylammonium (TEA)

In CHO-hOX_1-C1 cells, 70 mM TEA, a nonspecific potassium channel blocker, had an effect which was very similar to that seen with Mg^{2+}, clearly inhibiting the Ca^{2+} response induced by a low concentration of Ox-A, but having no significant effect on responses at higher concentrations of Ox-A (I, Fig. 1E). In patch clamp recordings, 70 mM TEA totally and reversibly blocked the membrane current induced by 0.3 nM Ox-A while not interfering with the basal current (I, Fig. 4F).

Dextromethorphan (Dex)

Dex was originally identified as a σ-opiate receptor ligand but was subsequently shown to reversibly block NMDA receptor channels and VOCs (Albers et al., 1989; Church and Fletcher, 1995; Shariatmadari et al., 2001). In CHO-hOX_1-C1 cells, it had a comparable inhibitory profile as Mg^{2+} and TEA. A concentration of 100 μM Dex totally inhibited Ca^{2+} elevation (I, Fig. 1A and 1E) and the membrane current (I, Fig. 4E) induced by a low concentration of Ox-A, but had no effect on peak response of higher Ox-A concentrations or thapsigargin (I, Fig. 1B, 1C and 1E). However, stable phases occurring after peak responses and the corresponding CCE activated by store release were partially inhibited by Dex (I, Fig. 1B, 1C and 1E). Similar effects of Dex were seen in IMR32 transiently expressing OX_1R. Dex almost completely blocked the sustained response to 1 nM Ox-A in IMR-32 cells (II, Fig. 2B and 2C), but had only a small inhibitory effect on the sustained elevation of $[Ca^{2+}]_i$ induced by thapsigargin (II, Fig. 4A and 4B).

SKF-96365 (SKF)

In CHO-hOX_1-C1 cells, 10 μM SKF, a known inhibitor of CCE (Merritt et al., 1990), had only a minor inhibitory effect on the Ca^{2+} elevation at a low concentration of Ox-A, but partially inhibited the response induced by higher concentration of Ox-A (I, Fig. 1E). When applied during the stable phase of the thapsigargin induced Ca^{2+} response, SKF achieved total inhibition (I, Fig. 1E).

2-Aminoethoxydiphenyl borate (2-APB)

In CHO-hOX$_1$-C1 cells, 10 μM 2-APB, an antagonist of IP$_3$Rs and an inhibitor of CCE (Wilcox et al., 1998), was able to reverse the response to thapsigargin when added during the stable phase (I, Fig. 1B, 2-APB). It had no effect on the Ca^{2+} oscillations induced by 1 nM Ox-A in HEK293OX$_1$R cells (III, Fig.3C and 3E) but blocked the oscillations induced by 100 nM Ox-A in the absence of extracellular Ca^{2+}, decreasing the oscillation frequency by 93 % (III, Fig. 3D and 3E).

Table 7. Effect of various inhibitors on different aspects of Ca^{2+} responses: peak responses (peak), stable phases (stable), Ca^{2+} oscillations (osc) and membrane current (current) induced by different Ox-A concentrations or by thapsigargin (thaps). The number of minus signs indicates the magnitude of the inhibitory effect (- low level of inhibition, -- intermediate inhibition and --- very strong inhibition). NE indicates no or only marginal effect.

Ox-A	Mg^{2+}	Dex	TEA	SKF	2-APB	Ni^{2+}
0.3 nM / 1 nM	--	---	--	-		
1 nM (osc)	---				NE	
0.3 nM (current)	-	---	---		NE	---
10 nM (peak)	NE	NE	NE	-		
10 nM (stable)	NE	--	NE	--		
100 nM (osc)	NE				---	
Thaps (peak)	NE	NE		-		
Thaps (stable)	-	-		---	---	

Abbreviations used: 2-APB = 2-aminoethoxydiphenyl borate, Dex = dextromethorphan, Mg^{2+} = magnesium ion, Ni^{2+} = nickel ion, SKF = SKF-96365, TEA = tetraethylammonium.

5.2.3 Dependence on Voltage

The present patch clamp recordings in CHO-hOX$_1$-C1 cells indicate that in addition to Ox-A induced membrane depolarization, also inward current and Ca^{2+} response are voltage-dependent. Using a voltage protocol introducing voltage ramps (-80 to +80 mV; 320 ms) every 5 or 7.5 s, an approximately linear increase in the inward current with an increasing negative intracellular polarity was recorded (I, Fig. 5A and 5B). The Ca^{2+} response to 0.3 nM Ox-A was clearly reduced by substitution of extracellular Na^+ with K^+, which caused a considerable depolarization of the cells, while the same treatment had little effect on the peak elevation at 3 nM Ox-A (I, Fig. 5F and 5G).

5.3 SIGNALLING PATHWAYS PARTICIPATING IN THE REGULATION OF THE OREXIN-A INDUCED ELEVATION OF THE INTRACELLULAR CALCIUM CONCENTRATION

5.3.1 Protein Kinase C

DAGs are produced following activation of various GPCRs by actions of PLC (reviewed in Rebecchi and Pentyala, 2000) or other phospholipases (Hodgkin et al., 1998; Wakelam, 1998) with the main target of these second messengers being PKC. The effect of PKC stimulation and inhibition was evaluated on Ca^{2+} responses induced by low (0.3 nM) and high (10 nM) concentrations of Ox-A and 100 nM thapsigargin in cell suspensions of CHO-hOX$_1$-C1. Different approaches used to activate PKC (30 μM dioctanoyl glycerol (DOG), 100 nM 12-O-tetradecanoylphorbol-13-acetate (TPA), inhibition of DAG kinase by

30 μM R59022) considerably lowered Ca^{2+} response induced by 0.3 nM Ox-A (I, Fig. 2A, 2B, 3A, and 3C). DOG was without any effect on the Ca^{2+} responses induced by 10 nM Ox-A and 100 nM thapsigargin (both peak and stable phases) and consistently TPA did not significantly alter the response induced by 10 nM Ox-A (I, Fig. 2C, 2D, 2E, 3B, and 3C). PKC inhibition by 10 μM GF109203X (GF-X) evoked a small increase in the response to 0.3 nM Ox-A and almost completely reversed the inhibitory effects of DOG and R59022 (I, Fig. 3A). It did not prevent store release and the CCE caused by thapsigargin treatment (I, Fig. 3B). Very similar results were obtained in single cell Ca^{2+} imaging experiments of IMR32 cells. A concentration of 30 μM DOG considerably decreased the elevation of $[Ca^{2+}]_i$ induced by a low concentration of Ox-A and the response was restored or even enhanced by 10 μM GF-X (II, Fig. 7A and 7B). When 100 nM TPA was applied during the the stable phase of Ca^{2+} response to 1 nM Ox-A, the level of $[Ca^{2+}]_i$ was clearly lowered. This inhibition was partially reversed by GF-X treatment (II, Fig. 7C and 7D). The effect of GF-X was also tested in HEK293OX_1R cells and it was found that the Ca^{2+} response to low concentration of Ox-A remained unaffected while GF-X decreased the responses to 10 nM and 100 nM Ox-A by over 40 % (IV, Fig. 3B). The main findings with PKC activators and inhibitor are also summarized in Table 8.

Table 8. Effect of various activators and inhibitors of PKC on Ca^{2+} responses induced by different Ox-A concentrations or by thapsigargin (thaps). The number of minus signs indicates the magnitude of the inhibitory effect (- low level of inhibition, -- intermediate inhibition and --- very strong inhibition). + sign indicates enhancement of the response and NE no or only marginal effect.

	0.3 nM / 1 nM	**10 nM (peak)**	**10 nM (stable)**	**Thaps (peak)**	**Thaps (stable)**
DOG	**---**	NE	NE	NE	NE
TPA	**---**	**-**	**-**		
DAGKI	**--**				
GF-X	**+**	**-**		NE	NE
DOG + GF-X	NE / **+**				
TPA + GF-X	**-**				
DAGKI + GF-X	NE				

Abbreviations used: DAGKI = diacylglycerol kinase inhibitor R59022, DOG = dioctanoyl glycerol, GF-X = GF109203X, TPA = 12-*O*-tetradecanoylphorbol-13-acetate.

5.3.2 Phospholipase A_2

OX_1R is believed to activate multiple phospholipases (Johansson et al., 2008). It was decided to investigate the potential role of PLA_2 in OX_1R signalling in HEK293OX_1R cells. Already a low concentration (1 nM) of Ox-A released a significant amount of AA, the main product of the activity of PLA_2 (III, Fig. 2A). Methylarachidonyl fluorophosphonate (MAFP), an inhibitor of PLA_2 (Lucas and Dennis, 2005), had a striking effect on Ox-A induced Ca^{2+} oscillations, decreasing the oscillation frequency by 76 % (III, Fig. 2B and 2E). Subsequent addition of AA rescued the Ca^{2+} elevation, but failed to rescue the oscillation pattern (III, Fig. 2C). The oscillatory responses induced by 100 nM Ox-A in the absence of extracellular Ca^{2+} were largely unaffected by MAFP (III, Fig. 2D).

5.3.3 Protein Kinase D1 and D3

The recent identification of activation of PKD1 and PKD3 by OX_1R stimulation was a reason to explore their possible roles in the regulation of Ox-A induced Ca^{2+} responses. First the responses of PKD1 and Ca^{2+} were compared. Ox-A induced phosphorylation of PKD1 at S916 and elevation of $[Ca^{2+}]_i$ in HEK293OX_1R cells were both very early responses detected at around 1 min after stimulation. A low concentration of Ox-A was sufficient to elicit these responses (IV, Fig.2A and 2B). The concentration-response relations of PKD1 S916 phosphorylation and $[Ca^{2+}]_i$ elevation were superimposable (IV, Fig. 2C). Secondly, cell fractionation experiments and fluorescence microscopy pictures demonstrated a clear Ox-A induced translocation of both PKD1 and PKD3 on the plasma membrane indicative of an active role for these kinases in this cell compartment (IV, Fig. 4B and 4C). Finally, in order to more directly test the effects of PKDs on Ox-A induced Ca^{2+} responses, HEK293OX_1R cells were transfected with EGFP-tagged kinase-dead PKD1 (PKD1kd) (Hausser et al., 2002) and EGFP-tagged kinase-dead PKD3 (PKD3kd) (Rey et al., 2006a) and single cell Ca^{2+} imaging experiments were conducted. Neither construct had any significant effect on peak amplitudes of Ca^{2+} responses induced by Ox-A (comparison of fluorescent cells expressing kinase-dead construct with non-fluorescent control cells on the same plate). However, the recorded oscillation patterns were considerably altered. Over-expression of PKD1kd increased the frequency of the oscillations by approximately 1 peak per every 10 min of stimulation (6.2 ± 0.4 mHz in PKD1kd expressing fluorescent cells (n = 62) versus 4.9 ± 0.3 mHz in control cells (n = 42), IV, Fig. 5A). The observed increase was statistically significant (IV, Fig. 5B). On the other hand, over-expression of PKD3kd in HEK293OX_1R cells completely disrupted the oscillation pattern (IV, Fig. 6A) with only a sustained $[Ca^{2+}]_i$ elevation remaining. The number of transiently oscillating cells was significantly decreased in this cell group compared to corresponding non-fluorescent control group (IV, Fig. 6B).

5.4 IDENTIFICATION OF THE CHANNEL ISOFORMS INVOLVED IN THE CALCIUM INFLUX INDUCED BY A LOW CONCENTRATION OF OREXIN-A

5.4.1 Voltage-gated Calcium Channels

Orexins have been reported to depolarize cells via several different mechanisms (reviewed in Kukkonen et al., 2002; Ferguson and Samson, 2003). Thus it seemed profitable to investigate if Ox-A induced $[Ca^{2+}]_i$ elevation was mediated via depolarization-activated VOCs in IMR32 cells. A high concentration of Mg^{2+}, which is able to block Ox-A induced Ca^{2+} response (II, Fig. 2B-E), did not have any effect on depolarization-induced Ca^{2+} influx (II, Fig. 3A). Differentiation of IMR-32 cells leads to an upregulation of mainly N-type VOCs, which can be blocked with ω–conotoxin (Carbone et al., 1990). Accordingly, the depolarization-induced Ca^{2+} influx was largely abolished by 0.5 μM ω–conotoxin (II, Fig. 3A), but the same treatment had no effect on the Ox-A induced Ca^{2+} response (II, Fig. 3C and 3D). Furthermore, the OX_1R response was still present and not apparently different from control in ω–conotoxin-treated cells that also receivedexposure to a depolarizing buffer before and during Ox-A application (II, Fig. 3C).

5.4.2 Na^+/Ca^{2+} Exchanger

The electrogenic Na^+/Ca^{2+} exchanger has been implicated in OxR-mediated neuronal excitation (Eriksson et al., 2001; Burdakov et al., 2003; Wu et al., 2004) and thus its

possible involvement in Ox-A induced Ca^{2+} influx was explored. KB-R7943 (KB-R), a potent blocker of the reverse operation mode of Na^+/Ca^{2+} exchange (Iwamoto et al., 1996), did not significantly alter the response to Ox-A in IMR-32 cells when averages of cells were plotted and compared (II, Fig. 4C and 4D). However, when analyzed at the single-cell level, the cells could be divided into three populations responding differentially: 1) about 10 % of the cells showed a decrease in $[Ca^{2+}]_i$ when KB-R was applied during the stable phase of Ox-A induced Ca^{2+} response, 2) the response in about 75 % of the cells did not alter and 3) about 15 % of the cells showed an increase in $[Ca^{2+}]_i$ in response to KB-R.

5.4.3 Canonical Transient Receptor Potential Channels

DAGs have been shown to activate some subtypes of TRP channels independently of receptor activation (Hofmann et al., 1999; Tesfai et al., 2001; Jung et al., 2002; Gamberucci et al., 2002). Evidence for DAG-activated Ca^{2+} entry in CHO-hOX_1-C1 cells and in differentiated IMR32 cells was found. DOG (30 μM), an analog of DAGs, caused an increase in $[Ca^{2+}]_i$ in both cell lines (CHO-hOX_1-C1: data not shown, IMR32: II, Fig. 6A and 6D). Further characterization of DOG-induced $[Ca^{2+}]_i$ elevation was performed in IMR32 cells. In a similar manner as the Ox-A induced increase in $[Ca^{2+}]_i$, this response was sensitive to a high concentration of Mg^{2+} (II, Fig. 6B and 6D) and to Dex (II, Fig. 6C and 6D). The PKC inhibitor (GF-X) significantly enhanced the response to DOG (II, Fig. 6D). Stimulation of the OX_1R during DOG application caused only a small, additional elevation of $[Ca^{2+}]_i$ (II, Fig. 7A and 7B) and, in a significant number of cells, no additional elevation at all.

The possible role of DAG-activated TRPC channels in Ox-A induced Ca^{2+} influx naturally is dependent on the channels being expressed in the cells. Thus the presence of the mRNA for different channel subtypes was investigated in CHO-hOX_1-C1 and differentiated IMR32 cells. The RT-PCR experiments confirmed the expression of mRNA for trpc1, trpc2, trpc3 and trpc4 in CHO-hOX_1-C1 cells (I, Fig. 6) and for all human trpc-type channels in differentiated IMR32 cells (II, Fig. 5). In addition, the third cell line used in this study, HEK293 cells, has been previously shown to express mRNA for several trpc subtypes (trpc1, trpc3, trpc4 and trpc6) (Garcia and Schilling, 1997; Wu et al., 2000).

In order to more directly test the involvement of TRPC channels in Ox-A induced Ca^{2+} responses, EGFP/EYFP-tagged truncated TRPC channel subtypes were designed having dominant negative effects on channel activity. These constructs were transfected into the cells and single cell Ca^{2+} imaging experiments were conducted. Transfected cells expressing truncated TRPC constructs were easy to identify based on their fluorescence and thus the Ox-A induced Ca^{2+} responses of fluorescent cells and non-fluorescent control cells from the same plates were compared. In CHO-hOX1-C1 cells, expression of trpc1N or trpc3N significantly attenuated Ca^{2+} responses induced by 0.3 nM Ox-A (I, Fig. 7B) while responses to a higher (3 nM) concentration of Ox-A remained unaffected (I, Fig.7A and 7B). After normalization of the responses against that observed with 100 mM BzATP (see chapter 4.5.4 Single Cell Ca^{2+} Imaging Experiments for further information), significant inhibitory effects of trpc1N and trpc3N, minor effects of trpc2N and trpc4N and no effects of EGFP and trpc7N were noted on the Ca^{2+} responses induced by 0.3 nM Ox-A (I, Fig. 6B). Trpc1N and trpc3N also reduced the number of responding cells by 21 % and 11 %, respectively. The effect of trpc1N and trpc3N was not due to their possible effect on membrane potential because transfected cells had similar resting potentials as their nontransfected counterparts.

In differentiated IMR32 cells, trpc3N had only a minor inhibitory effect on the Ca^{2+} response to 1 nM Ox-A. This was considered to be attributable to too low expression level or the inability of this truncated construct to interact sufficiently well with the endogenous related channel subunits. Therefore, a full-length, triple-mutated trpc6 dominant-negative construct ($trpc6^{DN}$) (Hofmann et al., 2002) was tested and this almost totally abolished the Ca^{2+} response induced by 60 μM DOG (II, Fig. 8A, 8B and 8E) and significantly inhibited the response to 1 nM Ox-A. In contrast, the peak responses to 10 nM Ox-A and to oxotremorine (an agonist of endogenous muscarinic receptors (Kukkonen et al., 1992) used to evaluate health and viability of the cells) remained unaltered (II, Fig. 8C, 8D and 8F).

In similar experiments in $HEK293OX_1R$ cells, trpc1N, trpc3N and trpc4N decreased the Ca^{2+} response to 1 nM Ox-A leaving the response to 100 nM Ox-A unaffected. The response to 100 nM Ox-A was used to normalize the responses to low concentration of Ox-A and this procedure revealed significant inhibitory effects of the channel constructs described above and no effects of $trpc6^{DN}$ and trpc7N (III, Fig. 1C). The effect of truncated channel constructs was also tested on Ca^{2+} oscillations induced by prolonged stimulation of $HEK293OX_1R$ cells by 1 nM Ox-A. The cells over-expressing trpc3N failed to produce the transient Ca^{2+} oscillations characteristically seen in the non-transfected cells (III, Fig. 1D). Statistical analysis established that expression of trpc3N significantly decreased the percentage of transiently oscillating cells as compared to the control cells (III, Fig. 1E).

In order to compare the characteristics of the primary current response to Ox-A and the TRPC3 mediated current, CHO-hOX1-C1 cells were transfected with trpc3FLAG and fluorescent cells were clamped at -80 mV. Under these conditions, TRPC3 was constitutively active. In general, inward currents were transient and were followed by a more steady current level, ranging around -250 to -600 pA (I, Fig.8A) (Zitt et al., 1997). Inhibitors, which had been previously shown to block Ox-A induced current were applied during the more steady current level. Three compounds, i.e. 70 mM TEA, 5 mM Mg^{2+} and 100 μM Dex, all blocked the TRPC3 current (I, Fig.8B). The magnitude of the block by TEA was on average found to be approximately 86 % with respect to zero current. Dex reduced the TRPC3 current by approximately 71 % and Mg^{2+} by approximately 50 %.

6 Discussion

In line with numerous previous studies, it was confirmed that membrane depolarization, elevation of $[Ca^{2+}]_i$, an increase in the ion current across the plasma membrane, and activation of several protein kinases were the cellular responses induced by stimulation of OX_1R by Ox-A. It was also possible to identify PKD1 and PKD3 as novel targets of OX_1R mediated activation. The present study intended to characterize in detail the Ca^{2+} signalling pathways utilized by the receptor. Previous studies have implicated two separate pathways as being involved in the elevation of $[Ca^{2+}]_i$ induced by Ox-A. Activation of PLCβ–pathway leading to release of Ca^{2+} from the intracellular Ca^{2+} stores at high nanomolar concentrations of Ox-A is firmly established by previous studies (Smart et al., 1999; Kukkonen and Akerman, 2001; Muroya et al., 2004; Ekholm et al., 2007; Johansson et al., 2007; 2008) and confirmed by these present investigations. Thus it was decided to concentrate on studying the Ca^{2+} responses induced by lower and probably physiologically more relevant concentrations of Ox-A, which are believed to be generated by receptor-operated Ca^{2+} influx from the extracellular space without detectable discharge of Ca^{2+} stores (Lund et al., 2000; Ammoun et al., 2003; Magga et al., 2006; Ekholm et al., 2007).

In addition to initial peak response in $[Ca^{2+}]_i$, Ca^{2+} oscillations were observed in two separate conditions: when HEK293OX_1R cells were stimulated by low nanomolar concentration of Ox-A for a prolonged period of time and when OX_1R was activated by higher concentration of Ox-A in the absence of extracellular Ca^{2+}. Ca^{2+} oscillations are recognized to be an important phenomenon in almost all cell types (Tsien and Tsien, 1990; Berridge, 1995; Parkash and Asotra, 2010). Different cellular processes such as the activation of protein kinases and transcription factors are regulated by Ca^{2+} oscillations at defined frequencies in the range between 1 and 100 mHz (reviewed in Boulware and Marchant, 2008). The frequency of Ox-A induced Ca^{2+} oscillations was about 7 mHz, which is known to activate protein kinases like ERK1/2 and PKC. Interestingly Ox-A activates these kinase pathways in non-excitable cells and in neurons in a Ca^{2+} dependent manner (Ammoun et al., 2006a).

6.1 LOW CONCENTRATION OF OREXIN-A ACTIVATES CALCIUM INFLUX PATHWAY DISTINCT FROM THE CAPACITATIVE CALCIUM ENTRY

Multiple lines of evidence presented in this study are in accordance with the current model of Ca^{2+} signalling of OX_1R. The Ca^{2+} responses (both transient and repetitive) at low concentrations of Ox-A were acutely dependent on $[Ca^{2+}]_o$ while higher Ox-A concentrations induced elevation of $[Ca^{2+}]_i$ also in the absence of extracellular Ca^{2+}. In CHO-hOX1-C1 cells, the Ox-A-activated current and membrane depolarization showed a steep dependence on a negative membrane potential. Similarly, the Ca^{2+} responses induced by 0.3 nM Ox-A were voltage-dependent while the responses to higher concentrations of Ox-A were unaffected by depolarization with high K^+. Transiently transduced IMR32 cells responded to 1 nM Ox-A with a robust elevation of $[Ca^{2+}]_i$ in the absence of measurable Ca^{2+} release from intracellular stores. All these results indicate that

the Ca^{2+} influx from the extracellular space is the primary response to low concentration of Ox-A instead of Ca^{2+} release from the intracellular stores.

As a first step to characterize this Ca^{2+} entry pathway, it was decided to explore if it was mediated via the same or different molecular entity as the CCE. It is difficult to conduct a pharmacological differentiation of non-store-operated channels from capacitative mechanisms due to the lack of specific blockers or other specific means to distinguish the pathways. Thus a panel of channel inhibitors was screened on the responses to Ox-A and thapsigargin, in order to identify compounds that would show a preference for blocking a putative non-capacitative Ca^{2+} entry over the typical CCE. From the results (see Table 7 in Results section), it is evident that the pharmacological profiles of the Ca^{2+} signalling pathways activated by low (0.3 nM or 1 nM) and high (10 nM or 100 nM) concentrations of Ox-A are distinct, suggesting that there are two separate molecular entities in the two cases. The first pathway is inhibited by known channel blockers Mg^{2+}, Dex, TEA and Ni^{2+} while the latter is mostly sensitive to inhibitors of CCE, SKF and 2-APB. Similar effects of Ni^{2+} and 2-APB on Ox-A induced Ca^{2+} responses have already been reported (Kukkonen and Akerman, 2001; Holmqvist et al., 2002). It should also be noted that the results with high concentrations of Ox-A and with thapsigargin, which is known to induce discharge of intracellular Ca^{2+} stores and the subsequent CCE via inhibition of ER Ca^{2+}-ATPase, are very similar providing further support for the concept of activation of PLCβ-pathway by high concentrations of Ox-A. These results are in line with the previous ones showing that SOCs are inhibited by SKF (Merritt et al., 1990) and by 2-APB (Wilcox et al., 1998; Kukkonen and Akerman, 2001), but not by Mg^{2+} (Voets et al., 2001).

Interestingly, also the Ca^{2+} oscillations in HEK293OX_1R cells shared the similar characteristics with the initial peak response observed in other types of cells. The Ca^{2+} influx pathway participating in the generation and maintenance of oscillatory response pattern has been often postulated to involve CCE via SOCs (Bootman et al., 1996; Sergeeva et al., 2000; Bird and Putney et al., 2005). However, these results with 1 nM Ox-A appear to indicate the involvement of some non-capacitative Ca^{2+} entry channel which is sensitive to elevated Mg^{2+} concentrations and is unaffected by 2-APB. At higher concentration of Ox-A, Ca^{2+} oscillations are only seen in the absence of extracellular Ca^{2+} ruling out the possible role of any channel type. The response pattern is most probably generated by repetitive release and reuptake of Ca^{2+} from the intracellular stores (reviewed in Tsien and Tsien, 1990; Taylor and Thorn, 2001; Berridge, 2005). In the present experiments, 2-APB was able to inhibit this process and to totally abolish the responses.

6.2 PROTEIN KINASE C, PHOSPHOLIPASE A_2, PROTEIN KINASE D1, AND PROTEIN KINASE D3 PARTICIPATE IN THE REGULATION OF OREXIN-A INDUCED ELEVATION OF INTRACELLULAR CALCIUM CONCENTRATION

6.2.1 Protein Kinase C

Next it was intended to identify specific signalling molecules participating in the regulation of Ca^{2+} entry induced by low concentration of Ox-A in order to obtain more clues about which of the possible channels are involved. PKC has been shown to tightly associate to Ca^{2+} signalling of the cells and to regulate the function of multiple ion channels on the plasma membrane (Tornquist, 1993; Miyakawa et al., 1998; van Rossum

and Patterson, 2009). The effects of activation and inhibition of PKC subtypes were explored on Ca^{2+} responses induced by different concentrations of Ox-A or by thapsigargin. It is evident from these results (see Table 8 in Results section) that the Ca^{2+} signalling pathways activated by low and high concentrations of Ox-A are regulated differentially. Thus, these results are in agreement with the findings presented above and provide still further support to the separate signalling pathways activated depending on the concentration of the stimulating agonist. The elevation of $[Ca^{2+}]_i$ induced by low nanomolar concentrations of Ox-A could be blocked in several ways which activate PKC (DOG, TPA and DAGKI) and enhanced by PKC inhibitor GF-X. Furthermore, GF-X was able to reverse the responses inhibited by DOG and DAGKI. It seems that PKC plays a pivotal role in the regulation of the Ca^{2+} influx pathway activated by Ox-A. One attractive explanation for the inhibitory effect of PKC is that PKC functions as a negative feedback to regulate Ca^{2+} influx in order to prevent massive elevation of $[Ca^{2+}]_i$ which might be harmful for the cell (Venkatachalam et al., 2003; Trebak et al., 2003). PKC has been already earlier shown to inhibit Ca^{2+} entry in response to receptor stimulation (Tornquist, 1993; Miyakawa et al., 1998). Several studies have identified PKC as a crucial intermediate effector of signalling pathways activated by orexins, often via a downstream effect linked to specific ion channels (Uramura et al., 2001; Xu et al., 2002: Kohlmeier et al., 2004) or less well defined ion conductances (Yang et al., 2003). In addition, a contradictory result has been reported. In hypothalamic neurons, van den Pol et al. (1998) observed a total block of the Ox-A induced $[Ca^{2+}]_i$ increase after inhibition of PKC.

Ca^{2+} responses induced by higher concentration of Ox-A or by thapsigargin were largely unaffected by PKC activators and/or inhibitors. Furthermore, the Ca^{2+} signalling pathways of high concentrations of Ox-A and thapsigargin displayed similar characteristics. Previously it has been shown that the capacitative pathway (the stable phase of the Ca^{2+} response) is unaffected by PKC activation (Venkatachalam et al., 2003). However PKC might have a role in regulating the events preceding activation of CCE, i.e. the release of Ca^{2+} from the intracellular stores (van Rossum and Patterson, 2009). The inhibitory effect of GF-X on Ca^{2+} responses induced by 10 and 100 nM Ox-A in HEK293OX_1R points to that direction. OX_1R has multiple consensus sites for PKC phosphorylation (Kukkonen et al., 2002) and one could argue that the effect of PKC is a direct phosphorylation of the receptor. However these results are contradictory to this hypothesis since the effects of PKC differs depending on the concentration of the stimulating agonist.

