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Intracellular Ca^{2+} Pools and Ion-channels Involved in Signal Transduction in Insulin-secreting Cells

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Abstract

Stimulus-secretion coupling in the pancreatic β-cell is mediated by different signalling molecules and involves a variety of ionic events including Ca^{2+}-influx through voltage-gated Ca^{2+} channels and Ca^{2+} mobilization from intracellular stores. Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) mobilizes Ca^{2+} from intracellular non-mitochondrial stores in both normal β-cells and insulin-secreting tumor cell-lines. In electropermeabilized RINm5F cell, subsequent to $Ins(1,4,5)P_3$-induced release of Ca^{2+}, there was an increased sequestration of the ion. Blocking the $Ins(1,4,5)P_3$ channel by heparin also had similar effect and Ca^{2+} sequestration by heparin and $Ins(1,4,5)P_3$ was not additive. $Ins(1,3,4,5)P_4$ did not induce Ca^{2+} sequestration. In permeabilized RINm5F cells, the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors thapsigargin and 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (tBuBHQ) released Ca^{2+} mainly from the $Ins(1,4,5)P_3$-sensitive pool. In this respect thapsigargin was more specific than tBuBHQ. Although the inhibitors released a large part of Ca^{2+}, complete depletion of the $Ins(1,4,5)P_3$-sensitive pool was difficult to achieve. After prolonged treatment by the inhibitors, further Ca^{2+} could still be released by $Ins(1,4,5)P_3$.

Caffeine, the usual pharmacological tool for activating ryanodine receptor (RyR), released little or no Ca^{2+} from permeabilized RINm5F cells and mouse β-cells. Also cyclic adenosine diphosphate ribose (cADPR), did not release Ca^{2+}. But in these cells, Ca^{2+} was released from an $Ins(1,4,5)P_3$-insensitive pool by the sulfhydryl(SH)-reagent thimerosal. The release was not inhibited by heparin, but was reversed by the reducing agent dithiothreitol (DTT). Ca^{2+} was released even after mobilization of Ca^{2+} from the $Ins(1,4,5)P_3$-sensitive pool by $Ins(2,4,5)P_3$ or thapsigargin. Ca^{2+}-release by $Ins(1,4,5)P_3$ and thimerosal was additive. Thimerosal-induced Ca^{2+} release was potentiated by caffeine. In contrast to the lack of effect in permeabilized cells, caffeine increased $[Ca^{2+}]_i$, as measured by dual wave-length excitation microfluorimetry in fura-2 loaded intact β-cells. This $[Ca^{2+}]_i$-rise by caffeine occurred only in the presence of extracellular Ca^{2+} and was blocked by D-600 or nifedipine, blockers of L-type voltage-gated Ca^{2+} channel. Depletion of agonist-sensitive intracellular Ca^{2+} pools by thapsigargin did not inhibit caffeine-induced increase in $[Ca^{2+}]_i$. Caffeine inhibited K_{ATP} channel activity in excised inside-out patches. Paradoxically, in glucose-stimulated β-cells caffeine (>10 mM) reduced $[Ca^{2+}]_i$ by inhibiting Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channel. Low dose of caffeine (5 mM), when added after glucose-stimulated increase in $[Ca^{2+}]_i$, induced fast $[Ca^{2+}]_i$ oscillations.

In excised inside-out patches of mouse β-cells, the SH-reagents, thimerosal and 2,2´-dithio-bis(5-nitropyridine) (DTBNP) inhibited K_{ATP} channel activity, an effect reversed by DTT. Thimerosal, which is hydrophilic, was effective only when applied to the cytoplasmic side of the plasma membrane whereas DTBNP, which is lipophilic, was effective from either side of the plasma membrane.

It is concluded that Ca^{2+} release by $Ins(1,4,5)P_3$ may be followed by increased re-sequestration of the ion. This phenomenon is possibly due to conversion of basally active $Ins(1,4,5)P_3$ receptors to a poorly conducting state following activation by the trisphosphate, thus reducing Ca^{2+} leakage and thereby increasing sequestration. In permeabilized RINm5F cells, thapsigargin and tBuBHQ mobilize Ca^{2+} predominantly from the $Ins(1,4,5)P_3$-sensitive pool but do not deplete the pool readily, suggesting possible division of this pool into uptake- and release-compartments. Pancreatic β-cells may have a ryanodine-receptor-like intracellular Ca^{2+} release channel, that can be experimentally activated by SH-reagents but is largely insensitive to caffeine or cADPR. Caffeine affects $[Ca^{2+}]_i$ in intact β-cells by mechanisms independent of its action on intracellular Ca^{2+} pools. It inhibits K_{ATP} channel activity, leading to cell-depolarization, opening of the L-type voltage-gated Ca^{2+} channel and increase in $[Ca^{2+}]_i$. A $[Ca^{2+}]_i$-lowering effect of caffeine, in glucose-stimulated β-cells, is due to inhibition of the L-type voltage-gated Ca^{2+} channel. The K_{ATP} channel of β-cells have functionally important SH-groups on the cytoplasmic side of the plasma membrane.