6.2.2 Phospholipase A_2

In addition to SOCs (Bootman et al., 1996; Sergeeva et al., 2000; Bird and Putney, 2005), a novel non-capacitative Ca^{2+} entry activated by AA (Akagi et al., 1997; Wu X. et al., 2002; Shuttleworth and Thompson, 1999) has been proposed to be responsible for the Ca^{2+} oscillations induced by GPCR stimulation. Thus it was decided to explore if AA participates in generation of oscillatory Ca^{2+} responses in HEK293OX_1R cells stimulated by 1 nM Ox-A. This concentration of agonist was able to induce a considerable release of AA in the cells and increasing the dose further elevated the release. Inhibition of PLA_2, the main source of AA in the cells (reviewed in Diaz and Arm, 2003; Hirabayashi et al., 2004), by MAFP (Lucas and Dennis, 2005) potently inhibited the Ca^{2+} oscillations induced by 1 nM Ox-A, but left oscillations at higher Ox-A concentration mostly untouched. AA was able to rescue the Ca^{2+} response inhibited by MAFP. These results indicate that PLA_2 participates in the regulation of Ca^{2+} influx induced by low concentrations of Ox-A while

at higher concentrations PLC dominates. Although the involvement of AA-activated influx pathway in the oscillatory response of OX_1R has been demonstrated, the involvement of an ARC as postulated by Shuttleworth et al. (2004) cannot be confirmed as other types of channels with distinct characteristics are also activated by AA (for review see for example van Rossum and Patterson, 2009).

6.2.3 Protein Kinase D1 and D3

It was found that ERK1/2, PKCδ and PKD1 were rapidly phosphorylated after treatment of HEK293OX_1R cells by Ox-A. The phosphorylation of ERK1/2 with the same characteristics as observed here have been reported previously by several research groups (Kukkonen et al., 2002; Milasta et al., 2005; Ammoun et al., 2006a; Ramanjaneya et al., 2009). As described above, PKC has been postulated to be of crucial importance in Ca^{2+} signalling of OX_1R. However, the specific PKC subtypes have not been identified and there is also a possibility that the effects seen with various inhibitors and activators are indirect and mediated via some downstream targets of PKC. The activation of PKCδ by Ox-A has been proposed earlier by Holmqvist et al. (2005) based on their pharmacological data and this present investigation confirmed the specific phosphorylation of this PKC subtype by Ox-A treatment. The phosphorylation of PKD1 by Ox-A was a novel finding and lead to the exploration of how this protein kinase and other members of the same kinase family participate in OX_1R signalling.

All known members of PKD family, PKD1, 2 and 3, were expressed in HEK293OX_1R cells and it was possible to demonstrate activation of PKD3 in the same conditions and at the same time as PKD1. Once activated, PKDs are usually translocated to specific cellular compartments: membrane (Matthews et al., 2000; Oancea et al., 2003), nuclei (Rey et al., 2001; Chen et al., 2008), mitochondria (Storz et al., 2005; Cowell et al., 2009), and golgi (Baron and Malhotra, 2002), where they can control downstream effectors. An increase of both PKD1 and PKD3 was observed in the membrane fraction as a result of OX_1R stimulation by Ox-A. This implies that PKDs might have a role in signalling events taking place in the membrane. One intriguing possibility is that PKDs participate in the regulation of Ca^{2+} influx, since PKD1 has been previously shown to control a number of ion channels present in the membrane via various mechanisms (Wang et al., 2004; Ase et al., 2005; McEneaney et al., 2008; Wen and Evans, 2009). So far, no comparable role for PKD3 in ion channel regulation has been identified.

In order to directly test the effect of PKDs on Ca^{2+} responses induced by Ox-A, kinase dead constructs were utilized to attenuate signalling via these kinases (Hausser et al., 2002; Rey et al., 2006a). Over-expression of PKD1kd or PKD3kd did not alter the amplitudes of Ca^{2+} responses to any concentration of Ox-A tested in the study (1 – 100 nM). However, the Ca^{2+} oscillations induced by 1 nM Ox-A were drastically changed. PKD1kd significantly increased the oscillation frequency while PKD3kd caused an apparent disruption of the oscillatory response pattern. This highlights the physiologically relevant consequence of the activation of PKD1 and PKD3 by Ox-A. One might wonder why the effects of PKDs on Ca^{2+} oscillations induced by Ox-A are so different from each other. However, this is quite conceivable in the light of recent studies emphasizing that the activities of the different PKD subtypes can be differentially regulated and targeted by the same stimulus. Each PKD subtype has a different affinity for the primary activator DAG (Chen et al., 2008), furthermore, PKD1 and 2 have an additional C-terminal auto-phosphorylation site missing from the shorter PKD3 (reviewed in Rykx et al., 2003) and finally, PKD3 lacks the C-terminal PDZ-domain,

which prevents it from binding to some of the partners of PKD1 and PKD2 (Sanchez-Ruiloba et al., 2006; Kunkel et al., 2009). The receptors are probably embedded inside large signalling complexes including many components, e.g. ion channels (Rey et al., 2006b; Woo et al., 2008) and anchoring proteins like filamin-A (Rey et al., 2005; 2006b) or Homer (Yuan et al., 2003) play a role in controlling Ca^{2+} responses induced by receptor stimulation. Assuming that the molecules participating in OX_1R signalling are similarly organized around the receptor, PKD1 and PKD3 could be anchored at different locations in the complex and thus be able to phosphorylate different proteins; this could well be part of the control mechanism responsible for the Ox-A induced oscillation pattern.

6.3 TRANSIENT RECEPTOR POTENTIAL CHANNELS ARE RESPONSIBLE FOR THE CALCIUM INFLUX INDUCED BY LOW CONCENTRATION OF OREXIN-A

The final goal was to identify the channel responsible for the Ca^{2+} influx induced by low concentration of Ox-A. The involvement of CCE is highly unlikely based on both these present results and earlier reports by other research groups. In excitable IMR32 cells, the Ca^{2+} response at low concentration of Ox-A could be a consequence of activation of VOCs or reversal of electrogenic Na^+/Ca^{2+} exchange. The present studies excluded the possible involvement of both of these Ca^{2+} entry mechanisms as the primary channels responsible for the Ca^{2+} influx. ω−conotoxin, a toxin known to block N-type VOCs (for review see Snutch, 2005), and KB-R, a blocker of the reverse mode of Na^+/Ca^{2+} exchange (Iwamoto et al., 1996), were both without any major effect. An effect on the residual non-N-type VOCs seems unlikely because Ox-A still activated Ca^{2+} entry during depolarization in the presence of ω−conotoxin. Conversely, elevated concentration of Mg^{2+}, which strongly blocked the response to Ox-A, did not affect the response to elevated K^+ concentration.

A large family of mammalian TRP channels, in particular the TRPC subfamily, is suggested to be involved in Ca^{2+} entry activated by GPCRs (for review see Minke and Cook, 2002; Clapham, 2003; 2007, Parkash and Asotra, 2010; Rowell et al., 2010). They appeared to be good candidates for the molecular entities responsible for OX_1R activated Ca^{2+} influx. Single cell reverse transcription-PCR in different brain loci has demonstrated that TRPC channels and OXRs are coexpressed in neurons (Sergeeva et al., 2003). All of our cell models expressed TRPC channels: mRNA for the trpc1-4 channel isoforms were detected in CHO-hOX1-C1 cells, transcripts of all members of the trpc subfamily were present in differentiated IMR32 cells and HEK293OX_1R cells have been previously shown to express trpc1, trpc3, trpc4 and trpc6 (Garcia and Schilling, 1997; Wu et al., 2000). The present experiments with pharmacological channel blockers revealed that Ca^{2+} influx induced by a low concentration of Ox-A was highly sensitive to Mg^{2+}, Dex and TEA but unaffected by 2-APB. Certain TRP channels are inhibited by Mg^{2+} (Hardie et al., 1997; Schaefer et al., 2000; Voets et al., 2001) and these results were strikingly similar to the effects of Mg^{2+} reported by Hardie et al. (1997) using *Drosophila* TRP and TRPL cation channels. When the TRPC3 channel was overexpressed in CHO-hOX1-C1 cells, a constitutive ion current was observed and this current displayed the same characteristics with inhibition by Mg^{2+}, Dex and TEA as Ox-A induced membrane current.

Some TRP channel subtypes are regulated by lipid products of phospholipase activity, such as DAG (Minke and Cook, 2002; Rowell et al., 2010). Johansson et al. (2008) reported that low concentrations of Ox-A led to the production of DAG although the generation of IP_3 could not be detected. Of the subtypes expressed in the tested cell models, TRPC3,

TRPC6 and TRPC7 have been shown to be activated by DAG (Hofmann et al., 1999; Tesfai et al., 2001; Jung et al., 2002). The present results showing that DOG, an analog of DAG, stimulated Ca^{2+} entry in both CHO-hOX1-C1 and differentiated IMR32 cells are evidence that these cell lines express DAG-activated Ca^{2+} channels. As data indicated from IMR32 cells, the DOG induced $[Ca^{2+}]_i$ elevation was similarly sensitive to Mg^{2+} and Dex as the Ca^{2+} response to low concentrations of Ox-A. DAG is the main activator of PKC and this kinase has been demonstrated to have an unexpected role in the regulation of TRPC channel function. TRPC3, TRPC5, TRPC6 and TRPC7 are inhibited after the activation of PKC (Venkatachalam et al., 2003; Trebak et al., 2003; 2005). Thus these present observations of the tight regulation of Ca^{2+} responses induced by low concentrations of Ox-A by PKC are in good agreement with the possible role of TRPC channels in Ox-A induced Ca^{2+} entry.

In order to more directly test the involvement of TRPC channels in Ox-A induced Ca^{2+} responses, the cells were transfected with truncated constructs of the channels exerting dominant negative effects on channel function. The responses at low concentrations of Ox-A were significantly lower in the cells overexpressing truncated channel constructs than in the control cells while the responses at higher concentrations of Ox-A remained unaffected. In CHO-hOX1-C1 and HEK293OX_1R cells, the main channel involved seemed to be TRPC3, but in both cell lines also trpc1N and in HEK293OX_1R cells trpc4N had significant effects. Ca^{2+} oscillations observed in HEK293OX_1R cells were disrupted by overexpression of trpc3N, pointing to a role of TRPC3 in the generation and maintenance of the oscillatory response pattern, especially when bearing in mind the sensitivity of the oscillations to Mg^{2+}, a known blocker of TRPC3 channel. Several studies have previously demonstrated Ca^{2+} oscillations activated by DAG and/or suggested an involvement of DAG-activated TRPC3 (or TRPC6/7) (Grimaldi et al., 2003; Shlykov and Sanborn, 2004; Bird and Putney, 2005; Wedel et al., 2007). Other members of TRPC subfamily have also been proposed to participate in the regulation of Ca^{2+} oscillations (Wu X. et al., 2002; Rey et al., 2006b). The high sensitivity of TRPC3 to changes in $[Ca^{2+}]_i$ (Zitt et al., 1997) makes it a feasible candidate for being the channel responsible for the oscillatory responses through feedback and feed-forward mechanisms. Although TRPC3 has not been reported to be activated by AA, which seems to play an important role in Ca^{2+} oscillations induced by Ox-A, there are studies pointing to the regulation of other mammalian TRPC channels and *Drosophila* TRP and TRPL channels by AA (Chyb et al., 1999; Wu X. et al., 2002; Harteneck et al., 2007). At first glance, the effects of trpc3N and PKD3kd on Ox-A induced Ca^{2+} oscillations seem to be very similar. However, a detailed analysis of TRPC3 sequence revealed no consensus sites for PKD phosphorylation. The effect of PKD3kd also differed from that of trpc3N, since no effect was observed on the magnitude of the peak $[Ca^{2+}]_i$ elevation which, on the other hand, was considerably attenuated by interference with TRPC3 function. Thus TRPC3 is unlikely to be the primary target of PKD3 phosphorylation.

In IMR32 cells, it was not possible to observe any significant effect of trpc3N overexpression on Ox-A induced Ca^{2+} responses. This may be due to a too low expression level or an inability of trpc3N to interact sufficiently well with the endogenous related channel subunits. Therefore a full-length, triple-mutated trpc6 dominant-negative construct ($trpc6^{DN}$) was tested, since this has been shown to inhibit ion fluxes through both TRPC6 and TRPC3 (Hofmann et al., 2002). There was a significant inhibition of elevation of $[Ca^{2+}]_i$ induced by low concentrations of both Ox-A and DOG. As TRPC channels are believed to form homo- and heterotetrameric channel complexes, especially

with the partners from the same subfamilies, the subfamily of DAG-activated TRPC3, TRPC6 and TRPC7 channels may well create the channel complexes involved in the OX_1R-mediated Ca^{2+} influx in IMR32 cells.

6.4 PHYSIOLOGICAL RELEVANCE OF THE RESULTS

Recombinant cell models were utilized to study exclusively OX_1R signalling in homogenous environments. Caution is necessary in extrapolating the results obtained in this study to neurons in their native environment, but the study has established a firm basis for future investigations. Similar basic signalling mechanisms, including activation of G-proteins and TRPC channel subunits, are functional in neuronal and non-neuronal cells, although in neurons, downstream pathways (e.g. different types of ion channels) may complicate the interpretation of data. Previous functional studies with recombinantly expressed orexin receptors in neuron-like cell lines (PC12 and Neuro2A) have revealed the same basic features as those described in CHO-K1 cells (Holmqvist et al., 2002). Despite explicit testing, Ca^{2+} release from the intracellular stores has not been demontrated in native neurons (van den Pol et al., 1998; Kohlmeier et al., 2004) indicating that Ca^{2+} influx from the extracellular space is the primary response to OX_1R stimulation also in neurons. The results obtained in IMR32 cells excluded the involvement of two previously postulated neuronal Ca^{2+} entry pathways, Na^+/Ca^{2+} exchange and VOCs (Kukkonen et al., 2002; Ferguson and Samson, 2003), in the OX_1R mediated Ca^{2+} influx, reinforcing the conclusions drawn from the investigations in CHO-hOX1-C1 and HEK293OX_1R cells. Usually with neurons, higher concentrations of Ox-A have been used and this complicates any comparison with the present results. However, the functions appearing at low concentrations of agonists can be hypothesized as being the primary responses of the receptor and thus more physiologically relevant (Kukkonen and Akerman, 2001). This provides extra value to the present investigations. It is also important to note that activation of the channel by Ox-A in CHO-hOX1-C1 cells was observed to depolarize the cells by about 10 mV, which implies that the same signalling mechanism may also be of significance in excitatory cells.

Experiments in native systems are complicated by several aspects. Analysis of responses has proven difficult due to the variations obtained in the data (Kukkonen et al., 2002; Ferguson and Samson, 2003). Furthermore, the majority of native cells appear to express OX_1R together with OX_2R, which are both activated by Ox-A (Kukkonen et al., 2002) and which, at least in some cells, couple to different messenger systems (Ferguson and Samson, 2003). Thus it is difficult to dissect the signalling attributable to a single receptor. Orexin receptors are promiscuous and may interact with several different G-proteins as well as other second messengers (Ferguson and Samson, 2003). The actions of orexins may be highly dependent on cellular microenvironments. Furthermore, the high concentrations of agonists required for visible responses in native neurons are indicative of spatially restricted areas of function. Although even a very low concentration of Ox-A can induce dramatic local responses in the cells, these are hard to observe in the current experimental setups. One must attempt to study the global changes induced by pharmacological (perhaps enormous) doses of agonists unless one is willing to accept a compromise and to simplify the environment at the expense of physiological relevance of the model.

7 Summary

This study sheds light on the previously poorly characterized signalling mechanisms of OX_1R. These results indicate that cellular responses induced by stimulation of OX_1R by Ox-A include membrane depolarization, elevation of $[Ca^{2+}]_i$, an increase in the ion current across the plasma membrane and activation of ERK1/2, PKCδ, and PKD1/3. It was decided to focus on exploring the signalling pathways leading to Ca^{2+} responses and elucidating the regulatory elements involved in these pathways. Ca^{2+} responses elicited by low and high nanomolar concentrations of Ox-A acting on OX_1R differed significantly from each other. High nanomolar concentrations of Ox-A activated the PLCβ-pathway leading to production of IP_3, release of Ca^{2+} from the intracellular stores and CCE. In HEK293OX_1R cells at these concentrations of Ox-A, Ca^{2+} oscillations were only seen in the absence of extracellular Ca^{2+} and the pharmacological profile of these responses indicated that the oscillations were being generated by the repetitive release and reuptake of stored Ca^{2+}. At lower concentrations of Ox-A, no clues for the release from intracellular Ca^{2+} stores were detected, but stimulation of OX_1R seemed to activate a Ca^{2+} permeable ion channel on the membrane, which was distinct from typical SOCs. The most probable molecular entity for the channel is DAG-activated TRPC3. PKC was found to be a pivotal regulator of the Ca^{2+} influx through the channel. In addition, the Ca^{2+} oscillations induced by a low concentration of Ox-A in HEK293OX_1R cells were found to be dependent on the function of TRPC3 but independent of store discharge. Three newly identified targets of OX_1R activation, PLA_2, PKD1 and PKD3, were shown to have a functional role in OX_1R signalling and to act as prominent modulators of the Ca^{2+} oscillations induced by Ox-A.

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ORIGINAL PUBLICATIONS (I-IV)

I

Orexin-A-induced Ca^{2+} entry: evidence for involvement of TRPC channels and protein kinase C regulation

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Orexin-A-induced Ca^{2+} Entry

EVIDENCE FOR INVOLVEMENT OF TRPC CHANNELS AND PROTEIN KINASE C REGULATION*

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The orexins are peptide transmitters/hormones, which exert stimulatory actions in many types of cells via the G-protein-coupled OX_1 and OX_2 receptors. Our previous results have suggested that low (subnanomolar) concentrations of orexin-A activate Ca^{2+} entry, whereas higher concentrations activate phospholipase C, Ca^{2+} release, and capacitative Ca^{2+} entry. As shown here, the Ca^{2+} response to subnanomolar orexin-A concentrations was blocked by activation of protein kinase C by using different approaches (12-*O*-tetradecanoylphorbol acetate, dioctanoylglycerol, and diacylglycerol kinase inhibition) and protein phosphatase inhibition by calyculin A. The Ca^{2+} response to subnanomolar orexin-A concentrations was also blocked by Mg^{2+}, dextromethorphan, and tetraethylammonium. These treatments neither affected the response to high concentrations of orexin-A nor the thapsigargin-stimulated capacitative entry. The capacitative entry was instead strongly suppressed by SKF96365. An inward membrane current activated by subnanomolar concentrations of orexin-A and the currents activated upon transient expression of trpc3 channels were also sensitive to Mg^{2+}, dextromethorphan, and tetraethylammonium. Responses to subnanomolar concentrations of orexin-A (Ca^{2+} elevation, inward current, and membrane depolarization) were voltage-dependent with a loss of the response around −15 mV. By using reverse transcription-PCR, mRNA for the trpc1–4 channel isoforms were detected in the CHO-hOX1-C1 cells. The expression of truncated TRPC channel isoforms, in particular trpc1 and trpc3, reduced the response to subnanomolar concentrations of orexin-A but did not affect the response to higher concentrations of orexin-A. The results suggest that activation of the OX_1 receptor leads to opening of a Ca^{2+}-permeable channel, involving trpc1 and -3, which is controlled by protein kinase C.

Orexins act via two G-protein-coupled receptors called OX_1R and OX_2R (1, 2). They activate neurons and secretory cells by mechanisms that are not fully understood (reviewed in Refs. 3 and 4). Interaction of orexin receptors with G-proteins of the $G_{q/11}$, G_i, and G_s families has been suggested based on second messenger assays and covalent labeling of G-proteins with azido-GTPγS[1] (5). The most typical responses to orexins in neurons include increased excitability, membrane depolarization (5–10 mV), and Ca^{2+} elevation (3, 4). Evidence for several different mechanisms have been proposed to explain these responses, including activation of a nonselective cation current, a decrease in K^+ conductance, and activation of Na^+/Ca^{2+} exchange. The orexin-stimulated Ca^{2+} elevation in neurons shows an explicit dependence on extracellular Ca^{2+} and is therefore likely to be due to Ca^{2+} entry into cells (5–7). Activation of recombinantly expressed orexin receptors in nonexcitable cells also leads to Ca^{2+} elevation (1, 8–11). High concentrations (>10 nM) of orexins induce intracellular Ca^{2+} release (9), but with lower concentrations of orexins the Ca^{2+} elevations observed are dependent on extracellular Ca^{2+} (8–11) and do not appear to involve measurable discharge from stores (9). Similar results are observed upon recombinant expression of orexin receptors in neuron-like excitable cells (PC12 and Neuro2A). Activation of a novel Ca^{2+} influx pathway was thus suggested. The existence of such a pathway is also indirectly suggested by the dependence of the Ca^{2+} response on a negative internal membrane potential and the activation of a robust influx of Mn^{2+} ions in the absence of store discharge (9). The identity of this Ca^{2+} influx pathway and the mechanisms involved in its activation remain unresolved. Because the orexin-stimulated $[Ca^{2+}]_e$-dependent Ca^{2+} elevation is relatively insensitive to blockers of capacitative Ca^{2+} entry, such as lanthanides and 2-APB, but blocked by Ni^{2+}, a different molecular entity was proposed (10). Several different pathways for receptor-activated Ca^{2+} entry have also been suggested based on functional studies with other receptors. These include store-operated Ca^{2+} channels and second messenger-operated channels as well as Ca^{2+}-activated Ca^{2+} channels (reviewed in Ref.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ566614, AJ566615 and AJ566613.

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[1] The abbreviations and trivial names used are: azido-GTPγS, azidoguanosine 5′-3-*O*-(thio)triphosphate; PKC, protein kinase C; TRPC, transient receptor potential channel; 2-APB, 2-aminoethoxydiphenyl borate; GF109203X, bisindolylmaleimide; R59022, diacylglycerol kinase inhibitor; SKF96365, 1-[β-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl]-1*H*-imidazole hydrochloride; BzATP, (2′,3′-*O*-(4-benzoyl-benzoyl)-ATP); DOG, dioctanoyl glycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; probenecid, *p*-(dipropylsulfamoyl)benzoic acid; Ox-A, orexin-A; CHO-K1, Chinese hamster ovary cells; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; ANOVA, analysis of variance; GFP, green fluorescent protein; EGFP, enhanced GFP; TEA, tetraethylammonium; Dex, dextromethorphan; RT, reverse transcription.

12). A large family of potential receptor-activated channels called transient receptor potential channels (TRP channels) has been identified (reviewed in Refs. 13–15). When recombinantly expressed, the different TRP channel subtypes produce currents that are, to various extents, dependent on extracellular Ca^{2+} and Na^+. The mechanism by which receptors couple to activate these channels has not been clarified, but recombinantly expressed TRP channels have been shown to modify receptor-activated Ca^{2+} influx, and it has thus been suggested that they represent the molecular entities of receptor-activated pathways (13). It has been shown by using single cell RT-PCR that both orexin receptors are co-expressed with several members of the TRPC channel family in *e.g.* rat aminergic neurons, but the expression profile varies significantly between different cells (16). Some TRP channel subtypes are regulated by lipid products such as diacylglycerol, *i.e.* exogenously applied diacylglycerol analogs activate trpc3 (17–20), although endogenously produced diacylglycerol appears to suppress the activation of some of these channels through a protein kinase C (PKC)-dependent mechanisms (21–23). Calyculin A, a protein phosphatase inhibitor, strongly suppresses the activity of TRP and TRPL channels in *Drosophila* (23) and causes internalization of trpc1, -3, and -4 in human neutrophils and overexpressed trpc3 in HEK293 (24, 25), which suggests that TRP channels are under the control of phosphorylation/dephosphorylation reactions. The TRP channels are widely distributed in different cells and the subtypes appear to represent subunits of larger channel complexes (26, 27). Interaction of expressed channels with endogenous channel complexes and constitutive activation further complicates the assessment of the functional properties of individual TRP channels (13, 14), whereas the identification of the specific function of endogenous TRP channels has, especially in neurons and endocrine cells, been hampered by the lack of specific pharmacological blockers.

The goal of this study was to investigate whether the primary orexin-A-activated Ca^{2+} entry (mediated by OX_1 receptor) involves TRPC channels using the CHO-hOX1-C1 cell line as an experimental system. Because excitable and nonexcitable cells express the same G-proteins and TRP channel subtypes (with the exception of TRPC5), their basic signaling mechanisms are expected to be the same or quite similar. A panel of channel blockers was used to distinguish the orexin-activated Ca^{2+} influx from store-operated influx, and patch clamp recordings were used to define the properties of the pathway. TRPC channel mRNA profiling was used to determine the best targets for interference with the function of the endogenous TRPC channel by using transient expression of truncated TRPC constructs and thus to assess their involvement in the orexin-stimulated Ca^{2+} entry. In addition the regulation of the orexin-activated pathway by PKC was tested by using PKC activation and inhibition.

EXPERIMENTAL PROCEDURES

The generation of CHO-hOX_1-C1 cells stably expressing the human OX_1R, has been described earlier (9). Cells were grown in Nutrient Mixture (Ham's F-12) medium (Invitrogen) supplemented with 100 units/ml penicillin G (Sigma), 80 units/ml streptomycin (Sigma), 400 μg/ml geneticin (G418; Invitrogen), and 10% (v/v) fetal calf serum (Invitrogen) at 37 °C in 5% CO_2 in an air-ventilated humidified incubator in 260-ml culture flasks (Nunc A/S, Roskilde, Denmark). For Ca^{2+} measurements in suspension, the cells were grown in 800-ml culture flasks (Nunc) in order to obtain a larger quantity of cells.

Materials—2-Aminoethoxydiphenyl borate (2-APB), GF109203X (bisindolylmaleimide), R59022 (diacylglycerol kinase inhibitor), and SKF96365 (1-[β-(3-(4-methoxyphenyl)propoxy)-4-methoxyphenethyl]-1*H*-imidazole-hydrochloride) were from Calbiochem. BzATP (2′,3′-*O*-(4-benzoyl-benzoyl)-ATP), calyculin A, dextromethorphan, dioctanoyl glycerol (DOG), 1,2-dicapryloyl-*sn*-glycerol, EDTA, EGTA, probenecid (*p*-[dipropylsulfamoyl]benzoic acid), ATP (Mg^{2+} salt), and GTP (Na^+ salt), $NiCl_2$, TPA (12-*O*-tetradecanoylphorbol-13-acetate), and TEA (tetraethylammonium) were from Sigma. Digitonin was from Merck. Fura-2-acetoxymethyl ester and fura-2-penta-potassium salt were from Molecular Probes (Eugene, OR). Thapsigargin was from RBI (Natick, MA). Human orexin-A was from Peninsula Laboratories Europe Ltd. (St. Helens, UK).

Media—The HEPES-buffered Na^+ medium (HBM) consisted of the following (in mM): 137 NaCl, 5 KCl, 1 $CaCl_2$, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 10 glucose, 1 probenecid, 20 HEPES, and 1.2 $MgCl_2$; and the pH was adjusted to 7.4 with NaOH. TEA was used by replacing the Na^+ in HBM. The desired TEA concentration was prepared by mixing the TEA-based HBM with Na^+-based HBM. In HBM prepared for electrophysiology, unless otherwise specified, $MgCl_2$ was in general excluded. The intracellular electrode solution used in the whole-cell voltage clamp recordings consisted of the following (in mM): 136 Cs^+ aspartate, 30 HEPES, 10 NaCl, 4 ATP, and 0.6 GTP. In current clamp recordings a similar intracellular solution was used but with 136 K^+ aspartate. The $[Ca^{2+}]$ in the intracellular electrode solution was optically measured with fura-2-pentapotassium salt and calibrated to ~140 nM by addition of 50 μM EGTA and 25 μM fura-2. The effect of a high intracellular Ca^{2+} buffer capacity was tested in some experiments by increasing the concentrations of EGTA and Ca^{2+} to 4 and 1 mM, respectively, or 10 and 2.8 mM, respectively. Finally, the pH was set to 7.25 with CsOH or KOH.

Ca^{2+} Measurements in Suspension—The fluorescent Ca^{2+} indicator fura-2 (28) was used to monitor changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as described previously (9). Briefly, the cells were harvested using phosphate-buffered saline containing 0.2 g/liter EDTA, spun down, and loaded at 37 °C in HBM, 1 mM probenecid, and 4 μM fura-2 acetoxymethyl ester for 20 min. The cells were washed once with Ca^{2+}-free HBM and stored on ice as pellets (medium removed). The measurement of $[Ca^{2+}]_i$ was carried out as follows: 1 pellet was resuspended in HBM at 37 °C and placed in a stirred quartz microcuvette in a thermostated cell-holder within a fluorescence spectrophotometer. Fluorescence was monitored with a PTI QuantaMaster fluorescence spectrophotometer at the wavelengths 340/360/380 (excitation) and 505 nm (emission). The experiments were calibrated by using 60 μg/ml digitonin, which gives the maximum value of fluorescence (F_{max}), and 10 mM EGTA, which gives the minimum value of fluorescence (F_{min}).

Combined Patch Clamp and Ca^{2+} Imaging—Orexin-A-evoked Ca^{2+} currents were studied in voltage clamp mode at 28 °C by using the standard whole-cell configuration (29) while concurrently monitoring the $[Ca^{2+}]_i$ by fura-2 imaging. The $[Ca^{2+}]_i$ in cells in the vicinity of the patched cell was monitored as controls. Cells were harvested from 260-ml cell flasks and replated on the day of use on 22 × 22-mm coverglasses (Warner Instruments Inc.) to a confluence of ~50–70% and rested for a minimum of 3 h before use. Cells on a coverglass were loaded with 2 μM fura-2 acetoxymethyl ester and 1 mM probenecid for 20 min at 37 °C in HBM. The coverglass was then attached to the bottom of an RC-24 fast exchange chamber (Warner Instruments Inc.) and positioned on top of the microscope. Cells were perfused with HBM (2.5 ml/min giving an exchange time of ~3 s) by a gravity-controlled drug delivery system. The perfusates were converging in a perfusion manifold and funneled through an SH-27B in-line heater (Warner Instruments Inc.) located just before the chamber inlet to obtain the desired temperature. Patch pipettes (model PG150T, Harvard Apparatus, UK) were prepared with a PC-10 puller and flame-polished with microforge MF-900 (Narishige, UK) to a resistance of 3.6–3.8 megohms measured in the bath solution. The patch clamp amplifier Axopatch 200A was connected to a computer via the AD/DA Digidata1320E SCSI interface (Axon Instruments). Voltage protocols and data acquisition were controlled with pClamp 8.1 (Axon Instruments). Cells were compensated for the pipette capacitance, whereas following whole-cell access the series resistance was analogically compensated to 60–70%. Liquid junction potential was calculated in pClamp 8.1 and subtracted from the recordings giving a more accurate clamping potential of −60 mV. In general, voltage ramps (−80 to +80 mV; 320 ms) were applied every 5 or 7.5 s. Data were digitally sampled at 3.8 kHz and filtered at 2 kHz by using the low pass Bessel filter on the recording amplifier. In current clamp experiments, data were digitally sampled at 5 kHz and filtered at 2 kHz. Combined fluorescence recordings were obtained with a second computer running the TILLvisION Multi-Color Ratio ImagingSystem (TILL Photonics GmbH, Gräfelfing, Germany), and saved for later analysis. The system consisted of a polychrome IV and a 12-bit IMAGO CCD camera under control of an external control unit. An inverted microscope (Nikon) was used to visualize the fluorescence. UV light was guided through an epifluorescence condenser, and cells were excited through a dichroic mirror (DM430, Nikon). The emission was measured

TABLE I
Primer pairs used for detection of TRP channels

Expected insert size and annealing temperatures (Ta (°C)) used for the reactions shown in Fig. 6. The sequences available from the GenBank™ database were used for primer design, as well as sequences initially obtained from TRP channel mRNA expressed in CHO-hOX_1-C1 cells.

Channel	Primer sequence	Product length	Ta
		bp	*°C*
TRPC1-5′	CTTGTTCTGTTTTCCTTCAC		55.0
TRPC1-3′	AAGCAGGTGCCAATGAACGA	139	
TRPC2-5′	TCATCCTGACTGCCTTCC		59.5
TRPC2-3′	CCAGGAACTGAGGCATGT	155	
TRPC3-5′	ACTACCTTGGGGCCAAAG		55.0
TRPC3-3′	CTACATCACTGTCATCCTC	248	
TRPC4-5′	GTGGAGAAGGGGGACTATGC		59.5
TRPC4-3′	CCACGGCTCCAACCACCT	219	
TRPC5-5′	TCCCTCTACCTGGCAACT		59.5
TRPC5-3′	AAAGAGCGTGGAGAAGGC	377	
TRPC6-5′	CTCTGAAGGTCTTTATGC		55.0
TRPC6-3′	TCATCCTCAATTTCCTGG	428	
TRPC7-5′	GCTGAAATACGACCACAA		55.0
TRPC7-3′	ATGAGGCACATCTTGATTC	278	

through a 510 nm cut-off filter (Nikon). The imaging protocol was designed to acquire images at 340 and 380 nm every 1–3 s. A TTL trigger pulse synchronized the patch clamp and imaging recordings; the TTL pulse was controlled by TILLvisION 4.0 to trigger the voltage clamp data acquisition by using the "digitizer start input" option in the pClamp 8.1. After ending the recordings, fluorescence from 340 and 380 nm of selected regions of interest were analyzed and converted into $[Ca^{2+}]_i$ as described previously (9). Voltage clamp and image data were then combined in Microcal Origin™ 6.0 for visualization and final analysis.

Identification of TRP Channel mRNA—For primer design, nucleotide sequences, retrieved from the GenBank™ data base, were aligned with MacMolly Tetra (version 3.10, align ppc program, Soft Gene GmbH). 0.5 μg of total RNA were reverse-transcribed using SuperscriptII cDNA synthesis kit (Invitrogen) and then amplified by using general trpc-specific degenerate primers 5′-nggvmchytgcagathtc-3′ and 5′-nckhgcaaayttccaytc-3′; the PCR conditions were as follows: 95 °C for 5 min, 50 °C for 30 s, 72 °C for 30 s, and 94 °C for 30 s, 30 cycles. Amplified DNA was gel-purified and inserted into PgemTeasy plasmid (Promega) and sequenced. PCR product identification was done using Blast program (30). For expression/comparison analysis, specific primers for each trpc mRNA subtype were designed and tested, PCR conditions were optimized (Table I). 1 or 0.5 μl (trpc1/trpc2) of the 20-μl cDNA reaction were amplified with channel-specific primers using optimized conditions. Identical amounts of PCRs were run on a 1.5% agarose TBE gel, stained with SYBRgreenI (Molecular Probes) according to manufacturer's instructions, and scanned on Storm 860 (Amersham Biosciences). Quantification of signal was done using ImageQuant program.

TRP Channel Constructs—Truncated forms, abbreviated (trpc1, -2, -3, -4, and -7)N, of five trp channels (trpc1, trpc2, trpc3, trpc4, and trpc7) were constructed. mtrpc1βN-EGFP-N3 (trpc1N) was constructed by subcloning a 1480-bp NsiI-BamHI fragment from pcDNAtrpc1βFLAG (see Ref. 31, gift of J. Frey) into BglII-PstI sites of EGFP-N3 (BD Biosciences). A fluorescent mtrpc1β was created by transferring a KpnI-BamHI fragment corresponding to mtrpc1β complete open reading frame from pcDNAtrpc1βFLAG into pEGFP-N3. mtrpc2N-EGFP-N1 (trpc2N) was constructed by subcloning a 2552-bp BamHI-PstI fragment from pcDNA-mtrpc2 clone 14 (see Ref. 32, gift of L. Birnbaumer) into BglI-PstI sites of pEGFP-N1. EYFP-hstrpc3N-C1 (trpc3N) was constructed by subcloning a 1620-bp BamHI-StuI (partial digest) fragment of human trpc3 cDNA (see Ref. 17, gift of C. Harteneck) into pEYFP-C1 BglII-SmaI. A functional trpc3 channel (TRPC3FLAG) was made by inserting BamHI-SpHI trpc3 cDNA fragment into pIRES-hrGFP1a (Stratagene, La Jolla, CA). In this construct the last three residues are replaced by a triple FLAG. EYFP-mtrpc4βdn-C1 (trpc4N) was constructed by subcloning a 1520-bp SalI-EcoRV fragment from mtrpc4β-stop-EYFP (see Ref. 34, gift of M. Nowycky) into pEYFP-C1 SalI-SmaI. mtrpc7αdn-EGFP-N1 (trpc7N) was constructed by subcloning a 1485-bp NheI-SacII fragment of PCIneomtrpc7α (see Ref. 35, gift of T. Okada), into pEGFP-N1.Verification that all constructs were correct and in-frame with GFP was done by automated sequencing.

Transfection and Ca^{2+} Imaging—For experiments, cells were seeded in 35-mm inner diameter Petri dishes (Nunc, Roskilde, Denmark) containing a coverslip (25 mm inner diameter, Merck Eurolab, Espoo, Finland) at a density of about 125,000 cells per plate in 2 ml of medium. After 18–24 h, cells were transfected with 3 μl of FuGENE 6 (Roche Applied Science) and 1 μg of DNA, according to the manufacturer's recommendations. Cells were used within 24 h of transfection. Expression of the GFP-tagged truncated channel isoforms was detected with 450–480 nm UV light and 520 nm barrier filter. The Ca^{2+} imaging experiments were performed, and the data were analyzed by using the intracellular imaging InCyt2™ fluorescence imaging system (Cincinnati, OH). In brief, the cells were perfused with HBM at 37 °C and excited by alternating wavelengths of 340 and 380 nm by using narrow band excitation filters, and the fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera. Fluorescence from 340 and 380 nm exposures were imported into Microcal Origin™ 6.0, and the ratios were calculated. Day to day variance in the orexin-A responses was cancelled out by normalizing Ca^{2+} responses in individual cells to a control response evoked by 100 μM 2′,3′-*O*-(4-benzoyl-benzoyl)-ATP (BzATP) at the end of an experiment. CHO-K1 cells have been shown previously to respond to BzATP via activation of P2X ($P2_Z$/$P2X_7$) receptors (36), and this response should not be affected by transfection. Cells were divided into responding and nonresponding groups, determined by their response to 0.3 nM orexin-A, and counted for statistical presentation. Nonresponding cells were then discarded in additional analysis, whereas the Δ peaks in responding cells were further processed.

Trp Co-immunoprecipitation—mTrpc1β-FLAG and trpc1N-EGFP (tarpon) were co-transfected into CHO-hOX1-C1 cells at a ratio of 0.5 μg/1.5 mg in a 60-mm inner diameter Petri dish. After 24 h, cells were harvested in PBS and lysed in RIPA 1% Triton X-100, incubated for 30 min on ice, and spun for 15 min at 15,000 × *g* at 4 °C. The supernatant was pre-cleared 15 min with protein G beads (Dynal, Oslo, Norway) and then mixed with 2 μg of polyclonal anti-full-length GFP antibody (BD Biosciences) cross-linked to protein G beads and mixed for 1 h at room temperature. Beads were captured and washed three times with RIPA and then eluted with SDS-PAGE sample buffer. Initially, 1.5 μg of trpc3FLAG was transfected into HEK293 cells using PEI50 (37) (Sigma) in a 35-mm inner diameter plate. The following day, 1.5 μg of trpc3N was transfected into the same cells, and successful transfection was determined by fluorescence microscopy (trpc3FLAG transfected cells are uniformly green; trpc3N transfected cells have localized fluorescence, and dually transfected cells have localized fluorescence in a fluorescent background). Cells were scraped in PBS and resuspended in 50 mM Tris, 120 mM NaCl, 0.5% IGEPAL, Complete™ protease inhibitors (Roche Applied Science) and spun 15 min at 15,000 × *g* at 4 °C. After preclearing and antibody-protein G incubation, beads were washed five times in high salt buffer (0.9 M NaCl) and 1 times in 0.1 M NaCl and eluted in SDS-PAGE buffer. Lysate and nonattached and eluted fraction were run on 7.5% acrylamide gel, blotted on polyvinylidene difluoride membrane, and probed with monoclonal anti-GFP (BD Biosciences) and anti-FLAG M2 (Stratagene). Detection was done with ECL-Plus (Amersham Biosciences) and visualized with Storm 860 (Amersham Biosciences) by using the program ImageQuant.

Data Processing—The differences in the responses between two groups were evaluated by the unpaired Student's t test. Between more than two groups the one-way ANOVA test was used followed by Scheffe's test. Significance is presented for $p < 0.05$ and $p < 0.01$. Data are expressed as means ± S.E., and n (where indicated) indicates the number of cells or experiments.

RESULTS

Effect of Mg^{2+} and Ion Channel Blockers on Orexin-A Evoked Ca^{2+} Elevation in Cell Suspensions—To distinguish the orexin-activated Ca^{2+} influx pathway from intracellular release and capacitative Ca^{2+} entry, we tested the effect of different inhibitors of cation channels on the response to low and high concentrations of orexin-A (Ox-A) and capacitative entry activated by thapsigargin in CHO-hOX_1-C1. Ca^{2+} measurements in suspension are shown in Fig. 1. Mg^{2+} ions have been shown previously to block a variety of Ca^{2+}-permeable channels including members of the TRP channel family (38–40). As shown

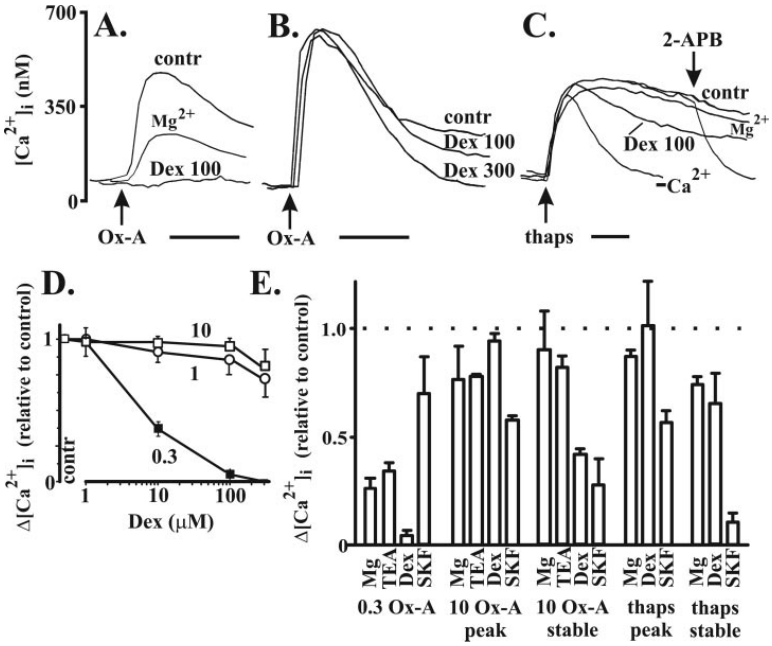

FIG. 1. **Effects of Mg^{2+}, dextromethorphan, tetraethylammonium, and SKF96365 on Ox-A or thapsigargin evoked Ca^{2+} elevation.** *A*, fura-2 recordings from cell suspensions are shown in response to addition of 0.3 nM Ox-A in the absence (*contr*) and presence of 5 mM Mg^{2+} or 100 μM dextromethorphan (*Dex 100*). Test substances were added 2 min prior to Ox-A. The response to Ox-A is completely abolished by Dex and partially by Mg^{2+}. *B*, similar recordings are shown as in *A*, but with 10 nM Ox-A and 300 μM Dex (*Dex 300*) additionally tested. *C*, 100 nM thapsigargin (*thaps*) was added in the presence of various substances. In Ca^{2+}-free conditions ($-Ca^{2+}$), no stable phase was observed. Addition of 10 μM 2-aminoethoxydiphenyl borate (*2-APB*) is shown to demonstrate the capacitative Ca^{2+} entry. 5 mM Mg^{2+} or 100 μM Dex is also shown following a 2-min preincubation. Mg^{2+} is not affecting the response, whereas Dex shows a reduction in the stable phase without affecting the peak. *Scale bars* in *A–C* represent 50 s. *D* shows the effect of increasing concentrations of Dex on Ox-A-evoked peak responses. The Ox-A concentrations tested are indicated by 0.3, 1, and 10 (nM). Data are obtained with similar recordings as in *A–C* and presented as means ± S.E. following normalization to control responses (*contr*). Each point is an average of 5–12 experiments. *E*, a *bar diagram* is shown for the fura-2 recordings performed as in *A–C* showing effects of 5 mM Mg^{2+}, 70 mM TEA, 100 μM Dex, and 10 μM SKF96365 (*SKF*). The cells were pretreated with the substances for 2 min before stimulation. The data are normalized to the respective control peak response or stable phase after 100 s, respectively, ($n = 6 \pm$ S.E.).

in Fig. 1*A* (*contr*) Ox-A at a concentration of 0.3 nM caused a robust elevation of $[Ca^{2+}]_i$. Increasing the extracellular Mg^{2+} from 1.2 to 5 mM caused a reduction in the response (Fig. 1*A*, Mg^{2+}, also see *bar diagram* in Fig. 1*E*). A higher concentration of Mg^{2+} (20 mM) did not cause a further inhibition of the response ($n = 5$, data not shown). The Ca^{2+} elevation seen at higher concentrations of Ox-A (3 nM or above) was unaffected by elevated Mg^{2+}.

Dextromethorphan was originally identified as a σ-opiate receptor ligand but was subsequently shown to reversibly block NMDA receptor channels and voltage-gated Ca^{2+} channels (41–43). As shown in Fig. 1*A*, this blocker at a concentration of 100 μM totally inhibited the Ox-A response to low Ox-A concentrations (0.3 nM Ox-A). In contrast, the peak Ca^{2+} elevation evoked by higher Ox-A concentrations (10 nM) was unaffected by dextromethorphan (Fig. 1*B*, *Dex 100*). A partial reduction of the magnitude of the stable phase of $[Ca^{2+}]_i$ elevation following the peak was seen however. A higher concentration of dextromethorphan (300 μM) caused a further inhibition of the stable phase of $[Ca^{2+}]_i$ elevation (Fig. 1*B*, *Dex 300*).

In order to test the effect of channel blockers on capacitative Ca^{2+} entry, the cells were exposed to 100 nM thapsigargin, which releases Ca^{2+} from intracellular stores and causes subsequent activation of store-operated pathways. When thapsigargin was added in the presence of extracellular Ca^{2+}, a long lasting elevation of $[Ca^{2+}]_i$ was observed (Fig. 1*C*, *contr*). Addition of 10 μM 2-APB, a blocker of capacitative Ca^{2+} entry, reversed the response to thapsigargin when added during the stable phase (Fig. 1*C*, *2-APB*). In line with this, removal of extracellular Ca^{2+} immediately prior to thapsigargin addition only evoked a transient Ca^{2+} elevation that returned to base line after ~100 s (Fig. 1*C*, $-Ca^{2+}$). Introduction of 5 mM Mg^{2+} did not significantly affect the response to thapsigargin-induced Ca^{2+} elevation (Fig. 1*C*, Mg^{2+}), whereas in the presence of 100 μM dextromethorphan only a small reduction in the stable phase was observed (Fig. 1*C*, *Dex 100*). The effect of 1–300 μM dextromethorphan was further investigated on peak responses evoked with 0.3, 1, and 10 nM Ox-A. As shown in Fig. 1*D*, dextromethorphan caused a significant inhibition of the response evoked with 0.3 nM Ox-A. This suggests that the effect of dextromethorphan is noncompetitive.

The mean responses (±S.E.) of the inhibitors tested are summarized in Fig. 1*E*. Elevated Mg^{2+} inhibited the response to 0.3 nM Ox-A by about 70% but had little or no effect on the peak or stable response to 10 nM Ox-A or 100 nM thapsigargin. Dextromethorphan at 100 μM strongly inhibited the effect of 0.3 nM Ox-A and had no effect on the peak response but partially inhibited the stable phase of the response to 10 nM Ox-A. It did not significantly affect the response to 100 nM thapsigargin. The nonspecific potassium channel blocker TEA was also tested under similar conditions and had, at 70 mM, an effect very similar to that seen with Mg^{2+}. SKF96365 (10 μM), a blocker of Ca^{2+} entry (43), had little effect on the response to 0.3 nM Ox-A, and it partially inhibited the peak and stable

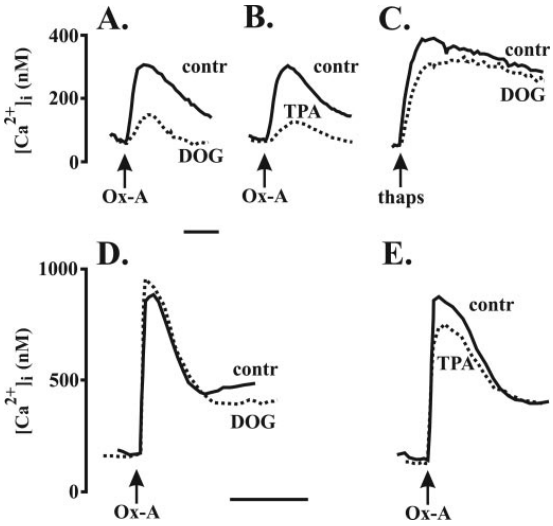

FIG. 2. **Effects of DOG and TPA on Ox-A or thapsigargin evoked Ca^{2+} elevation.** Fura-2 recordings were performed as in Fig. 1. The cells were preincubated with 30 μM DOG or 100 nM TPA for 2 min before challenge with 0.3 nM orexin-A (*Ox-A*) (*A* and *B*), 100 nM thapsigargin (*thaps*) (*C*), or 10 nM Ox-A (*D* and *E*), respectively. Controls traces are denoted as *contr*. *Scale bars* represent 50 s.

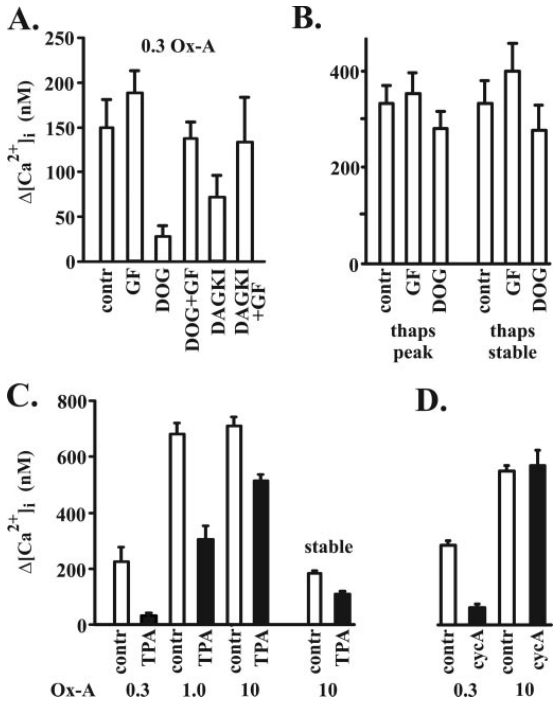

FIG. 3. **Effects of PKC modification on Ox-A and thapsigargin evoked Ca^{2+} elevation.** Results are obtained under same experimental condition as in Figs. 1 and 2. *A*, cell suspensions were challenged with 0.3 nM orexin-A (*0.3 Ox-A*), and the effects of the protein kinase C inhibitor 10 μM GF109203X (*GF*), 30 μM dioctanoylglycerol (*DOG*), and 30 μM of the DAG kinase inhibitor (R59022) (*DAGKI*), as well as the effect of GF on the two latter, are shown. The inhibitory effect of DOG and DAGKI is reversed by GF. *B*, the effect of GF and DOG on the peak (*thaps peak*) and stable response (*thaps stable*) to thapsigargin is shown. *C*, the effect of 100 nM TPA on the response to increasing concentrations of Ox-A is shown (0.3, 1, and 10 nM). *D*, cells were preincubated for 10 min in the absence or presence of 100 nM protein phosphatase inhibitor calyculin A (*cycA*), and the cells were challenged either with 0.3 or 10 nM Ox-A.

phase of the response to 10 nM Ox-A and strongly inhibited the stable response to thapsigargin.

Effect of Protein Kinase C Stimulation and Inhibition of Ox-A Evoked Ca^{2+} Elevation in Cell Suspensions—Ca^{2+} measurements in suspension were used to test the effect of diacylglycerols, which activate some subtypes of TRP channels independently of receptor activation (17–20). Addition of 30 μM DOG caused a slow increase in $[Ca^{2+}]_i$ by ~100 nM, which was dependent on extracellular Ca^{2+} (data not shown). This indicates the presence of diacylglycerol-activated Ca^{2+} entry in these cells. The $[Ca^{2+}]_i$ elevation in response to 0.3 nM Ox-A (Fig. 2*A*, *contr*) was considerably attenuated by the presence of DOG (Fig. 2*A*). At a concentration of 100 nM the phorbol ester TPA also attenuated the response to 0.3 nM Ox-A (Fig. 2*B*). The response to 100 nM thapsigargin was unaffected by DOG (Fig. 2*C*). Neither of these PKC activators significantly affected the response to 10 nM Ox-A (Fig. 2, *D* and *E*). The data summarized in Fig. 3*A* show that 10 μM GF109203X, an inhibitor of PKC, caused a small increase in the response to 0.3 nM Ox-A and almost completely reversed the inhibitory effect of DOG. A diacylglycerol kinase inhibitor R59022 (30 μM) (Fig. 3*A, DAGKI*) also reduced the response to 0.3 nM Ox-A. Likewise, this response was partially rescued by GF109203X. The peak $[Ca^{2+}]_i$ elevation and stable phase in response to 100 nM thapsigargin were unaffected by DOG or GF109203X (Fig. 3*B*). TPA inhibited the response to low concentrations of Ox-A (0.3 and 1 nM) but had little effect on the peak or stable response at 10 nM (Fig. 3*C*). As shown in Fig. 3*D*, preincubation with 100 nM calyculin A, a protein phosphatase blocker, for 10 min inhibited the effect of 0.3 nM Ox-A but did not affect the response to 10 nM Ox-A.

Orexin-activated Membrane Current and Channel Blockers Using Patch Clamp and Ca^{2+} Imaging—In order to further characterize the Ox-A activated pathway of Ca^{2+} entry, an effort was made to detect the Ca^{2+} influx pathway as a membrane current using whole-cell voltage clamp in combination with Ca^{2+} imaging. Cells were clamped at −60 mV, and Ox-A was introduced at a concentration of 0.3 nM. Basal currents in all experiments ranged from 8 to 30 pA. In experiments with a high intracellular Ca^{2+} buffer capacity added to the intracellular pipette solution, no currents were evoked by 0.3 nM Ox-A in 27/27 cells (data not shown). However, when the intracellular Ca^{2+} was buffered to a resting level similar to that in the intact cells (by addition of 50 μM EGTA and 25 μM fura-2 to the pipette solution), a large proportion (~45%) of the patched cells responded (n = 104/230) with an increase in inward current and Ca^{2+} elevations after a delay of minimum 15–30 s (Fig. 4*A*). The delay in the response was not due to patch conditions as a similar response time was observed in intact control cells. The delay in response time was significantly longer than the response time of ~6 s observed with 10 nM Ox-A. Removing extracellular Ca^{2+} rapidly reversed the Ox-A activated current response and Ca^{2+} elevation. Both responses were restored by re-addition of extracellular Ca^{2+}. The concentration response relation of the current as compared with the Ca^{2+} elevation is shown in Fig. 4*B*. The current increased steeply from 0.1 to 0.3 nM Ox-A, after which no further increase in the magnitude of current could be evoked even if the Ca^{2+} elevation continued to rise with increasing Ox-A concentrations.

A brief exposure to 5 mM Mg^{2+} reduced the current response by 43 ± 7% (n = 3), (Fig. 4*C*). Visa versa, when cells were exposed to 5 mM Mg^{2+} and subsequently challenged with Ox-A,

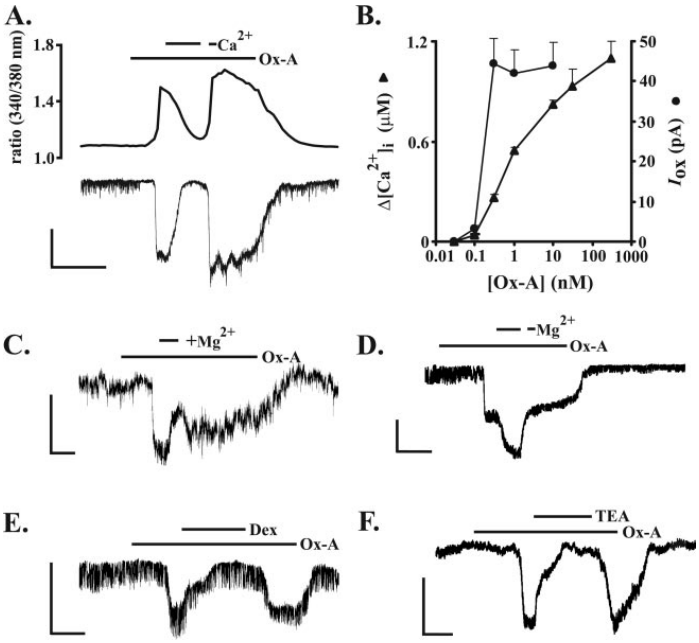

FIG. 4. **Orexin A-activated Ca^{2+} elevation and membrane current: effects of extracellular Ca^{2+}, Mg^{2+}, dextromethorphan, and tetraethylammonium.** The membrane potential was clamped in the whole-cell mode to −60 mV. Cs^+-based intracellular solution was used. The currents in unchallenged conditions ranged from 9 to 22 pA. *Scale bars* indicate 25 pA and 25 s, respectively. *A*, the effect of extracellular Ca^{2+} removal on the current and fura-2 response (340/380 nm ratio) to 0.3 nM Ox-A using combined patch clamp and Ca^{2+} imaging is shown. Removal of extracellular Ca^{2+} reversibly abolishes both responses. *B*, the relation of the Δ elevation in $[Ca^{2+}]_i$ (depicted on the *left y* axis) and the concomitant current response (depicted on the *right y* axis) is shown as a function of increasing Ox-A concentration. Points are an average of 5 experiments (Δ$[Ca^{2+}]$) whereas the average currents are obtained from 5 to 27 experiments. Data are presented as means ± S.E. *C* and *D*, the effect of Mg^{2+} on the current responses to 0.3 nM Ox-A is shown. *C*, current reduction when Mg^{2+} is elevated to 5 mM (in a HBM-buffer including 1.2 mM Mg^{2+}) is shown, and in *D* an increase in the current when 5 mM Mg^{2+} is removed is shown. *E*, the effect of 100 μM Dex and in *F* the effect of 70 mM TEA are shown. Dex and TEA completely and reversibly abolish the response to 0.3 nM Ox-A.

a current response could be observed that was rapidly and significantly enhanced upon removal of the extracellular Mg^{2+} (Fig. 4*D*). Under these conditions the Mg^{2+}-sensitive current corresponded to 44 ± 3% of the maximal evoked peak currents measured in the absence of Mg^{2+} (n = 6). On the other hand, we found that complete removal of extracellular Mg^{2+} from 1.2 mM or, visa versa, addition of 1.2 mM Mg^{2+} did not alter the Ox-A-evoked current. Mg^{2+} (5 mM) had no effect on the basal current in 5/5 cells under these conditions (data not shown). As shown in Fig. 4, *E* and *F*, dextromethorphan (100 μM) and TEA (70 mM) caused a total reversible inhibition of the current activated by 0.3 nM Ox-A. TEA and dextromethorphan did not affect the basal current in 7/7 cells. Exposure to 20 μM 2-APB (20–40 s), which totally blocked the stable response to thapsigargin (see above), had no effect on the current response to Ox-A or the basal current (n = 4, data not shown), whereas 5 mM Ni^{2+} caused an almost complete block of the Ox-A-evoked current response (92 ± 3%, n = 4, data not shown). Controls with K^+-based intracellular media did not alter the current response to 0.3 nM Ox-A (n = 14).