Key words: *Signal transduction, Ca^{2+} signalling, RINm5F cell, Mouse pancreatic β-cells, $Ins(1,4,5)P_3$, Sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase, Thapsigargin, 2,5,-di-(t-butyl)-1,4-benzohydroquinone, Intracellular Ca^{2+} pools, Thimerosal, Sulfhydryl reagents, Caffeine, Cyclic ADP-ribose, Calcium-induced calcium release, K_{ATP} channel, L-type voltage gated Ca^{2+} channel, Cyclic AMP, $[Ca^{2+}]_i$ oscillation, Bafilomycin A_1, Manoalide.*

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"A man would do nothing,
if he waited
until he could do it so well that
no one would find any fault
with what he has done"
-Cardinal Newman

To
Sayera Khatoon and
A. T. M. Shamsul Islam

Erratum
Paper II, p289, 3rd line from bottom: the line should read "Ryanodine (100 nM, final concentration)

ABBREVIATIONS

AMP	Adenosine monophosphate
b.p.	Base pairs
cADPR	Cyclic adenosine diphosphate ribose
CAM Kinase II	Calcium-calmodulin-dependent protein kinase II
cAMP	Adenosine 3′,5′-cyclic monophosphate
CCCP	Carbonyl cyanide *m*-chlorophenyl-hydrazone
CCK	Cholecystokinin
CICR	Calcium-induced Ca^{2+} release
D-600	Methoxyverapamil
DAG	Diacylglycerol
DHP	Dihydropyridine
DTBNP	2,2′-dithio-bis(5-nitropyridine)
DTT	Dithiothreitol
ER	Endoplasmic reticulum
ETH	Swiss Federal Institute of Technology
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like polypeptide-1
GSSG	Oxidized glutathione
$Ins(1,3,4,5)P_4$	Inositol 1,3,4,5-tetrakisphosphate
$Ins(1,4,5)P_3$	D-*myo*-inositol 1,4,5-trisphosphate
$Ins(2,4,5)P_3$	D-*myo*-inositol 2,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
K_{ATP} channel	ATP-sensitive potassium channel
K_{Ca}	Calcium-activated potassium channel
NAD^+	Nicotinamide adenine dinucleotide
NBD-Cl	7-chloro-4-nitrobenz-2-oxa-1,3-diazole
NEM	*N*-ethylmaleimide
NO	Nitric oxide
PtdIns-4,5-P_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLC	Phospholipase C
PVC	Poly(vinyl chloride)
*R*p-cAMPS	*R*p-adenosine-3′,5′-monophosphorothioate
RyR	Ryanodine receptor
SERCA	Sarco(endo)plasmic reticulum Ca^{2+}-ATPase
SH	Sulfhydryl
SR	Sarcoplasmic reticulum
tBuBHQ	2,5,-di(*t*-butyl)-1,4-benzohydroquinone
$[Ca^{2+}]$	Ambient free calcium concentration
$[Ca^{2+}]_i$	Cytoplasmic free calcium concentration

REPORTS CONSTITUTING THE THESIS

This thesis is a summary of the results presented in the following papers which in the text will be referred to by their Roman numerals.

I. **Interaction with the inositol 1,4,5-trisphosphate receptor promotes Ca^{2+} sequestration in permeabilized insulin-secreting cells.** Islam, M. S., Nilsson, T., Rorsman, P., and Berggren, P.-O. *FEBS Lett.* 1991. **288**, 27-29

II. **Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells.** Islam, M. S., Rorsman, P., and Berggren, P.-O. *FEBS Lett.* 1992. **296**, 287-291.

III. **Sulfhydryl oxidation induces rapid and reversible closure of ATP-regulated K^+ channels in pancreatic β-cells.** Islam, M. S., Berggren, P.-O., and Larsson, O. *FEBS Lett.* 1993. **319**, 128-132

IV. **Mobilization of Ca^{2+} by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone in permeabilized insulin-secreting RINm5F cells: evidence for separate uptake and release compartments in inositol 1,4,5-trisphosphate-sensitive Ca^{2+} Pool.** Islam, M. S., and Berggren, P.-O. *Biochem. J.* 1993. **293**, 423-429

V. **Cyclic ADP-ribose in β cells.** Islam, M. S., Larsson, O., and Berggren, P.-O. 1993. *Science*, **262**, 584-585

VI. **Cyclic ADP-ribose and pancreatic β cells.** Islam, M. S., Larsson, O., and Berggren, P.-O. 1993. *Science*, **262**, 1499