Ox-A-evoked Current-Voltage Relation, Depolarization, and Ca^{2+} Elevation Using Patch Clamp and Ca^{2+} Imaging—In order to analyze the voltage dependence of the Ox-A-activated membrane current, experiments were conducted with a voltage protocol introducing voltage ramps (−80 to +80 mV; 320 ms) every 5 or 7.5 s. Fig. 5*A* shows a current recorded at −60 mV (ramp traces not shown) in response to 0.3 nM Ox-A. The current-voltage profiles of ramps extracted before and during applications of Ox-A are shown in Fig. 5*B* (indicated by *numbers 1* and *2*). There was an approximately linear increase in inward current with increasing negative intracellular polarity.

The effect of 0.3 nM Ox-A was also investigated with whole-cell current clamp recordings with an intracellular K^+-based solution. As shown in Fig. 5*C*, Ox-A evoked a depolarization of the membrane. In agreement with the current-voltage profile, the magnitude of the depolarization was highly dependent on the resting membrane potential, and no or only a marginal response was seen at membrane potentials more positive than −20 mV. Fig. 5*D* shows the Ox-A-mediated depolarization as a function of the resting membrane potential.

Substitution of extracellular Na^+ with K^+ caused a considerable depolarization of the cells from a resting potential of −40 ± 5 to −13 ± 3 mV (n = *16*, Fig. 5*E*). The membrane depolarization was rapidly reversed when cells again were exposed to the Na^+-based external medium (data not shown). In line with this, Ca^{2+} imaging of intact cells showed that extracellular K^+ substitution considerably reduced the response to 0.3 nM Ox-A but had little effect on the peak elevation at 3 nM Ox-A (Fig. 5, *F* and *G*).

In order to exclude that the effects of the blockers used above would be due to changes in membrane potential, their effects were investigated on the resting membrane potential using current clamp that was found to be −41 ± 2 mV (n = 14). Neither 100 μM dextromethorphan (n = 3) nor 20 μM 2-APB (n = 3) affected the membrane potential, whereas 5 mM Mg^{2+} and 70 mM TEA caused marginal depolarization of 0.3 ± 0.6 mV (n = 4) and 0.4 ± 0.9 mV (n = 4), respectively.

Detection of TRPC Channel Isoforms Using RT-PCR—In order to investigate the possible role of TRPC channels in the responses to Ox-A, we first investigated the presence of the mRNA for different channel subtypes (subunits). For identification of trpc mRNA, primers were designed by using alignment of mammalian trpc1–7 nucleotide sequences, available from the GenBank™ data base. In the putative pore region, two very conserved sequences of 18 and 19 nucleotides, 350–400 bp apart (depending on the channel type), were identified, encompassing channel type-specific sequences that were conserved between different species. Initial RT-PCR amplification products were cloned and analyzed by restriction digestion. Several different clones were subsequently sequenced and identified as Chinese hamster homologues of trpc1, trpc2, and trpc3. In order to confirm that significant amounts of mRNA for trpc1, trpc2, and trpc3 were present in the cell line used, as well as to clarify whether other types of trpc channel mRNA

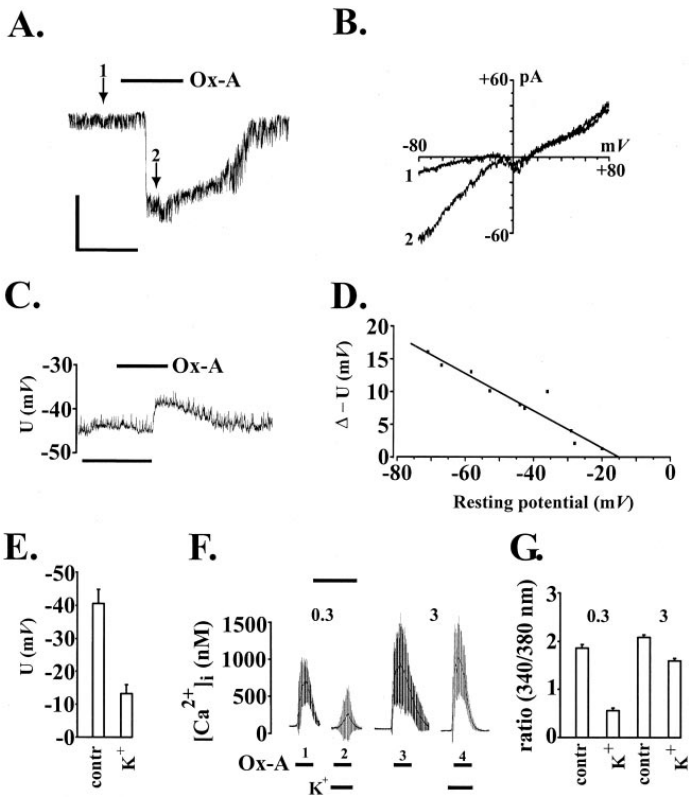

FIG. 5. **Current-voltage relationship and depolarization evoked by Ox-A.** Currents were recorded as described in Fig. 4, and voltage ramps were introduced every 5 or 7.5 s (−80 to +80 mV; 320 ms). *A*, current response to 0.3 nM Ox-A is shown. *Scale bars* indicate 30 pA and 50 s, respectively. *Arrows* with *numbers 1* and *2* indicate the position of extracted current-voltage traces shown in *B*. The current deflection during the ramp was removed from the trace for clarity. *B*, current-voltage traces are shown in the absence (*trace 1*) and presence of Ox-A (*trace 2*). *C*, whole-cell current clamp recording is shown in which 0.3 nM Ox-A evokes a reversible 7-mV depolarization. The intracellular solution was K^+-based. *Scale bar* represents 50 s. *D*, the correlation of the resting membrane potential and the Δ depolarization evoked by 0.3 nM Ox-A are shown. Note that the decline in depolarization with increasing membrane potential is correlating well with the current-voltage profile shown in *B*. The resting membrane potential and the depolarizing effect of substituting Na^+ with K^+ in the extracellular solution are shown in the *bar diagram* (*E*). Data are presented as means ± S.E. (n = 16). *F* and *G*, Ca^{2+} imaging on individual cells on coverslips is shown. *F*, Ca^{2+} responses to 0.3 and 3 nM are shown in cells on a coverslips in the absence and presence of a K^+ substitution. In this series the following were used: 1) 0.3 nM Ox-A was added as control; 2) Ox-A 0.3 nM was added in the presence of K^+; 3) 3 nM was added as control; and 4) 3 nM was tested in the presence of K^+. Washout between each application was 5 min. Data are presented as mean ± S.E. (n = 14 cells). *Scale bar* represents 100 s. *G*, summarizes the mean ± S.E. of six different experiments (98 cells) run under same conditions as in *F*. The majority of the response to low Ox-A (0.3 nM) disappeared during the K^+ depolarization, whereas the response to a high concentration (3 nM) was only marginally affected.

were present, channel specific primers were designed. All available sequences from each individual channel from a wide range of mammals (including partial sequences and our sequences) were aligned. Specific primers sequences were selected from the same pore region (except for trpc4 primers that were located at the 5′ end) and had the following features, the amplification product sequence would be highly conserved between organisms and channel type-specific sequences. This way trpc4 was detected, and the presence of trpc1, trpc2, and trpc3 mRNAs was confirmed. Quantification of relative amounts of the PCR product for each channel-specific primer pair (Fig. 6) indicates that trpc1 and trpc2 mRNA are the most abundant, whereas trpc3 and trpc4 mRNAs are present in lesser quantities. trpc5, trpc6 and trpc7 mRNAs were not detected, although several primer pairs were used (which produced fragments of the expected size from other cell lines and rat brain).

Effect of Overexpression of Truncated TRPC Channels on the Response to Ox-A Using Ca^{2+} Imaging—Splice variants of trpm1 and trpm2 encoding only for the N terminus cytosolic domain with 1 transmembrane domain in the case of trpm2 (44, 45) have been shown to be modulators of the full-length channel activity and, at least in some cases, to act by trapping functional channels inside the cells. TRPC channels have a similar coiled-coil domain, in their N-terminal region as TRPM channels and deletion of this region from mtrpc1β have been shown to prevent oligomerization of TRPC1 (31). C-terminally truncated Trpc1 channel constructs have been shown to have a dominant negative effect (46). We designed similarly truncated TRPC channel subtypes, and we tested the effect of their expression in the CHO-hOX1-C1 cell line on their response to Ox-A using Ca^{2+} imaging.

Fig. 7*A* shows a representative mean (±S.E.) of the Ca^{2+} response of the cells on a coverslip challenged with 0.3 and 3 nM Ox-A as well as 100 μM BzATP. In cells expressing the trpc1N construct (fluorescent cells), the response to 0.3 nM Ox-A was considerably attenuated. The response to BzATP was unaffected by the transfection. This suggests that the driving force (membrane potential) is comparable in transfected (fluores-

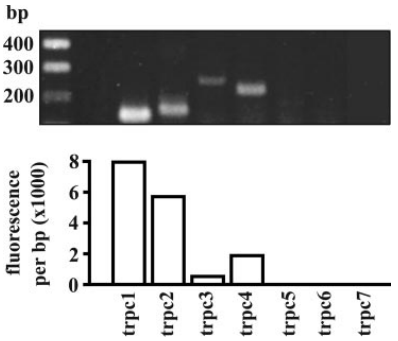

FIG. 6. **Expression of trpc channel mRNA in CHO-hOX1-C1 cells.** 6 μl of each PCR-amplified DNA was loaded on 1.5% agarose gel and post-stained with SYBRgreenI. Bands (*upper panel*) were visualized by UV transillumination (image captured using Bio-Rad Gel Doc 2000 with program Quantity One version 4.1.1). *Lower panel* shows the quantification of blue fluorescence from gel (as described under "Experimental Procedures"). Channel of trpc subtypes are listed on the *x* axis, and the *y* axis is the arbitrary units representing fluorescence per bp.

cent) and nontransfected cells (see below) because the response to BzATP mainly acts at endogenous expressed $P2X_7$ ion channels (36). BzATP was thus used as an internal control for the ability of the cell in question to respond. BzATP at this concentration gives a robust Ca^{2+} elevation in virtually all cells. Day to day variations and interference of the transfection procedure may also alter the quantification of the fura-2 signals. In some batches of cells a reduction in the responsiveness in fluorescent cells (regardless if the cells expressed fusion proteins or GFP alone) was observed in comparison to nonfluorescent cells. The responses were therefore normalized to the response to BzATP. No significant difference between GFP fluorescent and nonfluorescent cells was found, when the responses to 3 nM Ox-A were normalized to the BzATP response (Fig. 7*B*). Furthermore, the expression of GFP alone did not significantly affect the Ca^{2+} response to 0.3 nM Ox-A and was found to be 96.0 ± 1.5% of the nonfluorescent cells (one-way ANOVA test, $p = 0.34$, $n = 80$ experiments, 583 cells). Expression of the trpc7N construct evoked a similar response as expression of GFP alone. The trpc1N and trpc3N constructs caused a significant reduction of the Ca^{2+} response to 0.3 nM Ox-A, whereas trpc2N and trpc4N only had a marginal effects. The inhibitory effect of overexpressing truncated trpc channels was also reflected in the percentage of cells responding to 0.3 nM Ox-A. Although there was no difference in the number of cells responding to 3 nM Ox-A (and also BzATP) in the two groups (fluorescent *versus* nonfluorescent cells), the reduction in the number of cells responding to 0.3 nM Ox-A with the trpc1N construct was found to be 21%, trpc2N = 3%, trpc3N = 11%, trpc4N = 13%, and trpc7N = 5%.

As shown above, the magnitude of the Ox-A response to low Ox-A concentrations (0.3 nM) is obligatorily dependent upon the membrane potential. Thus, to rule out the possibility that the expression of truncated TRPC channel subtypes, *i.e.* trpc1N and trpc3N, evokes cell depolarization, we examined their effect on the membrane potential using whole-cell current clamp. Recordings showed that the trpc1N and trpc3N transfected cells have similar resting potentials compared with the controls (around −40 mV, see above). These were found to be −39 ± 2 mV ($n = 7$) and −42 ± 3 mV ($n = 6$), respectively. Further control experiments also showed that overexpression of truncated trpc constructs did not alter the basal current in voltage clamp recordings (data not shown).

The co-precipitation data in Fig. 7*C* demonstrates that

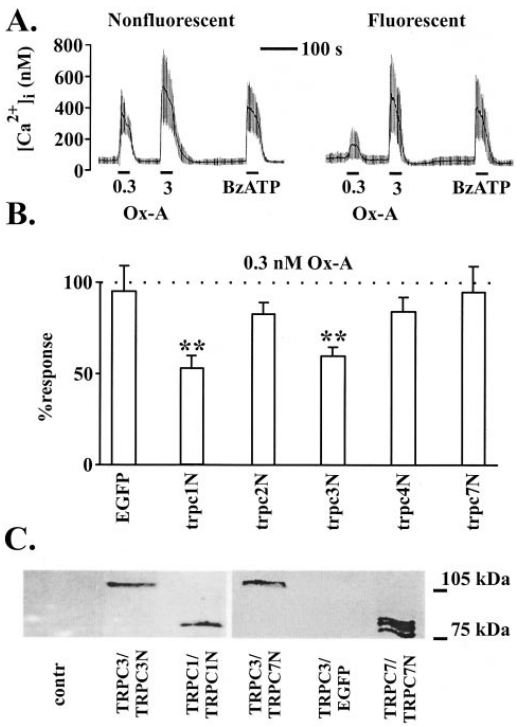

FIG. 7. **Effects of expression of truncated trpc channels in CHO-hOX1-C1 cells.** Cells were transfected with truncated constructs or GFP, and Ca^{2+} imaging was performed. Before each recording of the fura-2 responses, cells that were fluorescent at 450–480 nm excitations were identified, and the cells were on this basis divided into fluorescent and nonfluorescent groups, respectively. *A*, a typical experiment with cells transfected with the trpc1N construct is shown. The cells were challenged with 0.3 and 3 nM Ox-A and 100 μM BzATP as indicated. *B*, data obtained from experimental conditions from *A* are summarized showing the effects of transfection with the different trpcN constructs. The data were processed for 0.3 nM Ox-A by normalization of the response of the individual cells to BzATP. The results are presented as the ratio between the responses in fluorescent and nonfluorescent cells, respectively, and expressed as % response ± S.E. Statistical significance was established by the one-way ANOVA test and followed by the Scheffe's test. **, $p < 0.01$. *C*, co-immunoprecipitation of trpc3FLAG/eyfp-trpc3N, trpc1FLAG/trpc1N-egfp, trpc3FLAG/trpc7N-egfp, trpc3FLAG/egfp-n3, and trpc7NFLAG/trpc7N-egfp transfected cells with polyclonal anti-GFP antibody cross-linked to protein G-magnetic beads (*contr* = untransfected cells) and detection with anti-FLAG M2 antibody (dilution 1:2000).

TRPC1N, TRPC3N, and TRPC7N are capable of binding their intact counterpart and furthermore that TRPC7N can bind full-length TRPC3, but EGFP alone could not precipitate any TRPC channels. TRPC3N, TRPC7N, and TRPC2N were not co-precipitated with TRPC1 (data not shown).

Effect of Channel Blockers on TRPC3 Channel Current—Several TRPC channels are constitutively active when overexpressed in commonly used cell lines, and this asset has been used previously to characterize their properties (21). CHO-hOX1-C1 cells were thus transfected with TRPC-cDNA constructs, and cells expressing constructs were identified by GFP fluorescence. Cells were clamped at −80 mV. In patched cells, no change in the basal membrane current was observed upon overexpression of TRPC1 ($n = 6$), which is consistent with previous findings (47). Because the primary current response to Ox-A was highly sensitive to TEA, Mg^{2+}, and dextromethorphan, we tested their effect on cells expressing TRPC3FLAG.

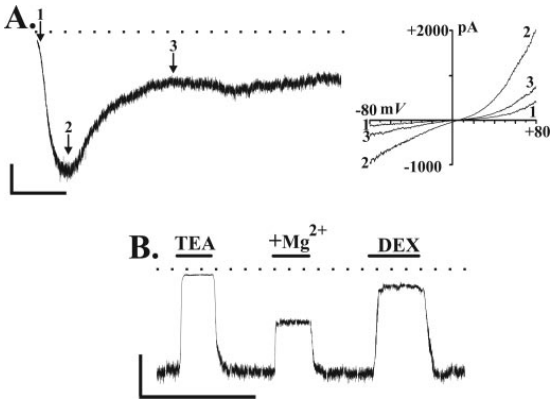

FIG. 8. **Effects of tetraethylammonium, Mg^{2+}, and dextromethorphan on membrane currents induced by TRPC3 overexpression.** CHO-hOX1-C1 were transfected with trpc3cDNA inserted into pIRES-hrGFP-1a, which transcribes trpc3 and GFP as a single bi-cistronic mRNA (TRPC3-expressing cells are fluorescent). The membrane potential was clamped in whole-cell mode to −80 mV, and voltage ramps were introduced every 5 or 7.5 s (−80 to +80 mV; 320 ms). A Cs^+-based intracellular solution was used. *A*, *left panel* shows a representative current response in a TRPC3-transfected cell. The current deflection during introduction of the ramps was removed from the trace for clarity. *Scale bars* indicate 200 pA and 100 s, respectively. *Arrows* with *numbers 1–3* indicate the position of extracted current-voltage traces shown in *A*, *right panel*. Reversal potential and current-voltage profiles were similar in all traces, and thus blockers were introduced during the more steady state current. *B* shows a TRPC3 steady state current recorded under same conditions as in *A*. The presented current trace shows the effect of 70 mM TEA, 5 mM Mg^{2+}, and 100 μM Dex in a HBM buffer including 1.2 mM Mg^{2+}. The *dotted line above* current traces in *A* and *B* indicates zero current.

Fig. 8*A* (*left panel*) shows a representative current of a cell expressing TRPC3FLAG. Inward currents were in general transient and were followed by a more steady current level ranging from around −250 to −600 pA (48). Ramp analysis (Fig. 8*A*, *right panel*, indicated by *numbers 1–3*) shows that the voltage profiles, following whole cell access and during the transient and the more steady phase of the currents, are similar. The reversal potential was found to be 6.6 ± 0.5 mV (n = 17) ranging from around 4 to 8 mV. Thus, to determine their blocking effect, TEA, Mg^{2+}, and dextromethorphan were applied during the more steady current level. Fig. 8*B* shows a representative recording in which TEA (70 mM) blocks the trpc3 current by 91%, Mg^{2+} (5 mM) by 47%, and dextromethorphan (100 μM) by 81%. TEA reduced the currents to less than 50 pA regardless of the magnitude of the basal steady current level. The magnitude of the block was in average found to be 85.6 ± 3.9% (n = 6) with respect to zero current. Dextromethorphan (100 μM) reduced the trpc3 current by 71.2 ± 4.5% (n = 6). Application of 5 mM Mg^{2+} was less effective and blocked the trpc3 current by 49.6 ± 2.0% (n = 6).

DISCUSSION

Orexins and their receptor can be found scattered in many brain areas at low density, but recently they have also been detected outside the central nervous system, particularly in organs involved in feeding and energy metabolism. The majority of native cells appears to express both OX_1 and OX_2 receptors (3), which at least in some cells couple to different messenger systems (4). Analysis of responses in native cells has proven highly variable (3, 4). This has made the investigation of physiological relevant responses to orexins difficult so far. We have utilized the CHO-hOX1-C1 cell line stably transfected with the OX_1 receptor to exclusively study a homogeneous environment.

The results of the present study suggest that the primary pathway for OX_1 receptor-mediated Ca^{2+} elevation is activation of a nonstore-operated Ca^{2+}-permeable channel. The molecular entity of this channel has, however, remained unresolved. A pharmacological distinction of nonstore-operated channels from store-operated mechanisms is difficult due to the lack of specific blockers or other specific means to distinguish the pathways. Thus, we screened a panel of channel inhibitors on the response to Ox-A and thapsigargin, in order to find compounds that would show preference for blocking a putative noncapacitative Ca^{2+} entry over typical capacitative entry. The Ca^{2+} response and inward currents evoked with 0.3 nM Ox-A were inhibited by Mg^{2+}, dextromethorphan, and 70 mM TEA. Because Cs^+ was used to substitute internal K^+ in voltage clamp experiments, the action of TEA would not be expected to stem from K^+ channel modulation but rather represents a direct channel block. This conclusion was further supported by our current clamp recordings that were unaffected by TEA. Sensitivity to Mg^{2+} has been demonstrated previously for certain TRP channels (38–40). We only observed the effects of extracellular Mg^{2+}, on the Ox-A evoked current, when Mg^{2+} was above ~1 mM. The Mg^{2+} block by 5 mM left a residual Ox-A current that was not further blocked even by a higher Mg^{2+} concentration of 20 mM. Ramp analysis of Ox-A-stimulated membrane currents indicated that the voltage profile of the Ox-A-dependent current does not change in the presence of Mg^{2+}, *i.e.* Mg^{2+} blocks in a voltage-independent manner.[2] The results presented here are strikingly similar to effects of Mg^{2+} reported by Hardie *et al.* (39) using *Drosophila* trp and TRPL cation channels. They showed a similar threshold at 1 mM Mg^{2+}, similar concentration dependence, lack of voltage dependence, and a residual current in the presence of high Mg^{2+}. They also demonstrated that the Mg^{2+} block was virtually voltage-independent. Like the Ox-A current, the constitutive current induced by TRPC3 overexpression was also sensitive to Mg^{2+}, dextromethorphan, and TEA, which also argues for an action at the level of channels rather than blocking the receptor mechanisms. The apparent lack of competition with respect to dextromethorphan is in agreement with this.

On the other hand the peak and stable phase of Ca^{2+} elevation seen with high concentrations of Ox-A and the thapsigargin-induced Ca^{2+} entry phase were relatively insensitive to these inhibitors. In line with this, the store-operated pathway has also been shown previously (40) to be insensitive to Mg^{2+}. Conversely, SKF96365 inhibited the stable phase of the Ox-A response and the thapsigargin-mediated Ca^{2+} entry more effectively than the response to subnanomolar concentrations of Ox-A. Thus, the data discussed above strongly suggest that the response to subnanomolar concentrations of Ox-A is due to activation of a pathway for Ca^{2+} entry, which is distinct from the store-operated entry. This is in agreement with previous data showing robust Ca^{2+} elevation with subnanomolar concentrations of Ox-A with no appreciable emptying of Ca^{2+} stores (9). It has been shown previously with fura-2 in suspension recordings that the $[Ca^{2+}]_e$-dependent response to orexins is inhibited by Ni^{2+} and is relatively insensitive to lanthanides or 2-APB, which in contrast strongly blocked the capacitative entry (10, 11). In line with this, voltage clamp recordings similarly showed that 0.3 nM Ox-A evoked an inward Ca^{2+}-dependent current, which were completely insensitive to 2-APB, whereas Ni^{2+} almost completely blocked the current.

[2] K. P. Larsson, H. M. Peltonen, G. Bart, L. M. Louhivuori, A. Penttonen, M. Antikainen, J. P. Kukkonen, and K. E. O. Åkerman, unpublished observations.

The involvement of a specific channel in the Ca^{2+} elevation by subnanomolar concentrations of Ox-A was further substantiated by the voltage dependence of the response. The Ox-A-activated current and membrane depolarization showed a steep dependence on a negative membrane potential. No appreciable current or depolarization was seen when the membrane potential was about −15 mV. In line with this, the Ca^{2+} elevation in intact cells showed a similar dependence on the membrane potential as depolarization with high K^+ almost totally abolished the response to 0.3 nM Ox-A but did not affect the response at higher concentrations of Ox-A. In previous reports, orexins have similarly been shown to induce depolarization in native cells of comparable magnitude as observed in this study (3).

Activation of PKC by DOG, the diacylglycerol kinase inhibitor R59022, or TPA caused a considerable reduction in the response to subnanomolar concentrations of Ox-A. In the same way as with some of the channel inhibitors Mg^{2+}, dextromethorphan, and TEA (as discussed above), PKC activation did not significantly affect the peak or stable phase of the response to high concentrations of Ox-A or to thapsigargin. PKC activation has been shown previously to inhibit Ca^{2+} entry in response to receptor stimulation (49, 50). It has recently also been shown that certain isoforms of TRPC channels, most notably TRPC3, are blocked by activation of PKC (21–23). The *Drosophila* TRP channels have also been shown to be sensitive to PKC activation as judged from stimulatory effects of PKC inhibitors and inhibition by protein phosphatase inhibitor calyculin A (23). In agreement with previous studies (21) the store-operated pathway (stable phase of the response to high Ox-A concentrations and thapsigargin) was unaffected by PKC activation. An attractive hypothesis may be that PKC functions as a negative feedback to regulate these Ca^{2+}-permeable channels and thus prevent massive intracellular Ca^{2+} elevation (21, 22). A negative feedback would explain the steep concentration dependence of the orexin-activated current response as compared with the far less steep concentration response curve for Ca^{2+} elevation. One possibility could be that the action of PKC is on the OX_1 receptor itself. However, this appears unlikely because the Ca^{2+} peak elevation with higher Ox-A concentration was relatively insensitive to PKC treatment. In the case of calyculin A, its action may be related to its ability to cause internalization of TRPC1, -3, and -4 channels (24).

A variety of nonstorage-activated Ca^{2+} channels are present in cells (12), and they are frequently observed when challenging cells with low agonist concentrations (51) as was also the case in this study. The characteristics of the pathway for Ca^{2+} entry described here including regulation by PKC, sensitivity to Mg^{2+}, and inhibition by low intracellular Ca^{2+} are similar to those observed with expressed TRP channels (13). RT-PCR suggests the presence of four functional TRPC channels subtypes in our cells (trpc1–4). Trpc1 and -2 have been identified previously in CHO-K1 cells (52, 53). Comparison of the amount of PCR product obtained with each primer pair also indicates trpc1 and trpc2 to be the major trpcs in CHO-hOX1-C1 cells. This is in line with our results. We have additionally detected trp1 and -4 mRNA, which currently nobody to our knowledge has studied in CHO cells.

In the present study, expressing truncated trpc1 and trpc3 subtypes caused a clear inhibition of the response to subnanomolar concentrations of Ox-A. None of the constructs had any effect on the response to higher concentrations of Ox-A. These data suggest that TRPC1 and TRPC3 have a central role in the signaling via the OX_1 receptor. TRPC1 may also interact with other members of the TRP channel family like TRPC4 and TRPP channels (54). RT-PCR shows the presence of mRNA for polycystin2, mucolipin1, trpm2–7, and trpv1, -2, and -4 in CHO-hOX1-C1 cells.[2] The data in this study thus indicate that the Ox-A-activated pathway for Ca^{2+} entry involves TRPC1. Interestingly, a physical interaction of TRPC1 with the mGlur1 receptor has also been demonstrated (55). The stimulation of Ca^{2+} entry by DOG indicates that the cells express diacylglycerol-activated channels. Of the TRPC channel subtypes expressed in these cells, only TRPC3 has been shown to be activated by diacylglycerol (13). These data taken together with the similar sensitivity of currents activated by TRPC3 overexpression to the Mg^{2+}, dextromethorphan, and TEA strongly suggest that TRPC3 channels are expressed in the membrane and are activated by orexin receptors. The sensitivity to protein kinase C activation (21–23) and calyculin A (24) is also a property typical of TRPC3 channels. As mentioned above TRPC3N expression also had a marked effect on the Ox-A response. We could not detect mRNA for the typical partners of TRPC3, namely TRPC6 and -7. Therefore, TRPC3 must be present as homomeric channels or then it interacts with as yet undefined partners. TRPC7N, which could interact with TRPC3, did also not affect the response. An explanation could be that the truncated channel subunits, even though they can bind the normal partners of their intact homolog, only have a dominant negative effect if they bind an intact endogenous homolog. Previous studies have also indicated that truncated channels may act by preventing insertion of native channel subunit into the membrane (45).

In some embryonic tissues, TRPC3 has been shown to be able to bind TRPC1 (27). A functional link between TRPC1 and TRPC3 is also suggested by findings demonstrating that these channel subtypes promote differentiation of hippocampal cells (56). Co-expression of TRPC1 and TRPC3 has further been shown to produce a novel membrane current indicating a functional interaction between these two channel subtypes (57). However, our co-immunoprecipitation data do not support a significant direct interaction. Because both TRPC1 and TRPC3 have been detected in caveolae and shown to bind caveolin-1, which is of importance for channel assembly (58, 59), the possibility also exist that TRPC1 and TRPC3 can collaborate without direct physical interaction. TRPC3 channels have in many studies been shown to be strongly stimulated by intracellular Ca^{2+} (13, 14, 22). Overexpression of TRPC3 produces constitutively active membrane currents in CHO-K1 cells (48). These currents are strongly regulated by Ca^{2+}. An attractive hypothesis may thus be that TRPC1 and TRPC3 are activated by subnanomolar concentrations of Ox-A, which subsequently evoke a delayed Ca^{2+}-dependent Ca^{2+} enhancement via subsequent TRPC3 channel stimulation. This hypothesis would also explain the delay in the response time and why the Ox-A-activated currents are abolished in a medium with strong intracellular Ca^{2+} buffer capacity. The lack of response to orexins in cells with high initial intracellular Ca^{2+}, which also do not respond to Ox-A, would also be explained as the TRPC3 current would be already active/inactive.

Whether the results obtained here have relevance for the action of orexins in neurons is difficult to prove at present. As discussed above, the same G-protein mechanisms and TRPC channel subunits are functional in neuronal and non-neuronal cells. Thus one would expect that the basic signaling mechanisms are similar, although in neurons downstream pathways (*e.g.* different types of ion channels) may complicate the interpretation of data. Functional studies with recombinantly expressed orexin receptors in neuron-like cells (PC12 and Neuro2A) show the same basic features as those described in CHO-K1 cells (10). An inward Ca^{2+}-dependent current and depolarization is also activated in PC12 cells.[2] In these cells

like in neurons, however, several other mechanisms are additionally activated. Several mechanisms have been proposed for the actions of orexins in neurons, *e.g.* nonselective cation channels, Na^+/Ca^{2+} exchange, and a reduction in potassium conductance or combinations of these (3, 4). The latter two mechanisms do not operate in CHO-K1 cells. It should be noted that the intracellular Ca^{2+} dependence as well as protein kinase regulation of the responses here may conceal signals in native cells. With neurons 1,000–10,000 higher, orexin concentrations have been used so the results may also not be directly comparable. Furthermore the orexin receptors are promiscuous and may interact with several different G-proteins (4). Therefore, the actions of orexins may be very dependent on the cellular microenvironment. The functions appearing at low concentrations of ligands are expected to be the primary responses of the receptor (33). Therefore, the mechanisms observed here are highly likely to be operating in neurons.

In conclusion, the data presented here show that the response to low concentrations of Ox-A acting at the OX_1 orexin receptor results in opening of a Ca^{2+}-permeable channel distinct from the typical store-operated channels. Activation of this channel is sufficient to depolarize the cells by about 10 mV, so this mechanism may be of significance in excitatory cells as well. This pathway of Ca^{2+} entry can be distinguished from other pathways of Ca^{2+} mobilization on the basis of its sensitivity to inhibitors, interference with TRPC1 and TRPC3 channels, and its regulation by PKC.

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II

The orexin OX_1 receptor regulates Ca^{2+} entry via diacylglycerol-activated channels in differentiated neuroblastoma cells

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The Orexin OX_1 Receptor Regulates Ca^{2+} Entry via Diacylglycerol-Activated Channels in Differentiated Neuroblastoma Cells

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We studied the cellular response to orexin type 1 receptor (OX_1R) stimulation in differentiated IMR-32 neuroblastoma cells. *In vitro* differentiation of IMR-32 cells with 5-bromo-2′-deoxyuridine leads to a neuronal phenotype with long neurite extensions and an upregulation of mainly N-type voltage-gated calcium channels. Transduction of differentiated IMR-32 cells with baculovirus harboring an OX_1R–green fluorescent protein cDNA fusion construct resulted in appearance of fluorescence that was confined mainly to the plasma membrane in the cell body and to neurites. Application of orexin-A to fluorescent cells led to an increase in intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$. At low nanomolar concentrations of orexin-A, the response was reversibly attenuated by removal of extracellular Ca^{2+}, by application of a high concentration (10 mM) of Mg^{2+}, and by the pharmacological channel blocker dextromethorphan. A diacylglycerol, dioctanoylglycerol, but not thapsigargin or depolarization with potassium, mimicked the OX_1R response with regard to Mg^{2+} sensitivity. A reverse transcription-PCR screening identified mRNAs for all transient receptor potential canonical (TRPC) channels, including TRPC3, TRPC6, and TRPC7, which are known to be activated by diacylglycerol. Expression of a dominant-negative TRPC6 channel subunit blunted the responses to both dioctanoylglycerol and OX_1R stimulation. The results suggest that the OX_1R activates a Ca^{2+} entry pathway that involves diacylglycerol-activated TRPC channels in neuronal cells.

Key words: **baculovirus; calcium; differentiation; neuroblastoma; orexin; TRP channel**

Introduction

Orexins/hypocretins are peptide transmitters synthesized by neurons in lateral hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998). These neurons project to multiple other brain loci and to the spinal cord (Peyron et al., 1998; van den Pol et al., 1998), with particularly dense innervation of areas involved in arousal, such as locus ceruleus (Horvath et al., 1999), raphe nucleus (Date et al., 1999; Liu et al., 2002), and tuberomammillary nucleus (Peyron et al., 1998; Eriksson et al., 2001). Evidence for orexin involvement in arousal and sleep/wake regulation has come from studies on animals with disrupted or modified orexin signaling system (Chemelli et al., 1999; Lin et al., 1999; Hara et al., 2001), which in some cases leads to the sleeping disorder narcolepsy. Orexins are also implicated in other physiological functions, such as regulation of food intake and metabolic processes and neuroendocrine functions (for review, see Kukkonen et al., 2002).

A central question is how orexins exert their actions at the cellular and molecular level. Orexins excite neurons by activating either or both of two identified G-protein-coupled orexin receptors, OX_1R and OX_2R (Sakurai et al., 1998). Investigations of the intracellular mechanisms for excitation have generated several plausible signaling pathways (for review, see Kukkonen et al., 2002; Ferguson and Samson, 2003). A useful indicator for OXR activation is an increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ can increase in cells by a variety of mechanisms, including depolarization-induced opening of voltage-gated calcium channels (VGCCs), opening of nonselective cation channels, release from intracellular stores, as well as reversal of electrogenic Na^+/Ca^{2+} exchanger. An increase in $[Ca^{2+}]_i$ during OXR stimulation is evident in neurons (van den Pol et al., 1998; Uramura et al., 2001; Kohlmeier et al., 2004), and it is also observable in different cell types used for heterologous expression (Sakurai et al., 1998; Smart et al., 1999; Holmqvist et al., 2002).

$[Ca^{2+}]_i$ measurements combined with patch-clamp recordings have demonstrated that the OX_1R activates a pathway for Ca^{2+} entry, which closely follows an inward current and depolarization, in Chinese hamster ovary (CHO) cells (Lund et al., 2000; Larsson et al., 2005). This Ca^{2+} entry pathway is well separated from store release at subnanomolar to low nanomolar concentrations of orexin-A. Inactivation of transient receptor potential canonical (TRPC) 1 and TRPC3 channels with dominant-negative (DN) constructs indicates involvement of

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these channels in the Ca^{2+} entry pathway in nonexcitable cells (Larsson et al., 2005).

Most TRP ion channels are Ca^{2+}-permeable, nonselective cation channels (for review, see Minke and Cook, 2002). The TRPC subfamily, TRPC1–TRPC7, has mostly been implicated in regulation by G-proteins and metabolites of phosphoinositide hydrolysis. TRPC channels are widely expressed in different tissues, including brain, and single cell reverse transcription (RT)-PCR in different brain loci has demonstrated that TRPC channels and OXRs are coexpressed in neurons (Sergeeva et al., 2003).

To characterize the OX_1R-induced Ca^{2+} entry in excitable cells, we have in this study used an *in vitro* differentiated neuroblastoma cell line, IMR-32, and a calcium imaging approach.

Materials and Methods

Materials. Orexin-A was from Bachem (St. Helens, UK). 5-Bromo-2′-deoxyuridine (BrdU), D-3-methoxy-*N*-methylmorphine (dextromethorphan), 1,2-dioctanoyl-*sn*-glycerol (DOG), and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) were from Sigma-Aldrich (Helsinki, Finland). Fura-2 AM was from Invitrogen (Paisley, UK). ω-Conotoxin GVIA (ωCTx) and 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate (KB-R7943) were from Tocris Cookson (Bristol, UK). Thapsigargin and *N*,*N*,*N*,-trimethyl-4-(2-oxo-1-pyrrolidinyl)-2-butyn-1-ammonium iodide (oxotremorine-M) was from Research Biochemicals International (Natick, MA) and bisindolylmaleimide I (GF109203X) was from Calbiochem (San Diego, CA).

Cell cultures. The human neuroblastoma IMR-32 cell line (Tumilowicz et al., 1970) was obtained from American Type Culture Collection (Manassas, VA) and grown in 80 cm^2 cell culture flasks (Nunc, Roskilde, Denmark) at 37°C in a humidified atmosphere (95% air/5% CO_2). The cells were cultured in standard MEM culture medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 100 U/ml penicillin–streptomycin (Invitrogen). The continuous cell culture (passages 53–63) was grown until a confluent monolayer appeared, then divided 1:8, and reseeded in new flasks. Cells for experiments were divided 1:3 or 1:4 and seeded onto coverslips kept in tissue culture dishes (35 mm diameter; Nunc). The following day, 5 μM BrdU was added, and thereafter the medium was exchanged with fresh medium containing BrdU every 2 or 3 d. After 5–8 d, the cells were transduced with baculovirus, and experiments were performed 1 or 2 d later.

Baculovirus and cell transduction. For mammalian cell transduction, we first designed a baculovirus construct to drive the expression of enhanced green fluorescent protein (EGFP). An *Ase*I (blunted)–*Not*I fragment from pEGFP–N1 (BD Biosciences Clontech, Palo Alto, CA), including the cytomegalovirus (CMV) promoter and the gene for GFP, was subcloned into an *Sna*BI–*Not*I gap in pFastBac1 (Invitrogen), removing the polyhedrin promoter. The resultant vector was called pFastBac–CMV–GFP. The human OX_1R cDNA in pcDNA3 (Invitrogen) was a gift from Dr. M. Detheux (Euroscreen SA, Bruxelles, Belgium). The OX_1R cDNA was processed by PCR to remove the stop codon and subsequently subcloned into pEGFP–N3 (BD Biosciences Clontech). The OX_1R cDNA fused to the cDNA for GFP was then transferred to pFastBac–CMV–GFP as an *Eco*RI–*Not*I fragment. An untagged OX_1R construct was generated by subcloning the whole coding sequence of OX_1R cDNA into pFastBac–CMV–GFP, the GFP from vector being cut out. For plasma membrane localization of the red fluorescent protein (RFP) Discosoma red (DsRed)-Monomer, a CAAX motif from K-ras, KKKKKSKTKCVIM, was added to the *Eco*RI–*Bam*HI gap of pDsRed-Monomer-C1 (BD Biosciences Clontech) by ligation of two complementary oligonucleotides, and subsequently the RFP fused to CAAX was transferred to pFastBac–CMV–GFP (GFP cut out) with *Bsh*TI and *Sph*I. The enhanced yellow fluorescent protein (EYFP)-tagged TRPC3DN was constructed by subcloning a 1620 bp *Bam*HI-*Stu*I (partial digest) fragment of hTRPC3 cDNA (Hofmann et al., 1999) (gift from C. Harteneck, Institute for Pharmacology, Freie Universität Berlin, Berlin, Germany) into pEYFP–C1 (BD Biosciences Clontech). The fused YFP–TRPC3DN was then transferred to pFastBac—CMV–GFP, and the GFP cDNA was replaced by complementary oligonucleotides encoding a V5 epitope, followed by a STOP codon. The hTRPC6DN fused to YFP in pcDNA3 (Hofmann et al., 2002) (gift from T. Gudermann, Institute for Pharmacology and Toxicology, Phillips Universität, Marburg, Germany) was subcloned into pFastBac–CMV–GFP with *Bam*HI and *Xba*I (GFP removed). All recombinant baculoviruses were obtained using the Bac-to-Bac expression system (Invitrogen).

Table 1. Primer pairs used for detection of TRPC channel transcripts

Channel subtype	Primer sequence	Expected size	Intron in amplicon	Gene location
TRPC1–5′	GGGTCCATTACAGATTTCAA	207 bp	1	chr 3
TRPC1–3′	AAGCAGGTGCCAATGAACGA			
TRPC3–5′	GTATGTGGACAGTTACGTC	553 bp	2	chr 4
TRPC3–3′	CTACATCACTGTCATCCTC			
TRPC4–5′	TGGGATGGCGGACTTCAG	391 bp	1	chr 13
TRPC4–3′	ATGCCTTTGCAGGTTAACCC			
TRPC5–5′	GTGGAGAAGGGGGACTATGC	525 bp	1	chr X
TRPC5–3′	CCTCACTTGATAAGGCAATG			
TRPC6–5′	CTCTGAAGGTCTTTATGC	428 bp	1	chr 11
TRPC6–3′	TCATCCTCAATTTCCTGG			
TRPC7–5′	AACCCAGCGTTTACAACG	361 bp	1	chr 5
TRPC7–3′	ATGAGGCACATCTTGATTC			

Primers were designed according to sequences available from the European Molecular Biology Laboratory database. At least one intron region was included in the amplicons to avoid amplification of genomic DNA and unprocessed RNA. Both 5′ primers and 3′ primers are written in 5′ to 3′ direction. chr, Chromosome.

For transient expression in IMR-32 cells, 0.5 ml of a high titer virus stock (10^7 pfu/ml), originating from Sf9 cell infection, was spun down in a microcentrifuge 12,000 rpm for 30 min. The pelleted viruses were resuspended in IMR-32 cell culture medium, added back to dishes with coverslips, and incubated until experimental use.

Confocal microscopy. Transduced cells were washed once with PBS and fixed for 30 min in 4% paraformaldehyde containing PBS. After removal of fixative, cells were washed four times with PBS, and then coverslips were mounted on glass slides. Confocal images were obtained using a Nikon (Tokyo, Japan) 100× (1.30 numerical aperture) Plan Fluor oil immersion objective with an Eclipse TE300 inverted microscope (Nikon) equipped with a Radiance 2100 confocal scanner (Bio-Rad, Hertfordshire, UK) under the control of LaserSharp 2000 software (Bio-Rad). GFP was excited at 488 nm with an argon laser, and RFP was excited at 543 nm with a green helium–neon laser. Images were acquired using a Kalman filter ($n = 8$).

Fura-2 imaging. Fura-2 AM was dissolved in dimethylsulfoxide to a concentration of 4 mM. Cells on coverslips were loaded with 4 μM fura-2 AM for 20 min and subsequently transferred to a perfusion chamber. The imaging experiments were performed using an InCyt2 fluorescence imaging system (Intracellular Imaging, Cincinnati, OH) essentially as described previously (Larsson et al., 2005). The perfusion HEPES-buffered Na^+ medium (HBM) consisted of the following (in mM): 137 NaCl, 5 KCl, 1 $CaCl_2$, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 10 glucose, 20 HEPES, and 1.2 $MgCl_2$, pH adjusted to 7.4 with NaOH. In the nominally Ca^{2+}-free HBM, no $CaCl_2$ was added. In the high K^+ HBM, NaCl was replaced by KCl, and the medium was diluted with HBM to achieve the desired K^+ concentration. The cells were perfused in HBM at 37°C and excited by alternating wavelengths of 340 and 380 nm using narrow band excitation filters. Fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu (San Diego, CA) CCD camera. One ratioed image was acquired per second.

Identification of TRP channel mRNA. Total RNA was extracted from differentiated cells using TRIzol (Invitrogen). Cells differentiated for 6, 8, and 10 d were used. Total RNA (5 μg) was used to make cDNA using SuperscriptII and oligo-dT (Invitrogen). An aliquot of the first-strand cDNA template (approximately the equivalent of 250 ng total RNA) was amplified with an annealing temperature of 55°C for 30 cycles with Dynazyme II (Finnzymes, Espoo, Finland) using specific primers (Table 1). The PCR reactions were electrophoretically analyzed on 2% agarose gels and stained with ethidium bromide, and images were collected using a GelDoc imaging system (Bio-Rad). Amplified DNA fragments were gel

purified and ligated into pGemTeasy (Promega, Madison, WI) and sequenced. Sequences were identified using the BLAST (basic local alignment search tool) program (Altschul et al., 1997).

Data analysis. Fura-2 imaging data were analyzed with Microcal Software (Northampton, MA) Origin 6.0 and given as absolute $[Ca^{2+}]$ levels, ($[Ca^{2+}]_i$), or as changes in $[Ca^{2+}]$ levels ($\Delta[Ca^{2+}]_i$). Traces show recordings from imaging of multiple cells simultaneously, and vertical lines indicate SDs. Every third or fifth SD is shown. Statistical significance between groups was determined with the unpaired Student's *t* test. Significance is depicted as $*p < 0.05$ or $**p < 0.01$ [not significant (ns), $p > 0.05$].

Results

Baculovirally expressed OX_1 receptors in differentiated IMR-32 cells

For this study, we sought a cell line that would resemble mature neurons and that would be prone to take up foreign DNA for OX_1R expression. The neuroblastoma cell line IMR-32 is an adrenergic human cell line that can be differentiated *in vitro* to extend long axon-like processes with numerous growth cones (Clementi et al., 1986; Carbone et al., 1990). After ~1 week of treatment of IMR-32 cell cultures with BrdU, the cell proliferation had ceased and an extensive network of processes was visible (data not shown). We found that the differentiated cells were very susceptible to baculovirus transduction. When transduced with the OX_1R–GFP baculovirus, the receptor colocalized with the CAAX motif fused to red fluorescent protein in the plasma membrane (Fig. 1*a–c*). Some GFP fluorescence was found in submembranous vesicle-like compartments, likely representing receptors being transported to or from the plasma membrane. The receptor fusion protein was also localized in cellular processes (Fig. 1*a*). The fluorescence observed when GFP was expressed alone was confined to the cytosol (data not shown).

Intracellular $[Ca^{2+}]$ elevation in response to OX_1R stimulation

Orexin-A application to transduced cells resulted in an elevation of $[Ca^{2+}]_i$ as determined with fura-2 (Fig. 1*d*,*e*). The $[Ca^{2+}]_i$ increase was dose dependent and consisted of store release as well as Ca^{2+} influx at orexin-A concentrations ≥3 nM (Fig. 1*e*,*f*). At 1 nM orexin-A, mainly an extracellular Ca^{2+}-dependent elevation was seen (Fig. 1*d*,*f*). The viral transduction efficiency was estimated by counting how many cells responded with a $[Ca^{2+}]_i$ elevation to orexin-A application. The efficiency reached >80% when tested with 100 nM (Fig. 1*g*). Of nontransduced or GFP-transduced cells, ~2% (6 of 303 cells) responded with a $[Ca^{2+}]_i$ elevation to 100 nM orexin-A. None responded to 10 nM (80 cells) or lower (220 cells).

Because our main interest lies on the Ca^{2+} entry activated by OXRs, we chose to use 1 nM orexin-A in subsequent experiments unless otherwise stated. Stimulation with 1 nM orexin-A resulted in most cells in a stable elevation of $[Ca^{2+}]_i$ (Fig. 2*a*). In some cells, this was preceded by a transient spike, indicating that some store release may occur also at this concentration. It should be taken into account that, whereas few cells showed an initial spiking at this agonist concentration, the absolute $[Ca^{2+}]_i$ increase in these cells was high enough to produce a spike in the average response in many experiments. The sustained $[Ca^{2+}]_i$ elevation lasted for >10 min. In some experiments, a decline of the sustained response over time was seen.

Previous studies on the OX_1R expressed in nonexcitable cells have shown that the activated Ca^{2+} entry and the inward Ca^{2+}-dependent current are significantly inhibited by high extracellular $[Mg^{2+}]$ and by dextromethorphan, a rather nonspecific calcium channel blocker (Larsson et al., 2005). To investigate whether the $[Ca^{2+}]_i$ elevation observed in IMR-32 cells was similar to that seen in CHO cells, cells were challenged with orexin-A in the presence of 10 mM Mg^{2+} (Fig. 2*b*). Only a small and transient response was observed under these conditions. Reduction of extracellular Mg^{2+} to 1.2 mM in the continued presence of orexin-A caused a sustained elevation of $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$ elevation was reversibly blocked by 10 mM Mg^{2+} and by 100 μM dextromethorphan, as well as by removal of extracellular Ca^{2+} (Fig. 2*b*). Statistical analysis of experiments similar to Figure 2*b* shows that high $[Mg^{2+}]$ and dextromethorphan almost completely block the sustained response to orexin-A (Fig. 2*c*). Similar types of experiments performed with higher doses of orexin-A indicated that the influx phase is partially Mg^{2+} sensitive at concentrations leading to store release as well (Fig. 2*d*) (see also Fig. 7*c*). Because the OX_1R–GFP receptor also was detected in neurites, it was of interest to test the response to orexin-A in these

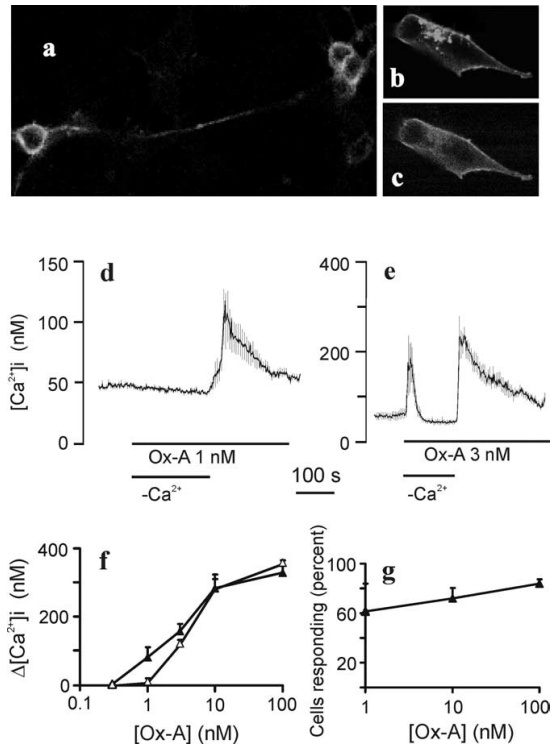

Figure 1. Expression of the OX_1R–GFP fusion protein in IMR-32 cells. ***a–c***, Confocal images of fusion proteins in IMR-32 cells. Cells were treated with BrdU and then transduced with recombinant baculovirus. One day after transduction, the cells were fixed and mounted for confocal imaging. ***a***, OX_1R–GFP fluorescence in differentiated cells with associated neurites. ***b***, Image of a cell that is not fully differentiated and thereby better illustrates the plasma membrane localization of OX_1R–GFP fluorescence. ***c***, The same cell as in ***b*** showing RFP fluorescence from RFP–CAAX. ***d***, ***e***, $[Ca^{2+}]_i$ increases in response to activation of the OX_1R 2 d after transduction. IMR-32 cells were perfused at 37°C with HBM. Where indicated, the cells were challenged with 1 nM (***d***) or 3 nM (***e***) orexin-A (Ox-A) in a nominally Ca^{2+}-free ($-Ca^{2+}$) or Ca^{2+}-containing HBM. The Ca^{2+} was removed simultaneously with application of orexin-A. The data represent averaged ± SD responses from 18 (***d***) and 36 (***e***) cells from single experiments. ***f***, Dose–response curves in normal conditions (filled triangles) and in nominally Ca^{2+}-free conditions (open triangles). The data represent averaged ± SEM responses from four experiments. ***g***, The percentages ± SD of fura-2-loaded cells responding to orexin-A applications in three to eight batches of cells.

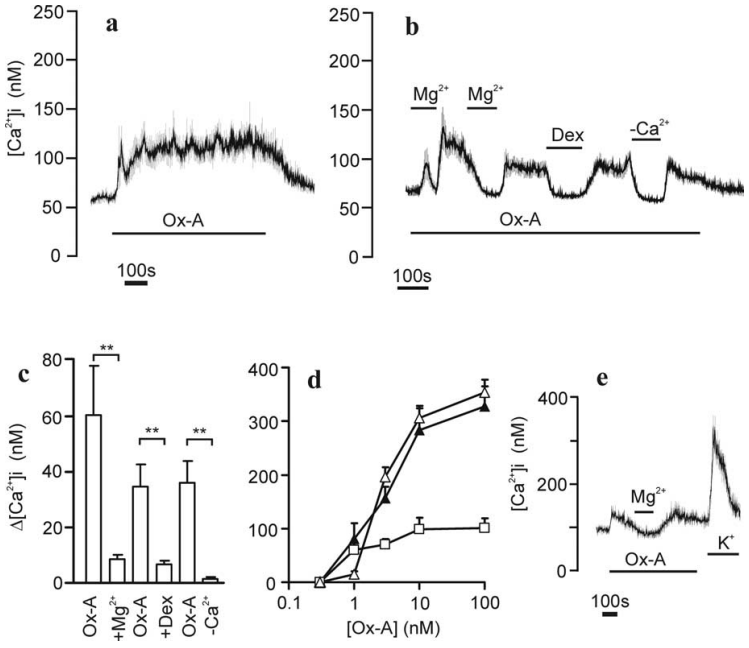

Figure 2. Effect of Mg^{2+} and dextromethorphan on the orexin-A-stimulated Ca^{2+} influx. Experimental conditions were as in Figure 1. ***a***, Cells (average ± SD of 32 cells) were continuously challenged with 1 nM orexin-A (Ox-A) to illustrate the stable $[Ca^{2+}]_i$ elevation. ***b***, A trace (average ± SD of 23 cells) of cells stimulated with 1 nM orexin-A and the effect of 10 mM Mg^{2+}, 100 μM dextromethorphan (Dex), or a nominally Ca^{2+}-free HBM ($-Ca^{2+}$). ***c***, The averages ± SEM from six experiments measured under similar conditions as in ***b***. ***d***, The dose–response relationships in the presence (open triangles) or absence (filled triangles) of high $[Mg^{2+}]$. The $\Delta[Ca^{2+}]_i$ increase after removal of 10 mM Mg^{2+} is additionally plotted (open squares). Data represent averages ± SEM from three to five experiments. ***e***, Areas containing only processes were monitored under similar conditions as in ***b*** with a subsequent depolarization with 70 mM K^+. Note that the regions of interest were larger than the areas of the processes and therefore the magnitude of the response is underestimated.

structures. Figure 2*e* shows a fura-2 recording from varicose-like structures on neurites. An Mg^{2+}- and dextromethorphan-sensitive $[Ca^{2+}]_i$ elevation was also apparent here.

Role of VGCCs in orexin-A-stimulated Ca^{2+} elevation

Orexins have been reported to depolarize cells via several different mechanisms (for review, see Kukkonen et al., 2002; Ferguson and Samson, 2003). Depolarization and a subsequent opening of VGCCs could potentially account for the OX_1R-mediated Ca^{2+} entry in IMR-32 cells. We thus performed a series of experiments that ought to reveal whether such mechanisms were involved. It is known that dextromethorphan also acts on VGCCs (Shariatmadari et al., 2001). We tested the effect of high $[Mg^{2+}]$ on the depolarization-induced Ca^{2+} influx (Fig. 3*a*). We could not detect any difference in the magnitude of $[Ca^{2+}]_i$ elevation when compared with control. It is of course possible that a minor component of VGCCs, which would not be detectable because of the robust $[Ca^{2+}]_i$ increase, could be blocked by high $[Mg^{2+}]$. Differentiation of IMR-32 cells leads to an upregulation of mainly N-type VGCCs, which can be blocked with ωCTx (Carbone et al., 1990). The depolarization-induced Ca^{2+} influx was to a large extent abolished in the presence of 0.5 μM ωCTx (Fig. 3*b*). ωCTx had no effect on the OX_1R-mediated Ca^{2+} influx (Fig. 3*c*,*d*). The residual depolarization-induced Ca^{2+} influx in the presence of ωCTx was partially attributable to nimodipine-sensitive VGCCs (presumably of L-type). Because nimodipine block of this residual Ca^{2+} influx was inconsistent and appeared to decrease with increased differentiation times, we chose to study the effect of orexin-A in ωCTx-treated cells that also received a depolarizing buffer before and during orexin-A application (Fig. 3*c*). The OX_1R response was still present and not apparently different from control, suggesting that the OX_1R response is not attributable to depolarization-induced opening of VGCCs.

Role of store release and Na^+/Ca^{2+} exchange in the OX_1R response

Ca^{2+} entry in response to depletion of intracellular Ca^{2+} stores occurs via the store-operated channels (for review, see Parekh and Putney, 2005). To test whether this type of channel is involved in Ca^{2+} elevation caused by orexin-A, cells were treated with thapsigargin to discharge Ca^{2+} from intracellular stores. Application of 100 nM thapsigargin caused a slowly developing, sustained elevation of $[Ca^{2+}]_i$ (Fig. 4*a*). Application of high $[Mg^{2+}]$ had no effect on the $[Ca^{2+}]_i$, suggesting an entirely different Ca^{2+} entry channel for capacitative store refilling compared with the OX_1R-activated channel. Dextromethorphan had a small inhibitory effect on the thapsigargin signal, and, as expected, removal of extracellular Ca^{2+} reversibly lowered the $[Ca^{2+}]_i$ (Fig. 4*a*,*b*).

The electrogenic Na^+/Ca^{2+} exchanger has been implicated in OXR-mediated neuronal excitation (Eriksson et al., 2001; Burdakov et al., 2003; Wu et al., 2004). It has also been shown to associate with TRPC3, a putative effector channel of OX_1R (Larsson et al., 2005), and to increase $[Ca^{2+}]_i$ as a consequence of TRPC3 activation attributable to the reverse mode of action (Rosker et al., 2004). A potent blocker of the reverse operation mode of Na^+/Ca^{2+} exchange, KB-R7943 (Iwamoto et al., 1996), did not significantly alter the response to orexin-A in IMR-32 cells when averages of cells were plotted and compared (Fig. 4*c*,*d*). It should be noted, however, that when analyzed on a single-cell level, there appeared to be three populations of responding cells: one (~10%) that showed a decrease in $[Ca^{2+}]_i$ when KB-R7943 was applied during the stable phase of OX_1R activation, a second population with unaltered response, and a third population (~15%) that showed an increase in $[Ca^{2+}]_i$ with KB-R7943 (data not shown). The reason for this variability is unknown at present, but, based on the average effect of KB-R7943, we do not consider the exchange mechanism as a main contributor to the Ca^{2+} influx pathway under investigation in the present study.

Diacylgycerol-activated $[Ca^{2+}]_i$ elevation and protein kinase C

Because TRPC channels were implicated in the OX_1R response in a previous study (Larsson et al., 2005), we turned our focus to these. Messenger RNAs for all human TRPC-type channels were detected in these cells as determined using RT-PCR (Fig. 5). The TRPC3/6/7 subfamily can be activated with DAG (Hofmann et

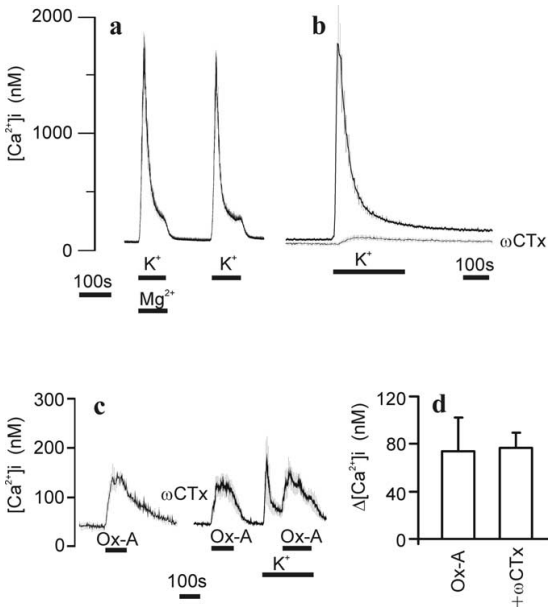

Figure 3. VGCCs in differentiated IMR-32 cells. Cells treated with BrdU were perfused at 37°C with HBM. Depolarization-induced $[Ca^{2+}]_i$ elevation with 70 mM K^+ (using an isotonic K^+-based HBM) in the presence or absence of 10 mM Mg^{2+} (***a***) and in the presence or absence of 0.5 μM ωCTx (***b***). Cells were pretreated with ωCTx for 20 min before the experiment. ***c***, Transduced cells were challenged with 1 nM orexin-A (Ox-A) in the presence or absence of 0.5 μM ωCTx. In the presence of ωCTx, the depolarizing buffer was used to activate residual VGCCs with a subsequent 1 nM orexin-A application. The data in ***a–c*** represent averaged ± SD responses from single representative experiments (10–24 cells). ***d***, Bar graph (averages ± SEM; $n = 4$) on the effect of ωCTx on orexin-A response.

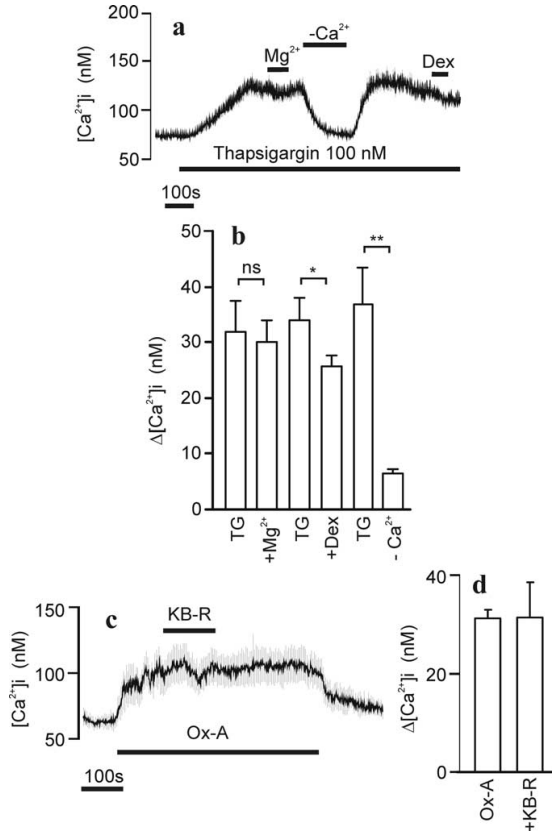

Figure 4. Ca^{2+} store release and Na^+/Ca^{2+} exchange in relation to orexin-A responses. Experimental conditions were as in Figure 1. ***a***, Cells (average ± SD of 40 cells) were challenged with 100 nM thapsigargin (TG), 10 mM Mg^{2+}, a nominally Ca^{2+}-free HBM ($-Ca^{2+}$), and 100 μM dextromethorphan (Dex) where indicated. ***b***, The averages ± SEM from four experiments treated under similar conditions as in ***a***. ***c***, Cells (average ± SD of 23 cells) were challenged with 1 nM Ox-A and 10 μM KB-R7943 (KB-R) transiently added as indicated. ***d***, Bar graph presentation of the average ± SEM effect of KB-R from three experiments.

al., 1999; Tesfai et al., 2001; Jung et al., 2002). Application of 30 μM DOG, a membrane-permeant DAG analog, caused a small irregular $[Ca^{2+}]_i$ elevation in a large proportion of the differentiated cells (Fig. 6*a*). In many of the cells, transient spiky $[Ca^{2+}]_i$ elevations were also seen (data not shown). The DOG-induced $[Ca^{2+}]_i$ elevation was sensitive to high $[Mg^{2+}]$ (Fig. 6*b*) and to dextromethorphan (Fig. 6*c*). Because DAGs also activate protein kinase C (PKC), which is known to inhibit several TRPC channel subtypes (Trebak et al., 2003; Venkatachalam et al., 2003), the effect of a PKC inhibitor, GF109203X, was tested. The inhibitor significantly enhanced the response to DOG. Data for DOG effects is compiled in Figure 6*d*.

Stimulation of the OX_1R during DOG application caused only a small, additional elevation of $[Ca^{2+}]_i$ (Fig. 7*a*,*b*) or, in a significant number of cells, no additional elevation at all (data not shown). In the presence of GF109203X, the OX_1R response was restored and even enhanced when compared with control conditions (Fig. 7*a*,*b*).

The results above suggested that the OX_1R response is linked to the DOG-activated channels and that these channels are inhibited by activated PKC. To activate PKC, without a concurrent $[Ca^{2+}]_i$ elevation, we used TPA, a mimic of DAG in activation of PKC. The OX_1R-activated Ca^{2+} entry was considerably reduced by TPA application, and the reduction was partially reversed by GF109203X (Fig. 7*c*,*d*).

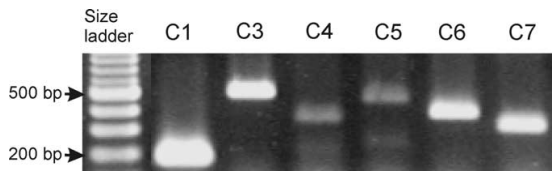

Figure 5. Identification of TRPC1–TRPC7 channel mRNAs in differentiated IMR-32 cells. The PCR-amplified DNA was separated on a 2% agarose gel and stained with ethidium bromide. Bands were visualized by UV transillumination and imaged using Bio-Rad Gel Doc 2000. The 200 and 500 bp bands of the 100 bp DNA size ladder are indicated.

Dominant-negative inhibition of the OX_1R response

To get additional evidence for TRPC channel involvement in the OX_1R response, we coexpressed a truncated dominant-negative TRPC3 (C3DN) construct, shown previously to inhibit OX_1R-mediated Ca^{2+} entry in CHO cells (Larsson et al., 2005). Because we found it more important to have a tagged C3DN construct rather than the receptor, we expressed an untagged OX_1R for these experiments. There was no observable difference between

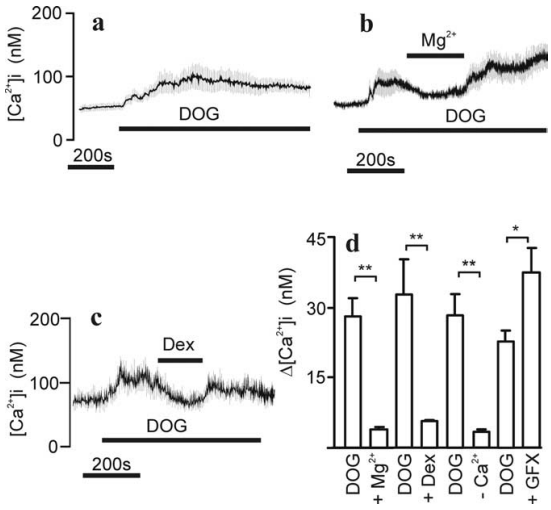

Figure 6. DOG-stimulated $[Ca^{2+}]_i$ elevation. Experimental conditions were as in Figure 1. ***a***, Cells (average ± SD of 33 cells) were challenged with 30 μM DOG, and, in ***b*** and ***c***, the effect of 10 mM Mg^{2+} and 100 μM dextromethorphan (Dex) is shown, respectively. ***d***, Statistical analysis (averages ± SEM; $n = 3–4$) of similar experiments as in ***a–c***. The DOG response varied somewhat between cell batches and was therefore always analyzed separately for each batch.

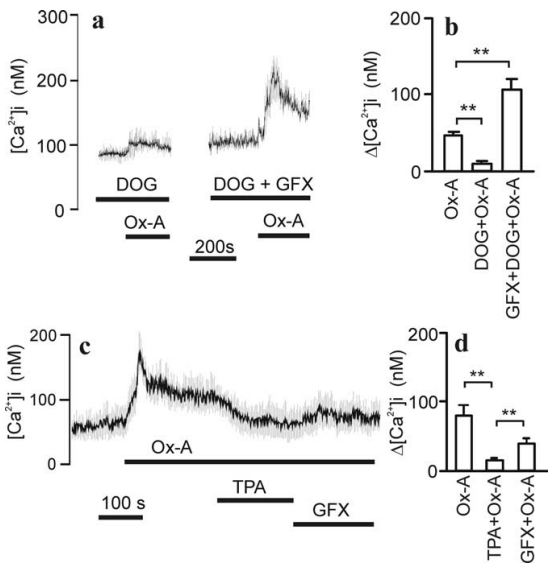

Figure 7. PKC-mediated inhibition of OX_1R response. Experimental conditions were as in Figure 1. ***a***, ***b***, The effect of DOG and GF109203X on orexin-A (1 nM) response. ***b***, Bar graph presentation of average ± SEM responses from three to four experiments. The $\Delta[Ca^{2+}]_i$ denotes changes from basal level or from the DOG response level when DOG was included. ***c***, TPA at 100 nM was used to test the effect of PKC activation on the sustained response to 1 nM orexin-A (average ± SD of 15 cells). ***d***, Statistical analysis was performed on data from three experiments.

the tagged and untagged receptor at the Ca^{2+} signaling level (data not shown). Quantitative determination of the effects of DN constructs on $[Ca^{2+}]_i$ elevation in differentiated IMR-32 cells was complicated by several factors. First, in coexpression experiments, only a proportion of the cells that responded to orexin-A expressed sufficient amount of the DN construct as determined by YFP fluorescence. Second, during differentiation, the cells tend to form aggregates whereupon the responses of neighboring cells, not expressing DN constructs, contaminated the observed response (as seen from the response profile). For that reason, we performed measurements only from areas containing individual cells. We also chose an experimental approach enabling measurement of the Ca^{2+} response sensitive to 10 mM Mg^{2+} to exclude signals attributable to Ca^{2+} discharge or capacitative entry. In addition, we set up two criteria to be fulfilled in the data processing: (1) the response to oxotremorine-M, acting on endogenous muscarinic receptors (Kukkonen et al., 1992), should exceed 300 nM $[Ca^{2+}]_i$ as a measure of healthy, viable cells, and (2) the peak response, arising from store release and not influenced by DN constructs, to 10 nM orexin-A should exceed 250 nM $[Ca^{2+}]_i$ as a measure of sufficiently high receptor expression to yield a "normal" response to 1 nM orexin-A. When these criteria were met, the C3DN had only a minor inhibitory effect on the response to 1 nM orexin-A ($\Delta[Ca^{2+}]_i$ of 68 ± 8 nM in control versus 48 ± 9 nM with C3DN; $p > 0.05$).

The dominant-negative strategy is based on incorporation of nonfunctional channel subunits into either homomeric or heteromeric channel complexes. This is governed, at least chemically, by the affinity between subunits and the availability of proteins. Given that the DAG-activated TRPC3/6/7 subfamily are able to form heteromeric complexes with each other (Hofmann et al., 2002), one would expect a DN construct from this subfamily to distort all DAG-activated channel complexes. Because the C3DN had so small an effect on the OX_1R response, we reasoned that this could be attributable to too low expression level or the inability of this truncated construct to interact sufficiently well with the endogenous related channel subunits. We therefore tested a full-length, triple-mutated TRPC6 dominant-negative construct (C6DN) (Hofmann et al., 2002). Using the same criteria as above, we found that the C6DN almost totally abolished the response to DOG (Fig. 8*a*,*b*,*e*). When tested with orexin-A applications, the response to 1 nM was significantly inhibited, whereas the 10 nM peak response was unaltered (Fig. 8*c*,*d*,*f*). The 10 nM steady-state response was also slightly inhibited, indicating, as shown previously, that the Mg^{2+}-sensitive Ca^{2+} entry is inherent to the OX_1R response regardless of agonist concentration.

Discussion

The IMR-32 neuroblastoma cells used in this study undergo a striking functional and morphological differentiation to mature neuron-like cells when treated with BrdU (Carbone et al., 1990). We found that the differentiated cells were surprisingly susceptible to baculovirus-mediated gene expression. It has been reported previously that baculovirus can enter mammalian cells, including undifferentiated neuroblastoma cells (Shoji et al., 1997; Sarkis et al., 2000; Tani et al., 2003), to drive expression of recombinant proteins (for review, see Kost and Condreay, 2002). It has also been demonstrated that baculovirus enters mature neurons *in vivo*, albeit with low efficiency (Sarkis et al., 2000). To our knowledge, this is the first report on baculovirus-transduced differentiated neuroblastoma cells. Although we did not perform a systematic study on the transduction efficiency, it was evident that, with an estimated 5–10 plaque forming units per cell, a large proportion of cells (≥80%) expressed the recombinant OX_1R–GFP protein as determined by counting of fluorescent cells and by counting of cells responding to orexin-A application.

When challenged with orexin-A, the OX_1R–GFP-transduced

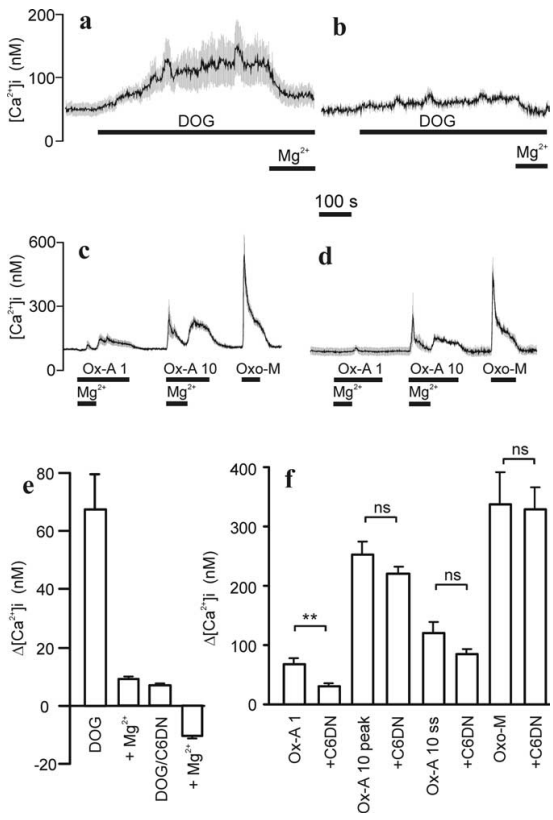

Figure 8. Effect of dominant-negative TRPC6 channel subunit expression. Experimental conditions were as in Figure 1 except that 10 μM GF109203X was included in HBM in all experiments. ***a***, ***b***, Representative traces of the effect of 60 μM DOG and 10 mM Mg^{2+} in control cells (***a***, average ± SD of 24 cells) and cells transduced with C6DN (***b***, average ± SD of 14 cells). ***e***, Compiled data from three experiments. ***c***, ***d***, Representative traces of cells challenged with orexin-A (Ox-A) and oxotremorine M (Oxo-M) in batches transduced with the OX_1R (***c***, average ± SD of 21 cells) or with the OX_1R and C6DN (***d***, average ± SD of 14 cells). ***f***, Bar graph presentation of data from 10–12 experiments. Data for 1 nM orexin-A (Ox-A 1) was collected from peak responses after removal of high $[Mg^{2+}]$. Data for 10 nM orexin-A was collected from both peak response in the presence of high $[Mg^{2+}]$ (Ox-A 10 peak) and steady-state responses after removal of high $[Mg^{2+}]$ (Ox-A 10 ss). Peak responses were collected for 30 μM Oxo-M.

cells responded with an increase in $[Ca^{2+}]_i$. At low agonist concentrations, a measurable Ca^{2+} release from intracellular stores was essentially absent. This is in agreement with previous studies (van den Pol et al., 1998; van den Pol, 1999; Lund et al., 2000; Uramura et al., 2001; Holmqvist et al., 2002; Willie et al., 2003; Kohlmeier et al., 2004) and indicates Ca^{2+} entry as a primary OX_1R response. In neuronal cells, a Ca^{2+} elevation in response to G-protein-coupled receptors could be the consequence of several different mechanisms, including activation of VGCC, reversal of electrogenic Na^+/Ca^{2+} exchange, or phospholipase C-linked Ca^{2+} store discharge with a consequent activation of store-operated channels. We excluded the involvement of N-type VGCC by the lack of an effect of ωCTx on the OX_1R response. An effect on the residual non-N-type VGCC seems unlikely because orexin-A still activated Ca^{2+} entry during depolarization in the presence of ωCTx. Conversely, elevated $[Mg^{2+}]$, which strongly blocked the response to orexin-A, did not affect the response to elevated $[K^+]$. OX_1R-activated Ca^{2+} entry was also apparent in the presence of 10 μM KB-R7943, a concentration at which this compound should selectively block the reverse mode of Na^+/Ca^{2+} exchange (Iwamoto et al., 1996). Ca^{2+} entry has been shown previously to be separated from release-induced Ca^{2+} entry at low agonist concentrations in CHO cells (Lund et al., 2000; Larsson et al., 2005). High Mg^{2+} concentrations or dextromethorphan inhibited the OX_1R-mediated Ca^{2+} entry in IMR-32 cells, whereas there was no or only a weak effect on thapsigargin-stimulated Ca^{2+} entry. This indicates that Ca^{2+} is entering through separate entities in the two cases.

The TRP channel family, in particular the TRPC channels, represents the most likely candidate as entities for Ca^{2+} entry activated by G-protein-coupled receptors (for review, see Minke and Cook, 2002; Clapham, 2003). In differentiated IMR-32 cells, we found transcripts of all members of the TRPC subfamily. An involvement of TRPC channels in the OX_1R response was indicated by the reduced Mg^{2+}-sensitive Ca^{2+} entry at low orexin-A concentrations on expression of the C6DN construct. In the same cells, the Mg^{2+}-insensitive $[Ca^{2+}]_i$ elevation (mainly Ca^{2+} release from stores) as well as $[Ca^{2+}]_i$ elevation by muscarinic receptor stimulation were essentially unaffected. Several other features of the Ca^{2+} response to orexin-A observed in this study suggest an involvement of TRP channels. The sensitivity to block by high $[Mg^{2+}]$ has been demonstrated for several TRPC channels (Schaefer et al., 2000; Larsson et al., 2005). Dextromethorphan, an NMDA receptor channel blocker, which also blocks a variety of other Ca^{2+}-permeable channels (Shariatmadari et al., 2001), including overexpressed TRPC3 channels (Larsson et al., 2005), also blocked the response to orexin-A in IMR-32 cells. DAG is a known activator of the TRPC3, TRPC6, and TRPC7 channel subunits (Hofmann et al., 1999; Tesfai et al., 2001; Jung et al., 2002). Functional evidence for the presence of these channels in differentiated IMR-32 cells comes from the DOG-induced $[Ca^{2+}]_i$ elevation, which was nearly abolished in C6DN-expressing cells. The DOG-induced $[Ca^{2+}]_i$ elevation was also sensitive to high $[Mg^{2+}]$ and to dextromethorphan in a manner similar to the response to low concentrations of orexin-A. The TRPC channels have been suggested to form both homotetrameric and heterotetrameric channel complexes, and the heteromeric complexes would be confined within certain subfamilies, such as TRPC3/6/7 (Hofmann et al., 2002). In agreement with this, the C6DN has been demonstrated to inhibit ion fluxes through both TRPC6 and TRPC3 channels (Hofmann et al., 2002). It thus appears likely that the TRPC3/6/7 subfamily makes up channel complexes engaged in the OX_1R-mediated Ca^{2+} influx in IMR-32 cells.

If the DAG-activated channels were the target for the OX_1R, one would expect DAG to modify the response to orexin-A. DAGs are, in addition to their ability to activate TRP channels, endogenous activators of PKC. TRP channels, in particular TRPC3, TRPC5, TRPC6, and TRPC7, are inhibited by activation of PKC (Trebak et al., 2003, 2005; Venkatachalam et al., 2003). This is considered to represent a feedback mechanism to fine tune the magnitude of the Ca^{2+} elevation and to prevent Ca^{2+} overload (Trebak et al., 2003). The reduced responsiveness to orexin-A in the presence of DOG and the enhanced response of DOG in the presence of the PKC inhibitor GF109203X are in agreement with this. A similar enhancement of DAG-stimulated TRPC3 channels with GF109203X has been demonstrated previously (Venkatachalam et al., 2003). In addition, TPA, an activator of PKC, which does not activate TRPC3/6/7 nor by itself increase $[Ca^{2+}]_i$ in IMR-32 cells, inhibited the response to 1 nM orexin-A, and this inhibition was partially reversed by GF109203X. Surpris-

ingly, the magnitude of the response to orexin-A in the presence of DOG and GF109203X was actually larger than that seen under control conditions, indicating that DOG potentiates the receptor response. This suggests that DAG is not necessarily the sole signal for channel activation but may rather function as a coactivator in addition to other more specific receptor-generated signals.

How does our finding relate to other studies performed on neurons? A $[Ca^{2+}]_i$ elevation in response to orexins has been described in several instances (van den Pol et al., 1998; van den Pol, 1999; Uramura et al., 2001; Kohlmeier et al., 2004; Muroya et al., 2004). One of the first reports on orexin action, which was performed on hypothalamic neurons, described an activated Ca^{2+} entry with no measurable membrane current (van den Pol et al., 1998). A difference between the Ca^{2+} entry in hypothalamic neurons and the Ca^{2+} entry evoked in IMR-32 cells is the apparent opposite regulation by PKC. van den Pol et al. (1998) found that the same inhibitor of PKC that we used totally blocked the $[Ca^{2+}]_i$ increase. In several other studies, PKC has been suggested to be a crucial intermediate effector in orexin action, often with downstream effects linked to specific ion channels (Uramura et al., 2001; Xu et al., 2002; Kohlmeier et al., 2004) or less well defined ion conductances (Yang et al., 2003). PKC has long been known to modulate VGCCs (Yang and Tsien, 1993) and K^+ conductances (Henry et al., 1996). In our current study, the pharmacological tools used to probe for PKC action all appeared to indicate a blocking role for PKC on the OX_1R-mediated Ca^{2+} entry. The PKC- and Mg^{2+}-sensitive Ca^{2+} entry shown here and previously in nonexcitable cells precedes other actions of orexin from a dose–response point of view and is thus expected to be a primary response (Kukkonen et al., 2002). It is possible that this signaling pathway is activated in parallel with VGCCs in native neurons as well but may be masked because of its sensitivity to PKC activity.

At orexin-A concentrations $\geq$3 nM, there was a clear release of Ca^{2+} from internal stores in IMR-32 cells. This is frequently observed in heterologous expression systems with both OX_1R and OX_2R (Smart et al., 1999; Holmqvist et al., 2002; Ammoun et al., 2003) and is likely to be a consequence of activation of phospholipase C (PLC) and the generation of IP_3. Some studies on native receptors in neurons have demonstrated sensitivity to inhibitors of phosphatidylinositol-specific PLC (Zhu et al., 2003; Muroya et al., 2004), suggesting that some store release may take place. Conversely, a Ca^{2+} release from intracellular stores has not been demonstrated in native neurons although explicitly tested for (van den Pol et al., 1998; Kohlmeier et al., 2004). It is possible that the OXRs are highly compartmentalized in native neurons and therefore the Ca^{2+} release may occur only in localized sparks, as would be the case also with the release-independent Ca^{2+} entry.

In conclusion, the primary response of the OX_1R at low concentrations of orexin-A in differentiated IMR-32 cells is dependent on the activation of a Ca^{2+}-permeable channel with many properties such as those described for defined TRPC channel subtypes, including block by Mg^{2+}, potentiation by DAG, and regulation by PKC. Higher agonist concentrations additionally activate Ca^{2+} store release with a subsequent pharmacologically different store-operated Ca^{2+} entry.

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III

Involvement of TRPC3 channels in calcium oscillations mediated by OX_1 orexin receptors

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Involvement of TRPC3 channels in calcium oscillations mediated by OX_1 orexin receptors

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ABSTRACT

Oscillations of intracellular Ca^{2+} provide a novel mechanism for sustained activation of cellular processes. Receptor-activated oscillations are mainly thought to occur through rhythmic IP_3-dependent store discharge. However, as shown here in HEK293 cells 1 nM orexin-A (Ox-A) acting at OX_1 receptors (OX_1R) triggered oscillatory Ca^{2+} responses, requiring external Ca^{2+}. These responses were attenuated by interference with TRPC3 channel (but not TRPC1/4) function using dominant negative constructs, elevated Mg^{2+} (a blocker of many TRP channels) or inhibition of phospholipase A_2. These treatments did not affect Ca^{2+} oscillations elicited by high concentrations of Ox-A (100 nM) in the absence of external Ca^{2+}. OX_1R are thus able to activate TRPC(3)-channel-dependent oscillatory responses independently of store discharge.

Repetitive calcium transients or oscillations are considered to be a novel mean for sustained activation of cellular processes such as gene expression [1,2], and they are often observed in response to G protein coupled receptor (GPCR) stimulation [3–5]. In general these oscillations are thought to be primarily derived from cyclic Ca^{2+} discharge from and reuptake into Ca^{2+} stores [2]. However, Ca^{2+} entry from the extracellular space also plays a critical role especially in the maintenance of oscillations by replenishing Ca^{2+} ions extruded from the cells [5–7]. The family of transient receptor potential (TRP) channels in particular the canonical (TRPC) subfamily, represent good candidates for receptor-activated pathways of Ca^{2+} entry [8]. Diacylglycerols (DAG) which activate defined canonical TRP channel subtypes (TRPC3/6/7) promote Ca^{2+} oscillations in some cell types [9,10]. Orexin-A (Ox-A) and -B (hypocretin-1 and -2) are hypothalamic neuropeptides/hormones, which regulate feeding and attention via two distinct GPCRs (OX_1R, OX_2R) [11–13]. Several lines of evidence indicate that low nanomolar concentrations of Ox-A acting on OX_1 orexin receptors (OX_1R) stimulate a pathway for Ca^{2+} entry, which is attenuated by expression of dominant negative TRPC1/3 channel constructs [14,15] and is independent of 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} discharge [16–18].

Since Ca^{2+} entry via TRPC channels is the dominant response to low concentrations of Ox-A our aim was to investigate whether oscillatory responses are mediated through the action of these channels. Oscillatory Ca^{2+} responses to GPCR activation has frequently been observed in human embryonic kidney (HEK293) cells [4–6] and we therefore decided to investigate this in HEK293 cells stable transfected with OX_1 receptors. Due to the lack of specific blockers for TRPC channels we used a dominant negative approach. Our data indicate that orexin receptor activation promotes two independent oscillatory responses depending on the concentration of Ox-A: low nanomolar concentrations of Ox-A trigger an oscillatory response by a TRPC3- and arachidonic acid- (AA-) dependent mechanism, independent of store discharge while higher concentrations of Ox-A promote oscillatory responses via repetitive Ca^{2+} discharge from the intracellular stores.

Materials and methods

Cell culture. HEK293 cells stably expressing OX_1R (HEK293OX_1R) were prepared as described in [19] and grown in standard Dulbecco's Modified Eagle's cell culture medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 100 U/ml penicillin–streptomycin (Invitrogen) and 0.05 mg/ml hygromycin (Invitrogen) at 37 °C in 5% CO_2 in an air-ventilated humified incubator.

Materials. 2-Aminoethoxydiphenyl borate (2-APB) was from Calbiochem (san Diego, CA). Arachidonic acid, and *P*-(dip-

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ropylsulfamoyl)benzoic acid (probenecid) was from Sigma (Helsinki, Finland). Fura-2-acetoxymethyl ester (fura-2) was from Molecular Probes (Eugene, OR, USA). Human orexin-A (Ox-A) was from Bachem (St. Helens, UK). FuGENE6™ was from Roche (Espoo, Finland). Methyl arachidonyl fluorophosphonate (MAFP) was from Cayman Chemical (Ann Arbor, MI, USA).

Experimental media. The HEPES-buffered Na^+ medium (HBM) consisted of the following (in mM): 137 NaCl, 5 KCl, 1 $CaCl_2$, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 10 glucose, 1 probenecid, 20 HEPES, and 1 $MgCl_2$; and the pH was adjusted to 7.4 with NaOH.

Dominant negative TRPC channel constructs and transfection. Dominant negative canonical transient receptor potential channel (TRPC) constructs trpc1N, trpc3N, trpc4N and trpc7N have been described earlier [14]. Dominant negative TRPC6, $TRPC6^{DN}$ [20] was a gift from T. Gudermann (Institute for Pharmacology and Toxicology, Philipps-Universität, Marburg, Germany). For single cell imaging experiments, cells were seeded in 35-mm inner diameter Petri dishes (Nunc, Roskilde, Denmark) containing a coverslip (25 mm inner diameter, Merck Eurolab, Espoo, Finland) at a density of 400,000 cells (unless otherwise specified) in 2 ml of medium. After 18–24 h, cells were transfected with 6 μl of FuGENE 6 (Roche Applied Science) and 1 μg of DNA, according to the manufacturer's recommendations.

Single cell Ca^{2+} imaging. For measurements, the cells were grown on coverslips and loaded with 4 μM fura-2 at 37 °C in HBM for approximately 30 min. Ca^{2+} imaging was performed as described previously [14]. Transfected cells over-expressing the enhanced green fluorescent protein (EGFP)-tagged dominant negative channel isoforms were identified by EGFP fluorescence with 450–480 nm light and 520 nm barrier filter. The data was imported into Microcal Origin™ 6.0 and further analysis was performed. Ca^{2+} measurements in cell suspensions were performed as described in [19].

Arachidonic acid release. Cells were cultured on poly-L-ornithine-coated 24-well plates (Nunc) to 50% confluence. 0.1 μCi [^{3}H]-AA ([5,6,8,9,11,12,14,15-^{3}H]-arachidonic acid, New England Nuclear Corp. GesmbH, Wien, Austria) was added in each well and the cells cultured for another 20 h. The incubation medium was removed and the cells were washed twice with the culture medium without serum but supplemented with 2 mg/ml bovine serum albumin (BSA). The stimulations with Ox-A were performed at 37 °C in 250 μl/well of this same medium. After 7 min stimulation, 200 μl of the medium from each well was transferred to an Eppendorf tube on ice. These samples were spun down for 1 min at 4 °C and 150 μl of the medium was transferred to a scintillation tube and the scintillation cocktail (HiSafe 3, Wallac-PerkinElmer, Turku, Finland) was added. Cell remnants on the 24 well plates were dissolved in 0.1 M NaOH and the scintillation cocktail was added. The radioactivity was counted in a scintillation counter after allowing the samples to set for 24 h.

Data processing. Data are expressed as means ± SE and significance is presented as ($^*p < 0.05$) or ($^{**}p < 0.01$). Where indicated n denotes the number of experiments. All the experiments were repeated at least three times. The significances were evaluated by unpaired Student's t-test.

Results

In 51% of HEK293 cells stably expressing OX_1R (HEK293OX_1R), 1 nM Ox-A produced a repetitive spike pattern of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The oscillation frequency varied from cell to cell, but in each individual cell, the frequency remained

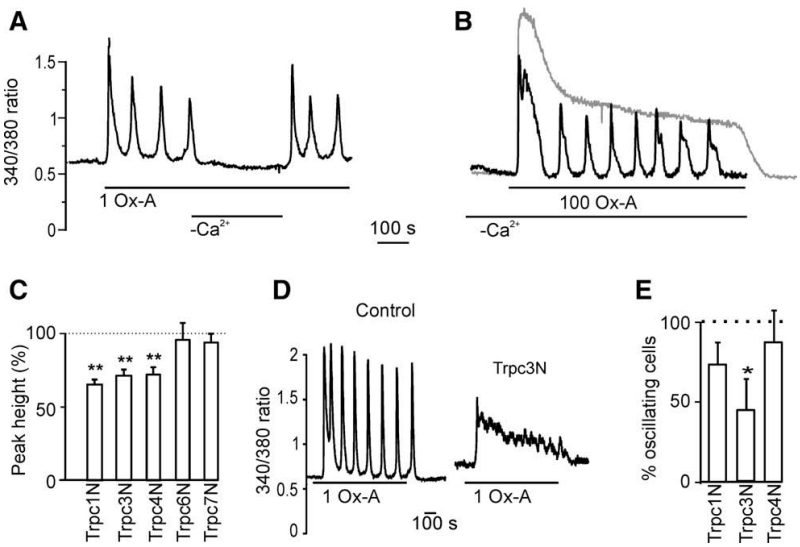

Fig. 1. Effect of extracellular Ca^{2+} and over-expression of dominant negative TRPC channel constructs on Ca^{2+} oscillations elicited by low (1 nM) and high (100 nM) concentrations of orexin-A (Ox-A). In (A) representative fura-2 recording from cells continuously treated with 1 nM Ox-A in the presence and absence ($-Ca^{2+}$) of extracellular Ca^{2+} and in (B) with 100 nM Ox-A in the presence (gray curve) and absence of external Ca^{2+}. In (C) cells (250,000 cells/dish) were transfected with EGFP-tagged dominant negative TRPC channel constructs and Ca^{2+} imaging was performed. The periods of stimulation with 1 and 100 nM Ox-A were 30 s. The summary of peak heights is shown. The responses of individual cells were normalized to the response of 100 nM Ox-A. The results are presented as the ratio between the responses to 1 nM Ox-A in fluorescent and non-fluorescent cells and expressed as Peak height (%) ± SE (n = 4–10). The responses of fluorescent cells were compared to the responses of non-fluorescent cells from the same coverslips and the statistical significance was established by the unpaired Student's t-test. **indicates $p < 0.01$. In (D) cells were transfected with dominant negative Trpc3N and Ca^{2+} imaging was performed while stimulating the cells with Ox-A for prolonged periods. A representative measurement from a non-fluorescent cell (left) and a fluorescent cell (right). The periods of stimulation with 1 nM Ox-A are indicated by horizontal bars. (E) Summary of the data obtained from experiments similar to (D) with different dominant negative TRPC channel constructs. The results are presented as the ratio between the proportions of transiently oscillating cells in fluorescent and non-fluorescent cells and expressed as Oscillating cells (%) ± SE (n = 3–5). The percentages of oscillating fluorescent cells were compared to the percentages of non-fluorescent cells from the same experiment.

the same for tens of minutes. The average frequency was 7.4 ± 0.2 mHz. Removal of Ca^{2+} from the perfusion solution caused an instantaneous termination of the Ca^{2+} oscillations and subsequent readdition of Ca^{2+} restored the oscillations (Fig. 1A, $n = 3$). A higher concentration (100 nM) of Ox-A induced a single biphasic $[Ca^{2+}]_i$ rise consisting of a transient spike followed by a sustained plateau (Fig. 1B, gray trace, $n = 3$). However, stimulation of the cells in the absence of extracellular Ca^{2+}, produced an oscillation pattern resembling the one induced by 1 nM Ox-A in the presence of extracellular Ca^{2+} (Fig. 1B, $n = 24$).

Since previous data indicates that TRPC channels are activated by Ox1 receptors we used a dominant negative approach to assess their role in Ox-A elicited oscillations. C-terminally truncated transient receptor potential channel (TRP) constructs, lacking pore-region and C-terminus have previously been shown to elicit a dominant negative effect on the corresponding channel activities [17,18,20,21]. HEK293 cells have been shown to have transcripts for several canonical TRP channel (TRPC) sub-types [22,23]. We transfected the HEK293OX_1R with dominant negative constructs of TRPC channels and the cells were then challenged with short pulses of 1 and 100 nM Ox-A. Cells over-expressing the constructs were identified by the co-expressed enhanced green fluorescent protein (EGFP) fluorescence. Over-expression of trpc1N ($n = 6$), trpc3N ($n = 10$), and trpc4N ($n = 5$) significantly decreased the initial peak height of Ca^{2+} responses to 1 nM Ox-A, whereas over-expression of $TRPC6^{DN}$ ($n = 5$) and trpc7N ($n = 4$) had no effect (Fig. 1C). None of the constructs affected the response to 100 nM Ox-A (data not shown). Fig. 1D shows typical response patterns in a non-fluorescent (left) and fluorescent (right) cell, from a coverslip transfected with trpc3N, to prolonged treatment with 1 nM Ox-A. While 1 nM Ox-A induced an oscillatory response in the nonfluorescent cell, the fluorescent cell over-expressing trpc3N failed to produce transient Ca^{2+} oscillations. The data obtained from the experimental conditions illustrated in Fig. 1D were analysed by counting the number of cells responding with transient Ca^{2+} oscillations to 1 nM Ox-A and comparing the percentages of transiently oscillating cells in each group (fluorescent and non-fluorescent) from the same coverslips. The results are summarized in Fig. 1E (trpc1N: $n = 3$, trpc3N: $n = 5$, trpc4N: $n = 4$). Expression of trpc3N but none of the other constructs was found to significantly decrease the percentage of transiently oscillating cells when compared to the control.

As Ca^{2+} entry in HEK293 cells is promoted by arachidonic acid (AA) activated channels [5] we investigated the role of AA in Ox-A induced Ca^{2+} oscillations. Fig. 2A shows the concentration dependence of Ox-A stimulated Ca^{2+} elevations in the presence and absence of external Ca^{2+}. As shown previously in other cell types [14,15] the response to low concentrations of Ox-A (1–10 nM) showed a strict dependence on extracellular Ca^{2+}, while Ca^{2+} discharge from stores required Ox-A concentrations above 10 nM. Fig. 2A also shows that a significant amount of AA release occured already at 1 nM Ox-A. Since AA was produced at concentrations of Ox-A producing sustained oscillations it was of interest to test whether blocking of phospholipase A_2 (PLA_2), the main source of AA, would affect the oscillatory response. We therefore used methyl arachidonyl fluorophosphonate (MAFP), which is a potent

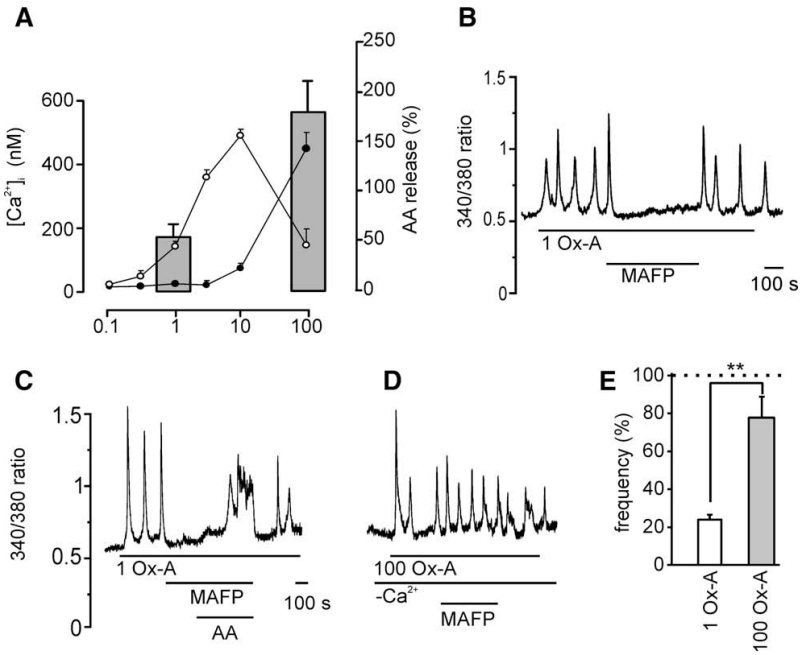

Fig. 2. Role of arachidonic acid in Ca^{2+} oscillations elicited by Ox-A. In (A) concentration–response curves for Ox-A stimulated Ca^{2+} elevations in the presence or absence of extracellular Ca^{2+} in cell suspensions. (○) denotes the portion of Ca^{2+} elevation blocked by removal of extracellular $Ca^{2+}$and (•) denotes the Ca^{2+} elevation seen in the absence of extracellular Ca^{2+}. Each datapoint was averaged from at least 4 similar experiments (±SE). The bars inserted in the graph represent ^{3}H-arachidonic acid (AA) release at the representative concentrations (see methods section for details). In (B) and (C) representative recordings from cells treated with 1 nM Ox-A and in (D) with 100 nM Ox-A in Ca^{2+} free conditions. The periods of stimulation with Ox-A, removal of extracellular Ca^{2+} and treatment with 10 μM MAFP or with 10 μM AA are indicated by horizontal bars. (E) The data obtained from experiments similar to (B) and (D) were quantified by calculating the oscillation frequencies as spikes per second (Hz). The frequency elicited by Ox-A before the treatment with MAFP was set as a control (100%). The results are presented as the ratio between the oscillation frequencies during and before treatments and expressed as Frequency (%) ± SE ($n = 3$–5). The effects of MAFP on the oscillation frequencies induced by 1 nM Ox-A and 100 nM Ox-A in the absence of extracellular Ca^{2+} were compared.

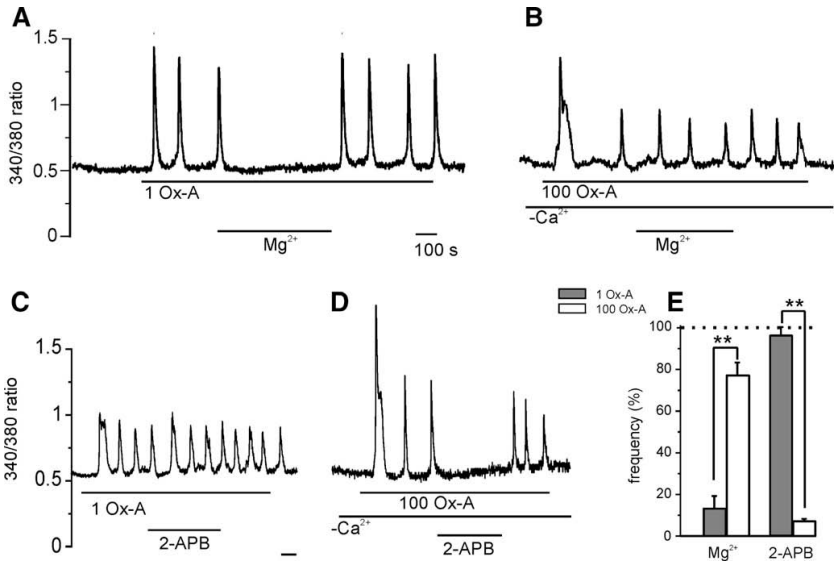

Fig. 3. Effect of elevated Mg^{2+} and 2-aminoethoxydiphenyl borate (2-APB) on Ca^{2+} oscillations elicited by low (1 nM) and high (100 nM) concentrations of Ox-A. In (A–D) representative recordings from cells treated with 1 nM Ox-A or with 100 nM Ox-A in Ca^{2+} free conditions. The periods of stimulation with Ox-A, removal of extracellular Ca^{2+} and treatment with 10 mM Mg^{2+} or with 1 μM 2-APB are indicated by horizontal bars. (E) The data obtained from experimental conditions similar to (A–D) were quantified by calculating the oscillation frequencies as spikes per second (Hz). The frequency elicited by Ox-A before the treatment with Mg^{2+} or 2-APB was set as a control (100%). The results are presented as the ratio between the oscillation frequencies during and before treatments and expressed as Frequency (%) ± SE (n = 3–5).

blocker of AA production in cells [reviewed in [24]]. We found that 10 μM MAFP potently inhibited the Ca^{2+} oscillations induced by 1 nM Ox-A (Fig. 2B, n = 5). Addition of 10 μM AA to cells treated with 1 nM Ox-A rescued the Ca^{2+} elevations inhibited by MAFP, but it failed to rescue the oscillation pattern (Fig. 2C, n = 3). The typical oscillatory pattern was resumed after washout of MAFP and AA. The oscillatory responses to 100 nM Ox-A in the absence of extracellular Ca^{2+} were largely unaffected by MAFP (Fig. 2D, n = 3). The effects of MAFP on oscillation frequencies are summarized in Fig. 2E. MAFP decreased the oscillation frequencies induced by 1 nM Ox-A and 100 nM Ox-A by 76% ($p < 0.01$) and 23% ($p < 0.01$), respectively. These effects of MAFP were significantly different from each other ($p < 0.01$).

In order to further resolve the mechanisms involved in the two oscillatory patterns seen we tested the effect of Mg^{2+} (a blocker of many TRP channels), on the Ca^{2+} oscillations. Ten mM Mg^{2+} blocked the Ca^{2+} oscillations induced by 1 nM Ox-A (n = 3) but had no visible effect on the Ca^{2+} oscillations induced by 100 nM Ox-A in the absence of extracellular Ca^{2+} (n = 3) (Fig. 3A and B). The inhibitory effect of Mg^{2+} on the oscillation frequencies in both conditions is summarized in Fig. 1C. Mg^{2+} decreased the oscillation frequency induced by 1 nM Ox-A by 87% ($p < 0.01$). 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of capacitative Ca^{2+} entry (CCE) and store discharge [reviewed in [25]], had no effect on the Ca^{2+} oscillations induced by 1 nM Ox-A (Fig. 3C, n = 5) but blocked oscillations induced by 100 nM Ox-A (Fig. 3D, n = 3). The oscillation frequency was decreased by 93% ($p < 0.01$).

Discussion

Different cellular processes such as the activation of protein kinases and transcription factors are regulated by Ca^{2+} oscillations at defined frequencies in the range between 1 and 100 mHz [reviewed in [1]]. As shown here Ox-A was able to elicit Ca^{2+} oscillations with a frequency of about 7 mHz, which frequency is known to activate protein kinases like ERK and protein kinase C. Interestingly Ox-A activates these kinase pathway in non excitable cells and neurons in a Ca^{2+} dependent manner [26].

Both the initial Ca^{2+} response induced by 1 nM Ox-A and the subsequent oscillations were acutely dependent on extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$). The possibility that Ca^{2+} permeable channels would function as an oscillators rather than replenishing stores is indicated by some previous reports. Diacylglycerols (DAG), which activate the TRPC3/6/7 subfamily of TRPC channels, activate Ca^{2+} oscillations in myometrial cells and astrocytes [9,10]. It is of interest in this context that low nanomolar concentrations of Ox-A causes the production of DAG independently of IP_3 production [18]. This together with the sensitivity of the oscillations to dominant negative trpc3N strongly suggests an involvement of TRPC3 channels. In addition elevated Mg^{2+}, which blocks over expressed TRPC3 channels [14] abolished the oscillatory response. Since TRPC3 is highly sensitive to changes in $[Ca^{2+}]_i$ it is a good candidate for generation of oscillatory responses through feedback and feed-forward mechanisms [27].

Arachidonic acid (AA) has previously been implicated to have a role in oscillatory responses [5]. We show here that 1 nM Ox-A caused a considerable production of AA and that MAFP, a potent inhibitor of PLA_2, inhibited the Ca^{2+} oscillations elicited by 1 nM Ox-A. This indicates that an AA activated pathway is involved in the oscillatory response. Mammalian TRPC channels and *Drosophila* TRP and TRPL channels, have been shown to be regulated by AA [28,29].

Removal of Ca^{2+} from the perfusion medium revealed an oscillatory pattern at high concentrations of Ox-A which was potently inhibited by 2-aminoethoxydiphenyl borate (2-APB), an inhibitor IP_3 mediated Ca^{2+} discharge [reviewed in [25]] and capacitative Ca^{2+} entry [30]. As these oscillations occur in the absence of $[Ca^{2+}]_o$ they would represent periodic Ca^{2+} discharge and reuptake of stored Ca^{2+} [2]. Previous studies have shown that higher concentrations of Ox-A activate the production of IP_3 and Ca^{2+} discharge from stores [14].

In conclusion, our studies reveal two separate pathways used by OX_1R to induce oscillation patterns of $[Ca^{2+}]_i$ depending on the agonist concentration stimulating the receptor. Low concentrations of Ox-A induce Ca^{2+} influx through Mg^{2+}-sensitive, AA-regulated Ca^{2+} channel, probably TRPC3. At higher concentration of Ox-A, Ca^{2+} oscillations are observed only in the absence of extracellular Ca^{2+} and are most probably derived from the periodic release and reuptake of Ca^{2+} from the intracellular Ca^{2+} stores.

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IV

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A role for PKD1 and PKD3 activation in modulation of calcium oscillations induced by orexin receptor 1 stimulation

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ABSTRACT

The neuropeptides orexin-A/hypocretin-1 (Ox-A) and orexin-B/hypocretin-2 play an important role in the control of energy metabolism via either of two G-protein-coupled receptors, orexin receptor 1 (Ox1R) and 2. Despite its significant physiological functions, signaling via orexin receptors is still poorly characterized. The aim of this study was to improve our understanding of early signaling events triggered by the binding of Ox-A to Ox1R. Using phosphospecific antibodies, we observed that early kinase activation by Ox-A in a HEK293 cell line stably expressing Ox1R (HEKOx1R) included ERK1/2, PKCδ, and PKD1. Elevation of intracellular Ca^{2+} is a well-characterized response to Ox1R activation. Comparison of Ox-A-induced calcium elevation and PKD1 activation demonstrated that both responses are detectable soon after stimulation and increase in a dose-dependent manner, but inhibition of protein kinase C, when low Ox-A concentrations are used, affects them differently. PKD family of protein kinases has 3 members: PKD1, 2, and 3, which are all expressed in HEKOx1R cells. In response to stimulation of the cells with 1 nM Ox-A, both PKD1 and PKD3 are activated and increased in the plasma membrane, pointing at a possible role for these kinases in that cell compartment. Overexpression of either kinase-dead PKD1 or kinase-dead PKD3 disrupts Ox-A-induced calcium oscillations demonstrating the functional role of these kinases in modulating physiological responses to Ox-A.

1. Introduction

Orexins/hypocretins, orexin-A (Ox-A) and -B, are hypothalamic neuropeptides [1,2] involved in multiple physiological functions including the regulation of feeding, wakefulness, breathing, reproduction, autonomic functions, and energy homeostasis (reviewed in references [3,4]). They activate two distinct G-protein-coupled receptors (GPCR), orexin receptor 1 (Ox1R) and 2 [2]. One well-characterized cellular response to binding of Ox-A to $G_{q/11}$-coupled Ox1R is an elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) [2,5–7]. More recently, we also characterized Ox-A-induced calcium oscillations in HEK293 cells stably expressing Ox1R (HEKOx1R) [8].

Stimulation of Ox1R by Ox-A has been shown to activate protein kinases: mitogen-activated protein (MAP) kinases [9–12], protein kinase C (PKC) [11,13], and Akt [14]. Activation of phospholipases by GPCR produces lipid second messengers, which are potent activators of novel PKC (reviewed in [15]) and protein kinase D (PKD) [16–18]. PKD1 has been shown to modulate ion channel trafficking [19] and activity [20–22].

In this study, we identified and characterized PKD1 and PKD3 responses to activation of Ox1R by Ox-A in HEKOx1R cells. We found that they are dose-dependent following a similar dose–response curve as Ox-A-induced calcium rise. Interfering with either PKD1 or PKD3 activity affects Ox-A-induced calcium oscillations, demonstrating the functional relevance of their activation in the physiological responses to Ox-A.

2. Materials and methods

2.1. Materials

P-(dipropylsulfamoyl)benzoic acid (probenecid), digitonin, and GF109203X (GF-X) and monoclonal anti-actin antibody were from Sigma-Aldrich (Helsinki, Finland). Fura-2-acetoxymethyl ester (fura-2 AM) was from Molecular Probes (Eugene, OR, USA). Human

Abbreviations: Ox-A, orexin-A; GPCR, G-protein-coupled receptor; Ox1R, orexin receptor 1; $[Ca^{2+}]_i$, intracellular calcium concentration; PKC, protein kinase C; PKD, protein kinase D; HEKOx1R, HEK293 cells stably transfected with Ox1R; GF-X, GF109203X; fura-2 AM, fura-2-acetoxymethyl ester; ERK1/2, extracellular signal-regulated kinase 1 and 2; EGFP, enhanced green fluorescent protein; DMEM, Dulbecco's modified Eagle's cell culture medium; HBM, HEPES-buffered Na^+ medium; RIPA, radioimmunoprecipitation assay buffer; TRPC, canonical transient receptor potential channel; DAG, diacylglycerol

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orexin-A (Ox-A) was from Neuropeptide (Strasbourg, France). FuGENE6™ was from Roche Diagnostics (Espoo, Finland). Phospho-PKC Antibody Sampler Kit, PKD1/PKCμ, Phospho-PKD1/PKCμ Ser744/748 and PKD1 Ser916 antibodies were from Cell Signaling (Danvers, MA, USA). Anti-active ERK1/2 was from Promega (Madison, WI, USA). Full-length A.v. polyclonal anti-GFP antibody and JL8 monoclonal anti-GFP antibody were from Clontech (Mountain View, CA, USA) and anti-Ox1R antibody from Alpha Diagnostic International (San Antonio, TX, USA). Dynabeads Protein G was from Invitrogen (Paisley, UK).

2.2. Cell culture

The generation of HEKOx1R cells has been described earlier [8,23]. The cells were grown in standard Dulbecco's modified Eagle's cell culture medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin–streptomycin (Invitrogen), and 0.05 mg/ml hygromycin (Invitrogen), at 37 °C in 5% CO_2 in an air-ventilated humidified incubator in 260-ml culture flasks (Nunc A/S, Roskilde, Denmark) or in 800-ml culture flasks for Ca^{2+} measurements in suspension.

2.3. Media for Ca^{2+} measurements

The HEPES-buffered Na^+ medium (HBM) consisted of the following (in mM): 137 NaCl, 5 KCl, 1 $CaCl_2$, 0.44 KH_2PO_4, 4.2 $NaHCO_3$, 10 glucose, 1 probenecid, 20 HEPES, and 1 $MgCl_2$; and the pH was adjusted to 7.4 with NaOH.

2.4. Detection and identification of PKD1, 2, and 3 mRNA in HEKOx1R cells

Primers were designed using eprimer3 (EMBOSS, CSC, Finland):

PKD1 5′-GCCAGCTTCGTAATGAGG-3′/5′-CCTGCCCTTTTCACTTGA-3′

PKD2 5′-CGCTCTTCCAGAACAACACG-3′/5′-ACGAAGTAGGTGG CATTGG-3′

PKD3 5′-CATGCCTGTTACTCCTCAAGC-3′/5′-AACTGGCCTGAAC CAAGC-3′

Total RNA was reverse-transcribed using Revertaid (Fermentas, Helsinki, Finland). cDNA was amplified using Dynazymes (Finnzymes, Espoo, Finland), 95 °C for 5 minutes, 94 °C/72 °C/55 °C 20 seconds each, 30 cycles, followed by 5 minutes at 72 °C. PCR products were purified and sequenced using ABI Prism system. Sequences were identified using Blast [24].

2.5. Transfection of kinase-dead PKD constructs

PKD1K612W-EGFP (PKD1kd) was a gift of A. Hausser [25], and EGFP-PKD3K605A (PKD3kd) from O. Rey [26]. For transfection, 200,000 HEKOx1R cells were seeded in 35-mm Petri dishes (Nunc A/S, Roskilde, Denmark) containing a coverslip (25-mm, Merck Eurolab, Espoo, Finland) in 2 ml of DMEM. After 18–24 hours, cells were transfected with 6 μl of FuGENE 6 (Roche Diagnostics, Espoo, Finland) and 1 μg of DNA.

2.6. Ca^{2+} imaging of single cells and cell suspension

For single-cell Ca^{2+} imaging experiments, the cells were loaded with 4 mM fura-2 AM at 37 °C in HBM for approximately 30 minutes. The coverslip was attached to the bottom of a thermostated (37 °C) perfusion chamber. Expression of the enhanced green fluorescent protein (EGFP)-tagged constructs was detected with 450–480 nm UV light and 520 nm barrier filter. The cells were excited at the wavelengths 340/380 nm under the control of an InCyt2™ system (Intracellular Imaging, Cincinnati, OH, USA). The emitted fluorescence was measured through a 430-nm dichroic mirror and a 510-nm barrier filter with a Cohu CCD camera. A new ratio image (340/380 nm) was collected every second. To investigate the effect of kinase-dead PKD constructs on peak heights induced by Ox-A, the cells were challenged by a short pulses of increasing concentrations (1, 10, and 100 nM) of Ox-A followed by 100 μM oxotremorine. Because EGFP fluorescence in transfected cells could interfere with the detected ratio (340/380 nm), response to oxotremorine was used as a control to normalize Ox-A responses. The results are expressed as percent of the peak height of the control response. Cells with no detectable response to 1 nM Ox-A were discarded in the analysis. Oscillation experiments were performed by stimulating the cells with 1 nM Ox-A for a prolonged period of time. The number of peaks appearing during the stimulation was calculated and divided by the duration of the stimulation. The oscillation frequencies are expressed as peaks per second (Hz). The data from single cell Ca^{2+} imaging experiments were imported into Microcal Origin™ 6.0, and further analysis was performed. Ca^{2+} measurements in cell suspension were performed as described earlier [23].

2.7. Data processing

The differences in the responses between two groups were evaluated by the unpaired Student's *t*-test. $p<0.05$ (*) was considered significant. Data are expressed as means ± SE.

2.8. Screen for active protein kinase

Cells were plated in 35-mm plates and grown to near confluency, then treated with 5 nM Ox-A in HBM at 37 °C at indicated times, buffer was removed, dish was transferred on ice, and cells were lysed in radioimmunoprecipitation assay buffer (RIPA). Protein concentration was determined using BCA™ Protein Assay Kit (Thermo scientific, Rockford, IL, USA). About 5–10 μg of protein was run on 7.5% or 10% acrylamide gel, transferred to polyvinylidene difluoride (PVDF) membrane, and probed with antibodies according to the manufacturer's instructions. Positive bands were detected with ECL + and scanned on STORM (GE, Uppsala, Sweden).

2.9. PKD3 microscopy and immunoprecipitation

HEKOx1R cells were transfected with EGFP-PKD3 [26], selected with 400 μg/ml geneticin (Invitrogen, Paisley, UK), and then plated on coverslips in 12-well plates. At near confluency, medium was replaced with HBM with 1 or 50 nM Ox-A. At selected time, cells were fixed in formalin, coverslips were mounted on glass slides with anti-fade and DAPI stain. Fluorescent cells were observed with fluorescence microscope, Olympus IX71. Images were captured using Olympus DP controller software. For immunoprecipitation, cells were plated to 60-mm plates, treated in HBM for 5 minutes, then lysed, and immunoprecipitated with full-length A.v. polyclonal anti-GFP antibody cross-linked to Dynabeads protein G. Western blots of immunoprecipitated proteins were probed with anti-phospho-PKD1/PKCμSer744/748 and JL8 monoclonal anti-GFP antibody.

2.10. Cell fractionation

HEKOx1R or HEKOx1R-EGFP-PKD3 cells were treated with Ox-A in HBM. Cytosolic and membrane fractions were isolated according to Brott et al. [27].

3. Results

To identify protein kinases activated by Ox-A stimulation of Ox1R, we treated near-confluent HEKOx1R cells with 5 nM Ox-A in HBM and lysed them at different time points in RIPA buffer. Total protein lysates were tested by Western blotting and detection with antibodies against selected phosphorylated PKC subtypes, PKD1, and extracellular signal-

regulated kinases 1 and 2 (ERK1/2). Phosphorylated forms of ERK1/2, PKCδ, and PKD1 were increased in a time-dependent manner in Ox-A-treated samples compared to untreated controls, but phosphorylation of PKCα/β T638/641 was unaffected by Ox-A treatment (Fig. 1A). The experiment was repeated three times with similar results. The first detectable response in this assay was the phosphorylation of PKD1 S916, which was always observed already in the first lysate (75 s or less). The phosphorylation of ERK1/2 was detected after about 90 seconds of treatment. The maximum detectable phosphorylation level was reached after 3 minutes in the case of ERK1/2 and around 5 minutes in the case of PKD1. ERK1/2 were only transiently phosphorylated. ERK2 phosphorylation remained detectable longer, but both started to decline towards baseline after 5 minutes. Phosphorylation of PKD1 was sustained for at least 1 hour (Fig. 1B). PKCδ phosphorylation was also detected in the first lysate but was less sustained after 30 minutes (Fig. 1B).

Phosphorylation of residue serine 916 in the active loop correlates well with the active state of PKD1 [28] (Fig. 1A). We could therefore use Western blot to characterize PKD1 response to Ox-A. We aimed to compare the newly identified PKD1 response with $[Ca^{2+}]_i$ elevation, which is a well-characterized response of Ox1R activation. Both responses were detectable very early (around 1 minute) after the stimulation. HEKOx1R cells responded to stimulation of Ox1R with intracellular calcium rise already when as little as 0.3 nM Ox-A was used. A stepwise increase in stimulating Ox-A concentration to 100 nM caused a concentration-dependent increase in this response (Fig. 2A). Similarly, already a low concentration of Ox-A (1 nM) was sufficient to induce detectable phosphorylation of PKD1 S916 residue (Fig. 2B). The peak of the response to 1 nM Ox-A occurred at 5 minutes. This time point was chosen to establish the dose-response curve presented in Fig. 2C (solid line). An apparent saturation of the response was reached with Ox-A concentration of 20 nM. The dose responses of Ox-A-induced PKD1 phosphorylation and $[Ca^{2+}]_i$ elevation were superimposable (Fig. 2C).

Because of the similarity between the two responses, further investigations were made to determine whether they are linked to

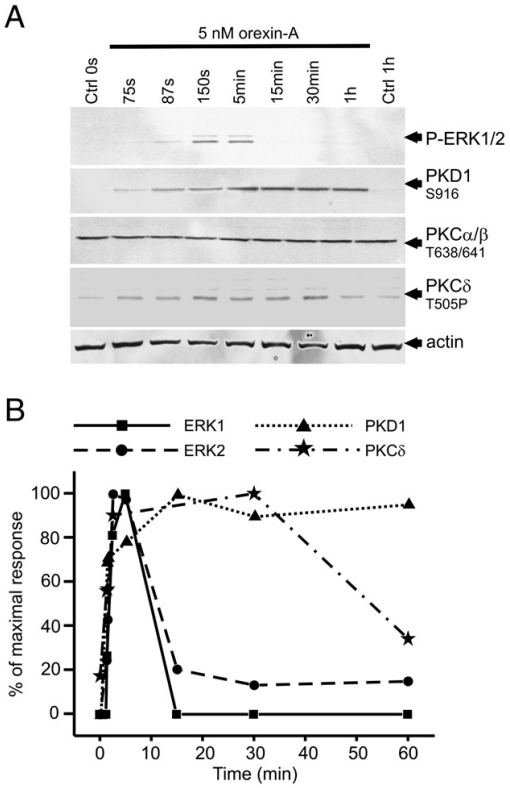

Fig. 1. Ox-A-induced phosphorylation of protein kinases. (A) Representative Western blot of HEKOx1R cells treated with 5 nM Ox-A for various times (indicated in the figure) and probed with anti-active ERK, anti-PKD1 S916p, anti-PKCα/β T638/641p, anti-PKCδ T505p, and anti-actin (positive control). Ctrl 0 s (first lane) and Ctrl 1 h (last lane) represent control samples treated with vehicle 0 second and 1 hour, respectively. (B) Time profiles of ERK1/2, PKD1 and PKCδ phosphorylation obtained by quantifying the scanned blots from experimental conditions similar to (A) with ImageQuant ($n=3$). Data are presented as percent of maximal response.

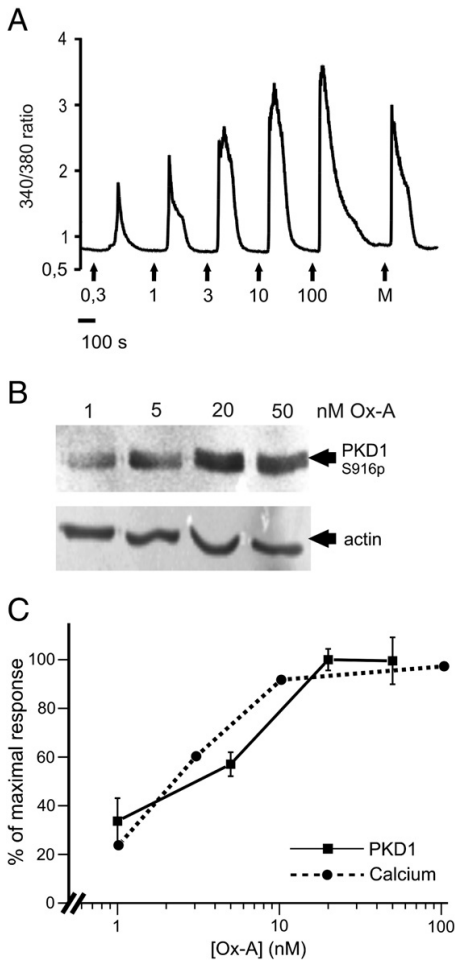

Fig. 2. Comparison of Ox-A-induced calcium and PKD1 responses. (A) Representative Ca^{2+} imaging recording with fura-2 AM from single HEKOx1R cell stimulated by increasing nM concentrations of Ox-A indicated in the figure and by 100 μM oxotremorine (M, positive control) at time points indicated by arrows. (B) Representative Western blot of HEKOx1R cells treated with increasing nM concentrations of Ox-A indicated in the figure and probed with anti-PKD1 S916p and anti-actin (positive control). (C) Dose–response curves of $[Ca^{2+}]_i$ elevation obtained from Ca^{2+} imaging recordings with fura-2 AM from cell suspensions of HEKOx1R cells stimulated by increasing concentrations of Ox-A ($n=9$–40) and of PKD1 activation obtained by quantifying the scanned blots from experimental conditions similar to (B) with ImageQuant ($n=2$). Data are presented as percent of maximal response ± SE.

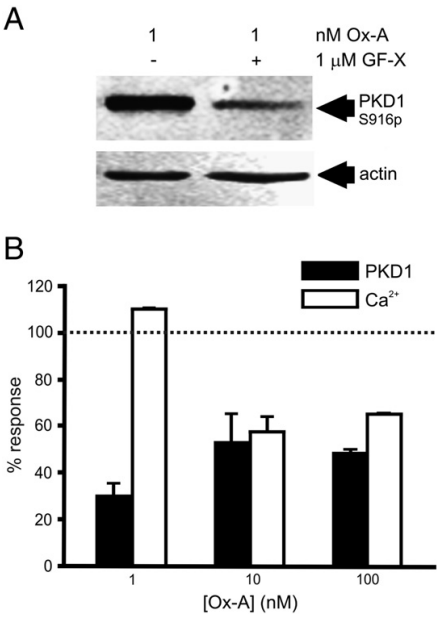

Fig. 3. Effect of PKC on Ox-A-induced calcium and PKD1 responses. (A) Representative Western blot of HEKOx1R cells treated with 1 nM Ox-A for 5 minutes in the absence and presence of PKC inhibitor GF-X (1 μM) as indicated in the figure and probed with anti-PKD1 S916p and anti-actin (positive control). (B) Comparison of the effects of GF-X on calcium and PKD1 responses. $[Ca^{2+}]_i$ elevations in the absence and presence of 1 μM GF-X were determined in Ca^{2+} imaging recordings with fura-2 AM from cell suspensions of HEKOx1R cells stimulated by increasing concentrations of Ox-A indicated in the figure ($n=2$–18). PKD1 activation was obtained by quantifying (ImageQuant) the scanned blots ($n\geq 3$) from experimental conditions similar to (A) with increasing concentrations of Ox-A. Responses in the presence of GF-X were compared with the responses without GF-X, and data are presented as percent of control response ± SE.

each other, keeping in mind that PKD1 activation has usually been described as being calcium-independent [29,30]. Most studies have found that PKD1 activation by GPCR is PKC-dependent [16,17,31,32]. We tested the importance of PKC using the PKC inhibitor GF-X and found that PKD1 phosphorylation induced by 1 nM Ox-A was clearly (approximately by 70%) inhibited by the treatment (Fig. 3A). In contrast, calcium response to low Ox-A concentration was unaffected by GF-X treatment. At higher concentrations (10 nM and above), GF-X showed a comparable inhibitory effect on both PKD1 and calcium responses (Fig. 3B).

Different cell types express different subtypes of PKDs. Using RT–PCR with subtype specific primers, we detected PKD1, PKD2, and PKD3 mRNA in HEKOx1R cells (confirmed by sequencing). PKD3 does not have the S916 phosphorylation site, but activation of all three PKD subtypes can be detected using an antibody against phosphorylated serine residue 744/748 in the active loop (reviewed in [33]). To be certain of the subtype identity, we used HEKOx1R cells stably transfected with an EGFP-tagged PKD3 construct [26]. Immunoprecipitation with anti-GFP antibody of control and Ox-A-treated cells, and subsequent Western blot detection with anti-active PKD(744/748) showed increased phosphorylation of PKD3 after stimulating HEKOx1R cells with 1 nM Ox-A for 5 minutes (Fig. 4A). Higher concentrations of Ox-A increased the detectable level of phosphorylated PKD3.

Activation of PKD1 and PKD3 leads to their translocation to other cell compartments [32,34–37]. To confirm that both PKD1 and PKD3 are activated by Ox-A, we investigated if they were translocated and where. Cell fractionation experiments indicated that the quantity of PKD1 was increased in a dose-dependent manner in membrane fraction after a 5 minutes treatment of the cells with Ox-A (Fig. 4B; anti-Ox1R antibody was used as a control to confirm loading of the membrane fraction). The membrane localization of PKD3 was also demonstrated by visualization of EGFP-tagged PKD3 by fluorescence microscopy. In untreated cells, the fluorescence was uniformly distributed throughout the cells. Treatment of the cells with Ox-A (1 nM or 50 nM) markedly brightened the fluorescence at the periphery of the cells (Fig. 4C).

When stimulated by 1 nM Ox-A for prolonged periods of time HEKOx1R cells show an oscillating response pattern which is independent of calcium discharge from intracellular calcium stores and involves canonical transient receptor potential channel 3 (TRPC3) channels [8]. To test if PKD1 or PKD3 had a role in the modulation of this type of oscillating calcium entry, we transfected HEKOx1R cells with EGFP-tagged kinase-dead PKD1 (PKD1kd) [25] and EGFP-tagged kinase-dead PKD3 (PKD3kd) [26] and conducted single-cell Ca^{2+} imaging experiments. Neither construct had any significant effect on peak amplitudes of calcium responses induced by Ox-A (comparison of fluorescent cells expressing kinase-dead construct with nonfluorescent control cells on the same plate). When stimulating the cells with 1 nM Ox-A, the peak height of PKD1 kinase-dead expressing cells ($n=48$) was 125 ± 12% and control cells (nonfluorescent, $n=53$) 145 ± 14% (peak heights expressed as % of the peak height of a subsequent addition of 100 μM oxotremorine). Corresponding values for PKD3kd ($n=63$) and its control ($n=71$) were 147 ± 6% and 137 ± 5%. The recorded oscillation patterns were, however, considerably altered. Overexpression of PKD1kd increased the frequency of the oscillations by approximately 1 peak for every 10 minutes of stimulation (6.2 ± 0.4 mHz in PKD1kd-expressing fluorescent cells ($n=62$) versus 4.9 ± 0.3 mHz in control cells ($n=42$); Fig. 5A). The observed increase was statistically significant (Fig. 5B). On the other hand, overexpression of PKD3kd in HEKOx1R cells completely disrupted the oscillation pattern (Fig. 6A). Only a sustained $[Ca^{2+}]_i$ elevation remained. The number of transiently oscillating cells was significantly decreased in this cell group compared to corresponding nonfluorescent control group (Fig. 6B). The oscillation frequency of the fluorescent cells showing oscillations did not differ from the controls (6.7 ± 0.6 mHz in PKD3kd-expressing fluorescent cells ($n=15$) versus 6.5 ± 0.4 mHz in control cells ($n=38$)).

4. Discussion

In spite of their high potential as drug targets (reviewed in references [3,4]), cellular events triggered by the activation of orexin receptors remain poorly understood. One of the most notable responses elicited by low concentrations of Ox-A is an entry of calcium into the stimulated cells [7,8,23,38–40]. This response is modified by the activation of protein kinases [5,6,39–41]. Ox-A has previously been shown to activate ERK1/2 [9–12]. We show here a fast Ox-A-induced phosphorylation of PKCδ and PKC-dependent activation of PKD1. All three members of PKD family (PKD1, 2, and 3) were detectable in HEKOx1R cells by RT–PCR, and in addition to PKD1, we observed an activation of PKD3 in the same conditions and at the same time.

PKD activation is a two-step event starting with diacylglycerol (DAG) binding to the C1 domain followed by phosphorylation by PKC [36,42]. Ox-A-induced activation of PKD1 follows the same pattern, which, in this study, was demonstrated by the inhibition of PKD1 activation by a broad-spectrum PKC inhibitor: GF-X. PKCε, which has been shown to be rapidly translocated to the membrane in response to Ox-A [11], and PKCδ, which, in this study, was activated following a similar time pattern as PKD1 and has been shown to be associated with PKD1 [43,44], are two likely candidates for the control of Ox-A-induced PKD1 activity. Once activated, PKDs are usually translocated to specific cellular compartments: membrane [34,36], nuclei [35,45],

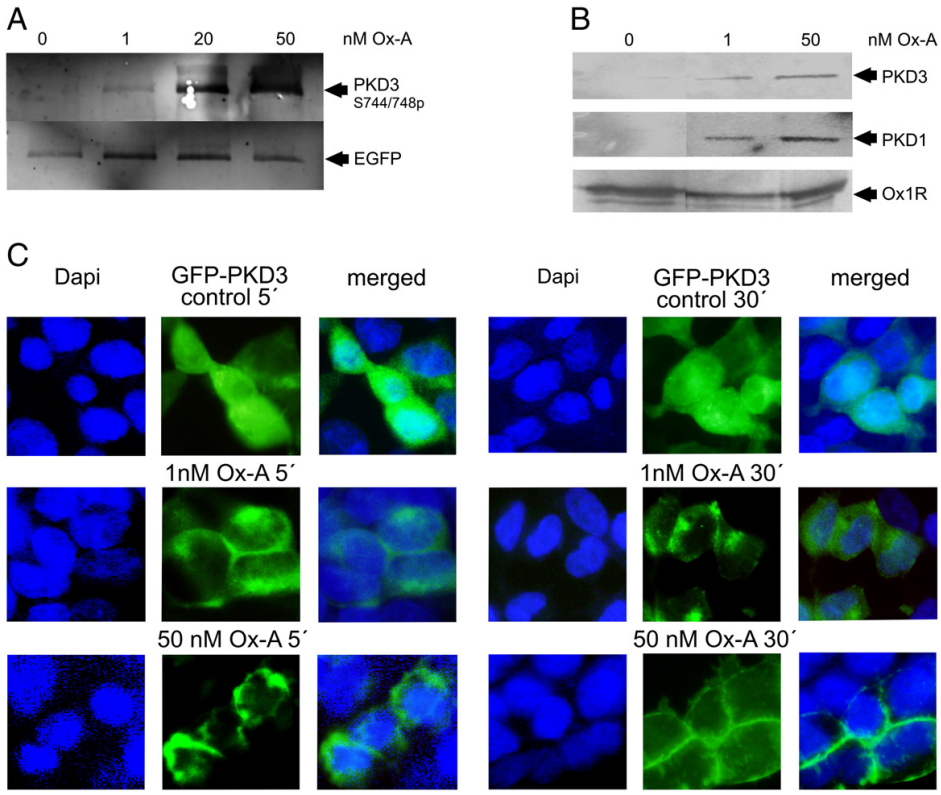

Fig. 4. Ox-A-induced activation and localization of PKD1 and PKD3. (A) Representative Western blot of HEKOx1R-EGFP-PKD3 cells treated with vehicle, 1, 20, or 50 nM Ox-A for 5 minutes, immunoprecipitated with polyclonal anti-GFP antibody, and probed with anti-active PKD (S744/748P) ($n=4$) and with anti-GFP (positive control). (B) Representative Western blots of cell membrane fraction of HEKOx1R and HEKOx1R-EGFP-PKD3 cells treated with vehicle, 1 nM or 50 nM Ox-A for 5 minutes and probed with monoclonal anti-GFP in the case of PKD3 ($n=2$), with anti-PKD1/PKCμ in the case of PKD1 ($n=3$), and with anti-Ox1R as a positive control ($n=3$). (C) Epi-fluorescence microscopy images of HEKOx1R-EGFP-PKD3 cells treated with vehicle (control), 1 nM or 50 nM Ox-A for 5 and 30 minutes.

mitochondria [43,46], and Golgi [47], where they can control downstream effectors. Here we show an increase of both PKD1 and PKD3 in membrane fractions as a result of Ox1R stimulation by Ox-A.

The primary activators of PKDs, DAG and PKC, also play important roles in the control of Ox-A-induced calcium response [5,6,39–41]. TRPC3, the main channel involved in Ca^{2+} influx activated by Ox1R

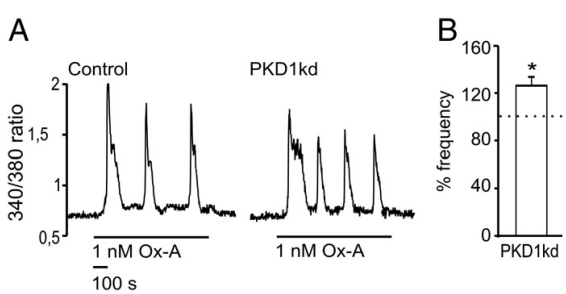

Fig. 5. Effect of kinase-dead construct of PKD1 (PKD1kd) on Ox-A-induced calcium oscillations. (A) Representative single cell Ca^{2+} imaging recordings with fura-2 AM from a control cell (left) and a PKD1kd-expressing cell (right) stimulated by 1 nM Ox-A for times indicated by horizontal bars. The cells successfully transfected by PKD1kd were identified based on EGFP fluorescence. (B) Summary of oscillation frequency data obtained from experimental conditions similar to (A). The oscillation frequencies of transiently oscillating PKD1kd-expressing ($n=62$) and control (nonfluorescent) cells ($n=42$) from same experiments were calculated as spikes per second (Hz) and compared. The oscillation frequency of control group was set as 100%, and the results are presented as percent frequency ± SE.

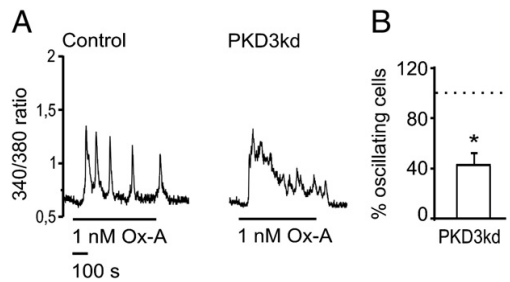

Fig. 6. Effect of kinase-dead construct of PKD3 (PKD3kd) on Ox-A-induced calcium oscillations. (A) Representative single cell Ca^{2+} imaging recordings with fura-2 AM from a control cell (left) and a PKD3kd-expressing cell (right) stimulated by 1 nM Ox-A for times indicated by horizontal bars. The cells successfully transfected with PKD3kd were identified based on EGFP fluorescence. (B) Summary of proportions of transiently oscillating cells obtained from experimental conditions similar to (A). The percentages of transiently oscillating cells from all fluorescent cells expressing PKD3kd ($n=65$) were calculated and compared with the corresponding percentages from nonfluorescent control cells ($n=74$) from the same experiments. The proportions of oscillating cells in control groups were set as 100%, and the results are presented as percent oscillating cells ± SE.

stimulation with 1 nM Ox-A [8,39,40], is both DAG-activated [48] and inhibited by PKC [49,50], which likely explains the absence of effect of PKC inhibitor GF-X in this case. Ca^{2+} responses to higher concentrations of Ox-A result from the activation of multiple signaling pathways, including PLCβ pathway, leading to discharge of Ca^{2+} from the intracellular stores [51], and PLA_2 pathway, leading to generation of arachidonic acid [8]. PKC is known to phosphorylate and regulate the function of for example IP_3R and PLA_2 among a plethora of other proteins participating in the regulation of Ca^{2+} signaling in cells (for review, see [52]). Thus, the exact mechanism of GF-X-induced inhibition is difficult to pinpoint, although many possible targets exist.

The specific PKC subtypes involved in Ox-A-induced Ca^{2+} response have not been identified and the modulatory effects resulting from PKC activation could also be indirect. Our initial investigation of the role of PKD subtypes showed that neither overexpression of kinase-dead PKD1 (PKD1kd) [25] nor kinase-dead PKD3 (PKD3kd) [26] significantly affected the amplitudes of calcium responses, but recent characterization of Ox-A-induced calcium oscillations [8] opened other avenues for the investigation of the role of PKDs in orexin signaling.

Calcium oscillations in response to GPCR stimulation are recognized to be an important and widespread signaling mechanism [53]. The pattern and frequency of calcium oscillations encode for different regulation of cellular functions, for instance, gene regulation and metabolism [54–56]. Calcium oscillations induced by 1 nM Ox-A in HEKOx1R cells are dependent on extracellular Ca^{2+} and sensitive to Mg^{2+} but not dependent on intracellular calcium store release and capacitative calcium entry [8]. In addition, phospholipase A_2 and the ion channel TRPC3 were shown to be essential for the generation of these specific oscillations. Both PKD1kd and PKD3kd affected 1 nM Ox-A-induced calcium oscillations but not in a similar manner: PKD1kd significantly increased the oscillation frequency while PKD3kd caused an apparent disruption of the oscillatory pattern. Although the effect of PKD3kd appeared very similar to that of dominant-negative TRPC3 [8], TRPC3 is unlikely to be the primary target of PKD phosphorylation because our careful analysis of TRPC3 sequence revealed no consensus sites for PKD phosphorylation. The effect of PKD3kd also differed from that of the dominant-negative TRPC3 since no effect was observed on the magnitude of the peak $[Ca^{2+}]_i$ elevation, which, on the other hand, was considerably attenuated by interference with TRPC3 function.

PKD1 has been shown to control a number of ion channels by different mechanisms [19–21,57]. Unlike for PKD1, specific roles for PKD3 in ion channel regulation have not been reported so far. Several recent reports may explain how activities of the different PKD subtypes can be differentially regulated and targeted by the same stimulus: each subtype has a different affinity for DAG [45] and PKD1 and 2 have an additional C-terminal autophosphorylation site missing from the shorter PKD3 (reviewed in [33]). Finally, PKD3 lacks the C-terminal PDZ-domain, which prevents it from binding certain partners of PKD1 and PKD2 [58,59]. Receptors are probably embedded inside big signaling complexes including many components, among which TRPC channels [60], anchoring proteins like filamin-A [61,62] or Homer [63] play a role in controlling Ca^{2+} responses induced by receptor stimulation. Assuming that molecules participating in Ox1R signaling are similarly organized around the receptor, PKD1 and PKD3 anchored at different locations in the complex and phosphorylating different proteins are likely to be part of the control mechanism responsible for Ox-A-induced oscillation pattern.

5. Conclusions

In conclusion, we describe a novel signaling pathway activated by Ox1R stimulation in well-characterized conditions. This pathway involves activation and membrane translocation of PKD1 and PKD3 followed by modulation of Ox-A-induced calcium oscillations.

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Signalling Mechanisms Used by the Orexin-1 Receptor

The orexin system consisting of two hypothalamic peptides, orexin-A and orexin-B, and two G-protein coupled receptors, orexin-1 and orexin-2 receptor, has multiple important physiological effects and is implicated in many disease states. If one wishes to understand these effects and to develop efficient therapies for orexin-related disorders the detailed knowledge of the signalling inside the cell is critical. This study sheds light on the previously poorly characterized cellular signalling mechanisms used by the orexin-1 receptor.

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