VII. **Caffeine induces changes in cytoplasmic free calcium concentration by mechanisms independent of its action on intracellular Ca^{2+}-pools in pancreatic β-Cells.** Islam, M. S., Larsson, O., and Berggren P.-O. (*Manuscript*)

CONTENTS

INTRODUCTION

Under physiological conditions, secretion of insulin from the pancreatic β-cell is regulated by a complex interplay between multiple signal transduction pathways (1). In the β-cell, metabolism of glucose is coupled to an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) through the participation of at least two different types of ion-channels: the ATP-sensitive potassium channel (K_{ATP}) (2) and the L-type voltage gated Ca^{2+} channel (3). According to the current model (Fig.1), metabolism of glucose or other nutrients increases the cytoplasmic ATP/ADP ratio leading to closure of the K_{ATP} channel,

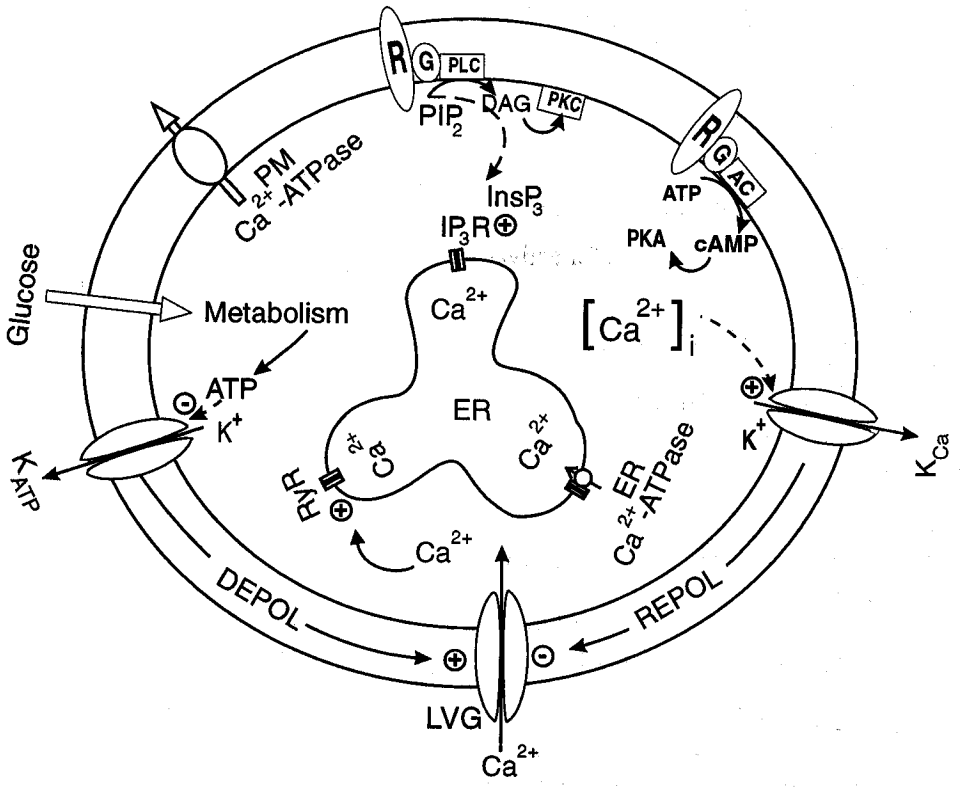

Fig. 1. Schematic diagram illustrating some of the components of β-cell signal-transduction system. **R**, receptor; **G**, GTP-binding protein; **AC**, adenelyl cyclase; **PIP_2**, phosphatidyl inositol 4,5-bisphosphate; **$InsP_3$**, inositol 1,4,5-trisphosphate; **depol**, depolarization; **repol**, repolarization; **LVG**, L-type voltage-gated Ca^{2+} channel. For details refer to the text.

cell depolarization, opening of the L-type voltage-gated Ca^{2+} channel, rise in $[Ca^{2+}]_i$ and finally exocytosis of insulin (4,5).

However, it must be noted that glucose *per se*, in its physiological concentration, is not a

strong stimulator of insulin secretion (6). Gastrointestinal hormones and neurotransmitters also play important roles in the modulation of insulin secretion (7,8). These include gastric inhibitory polypeptide (GIP, also called glucose-dependent insulinotropic polypeptide), glucagon-like polypeptide-1 (GLP-1), acetylcholine and cholecystokinin (CCK). GLP-1 and probably also GIP stimulate the formation of cAMP (8,9). Acetylcholine and CCK activate phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P_2) in the plasma membrane and forms two signalling molecules: Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG) (10,11). Ins(1,4,5)P_3 releases Ca^{2+} from intracellular stores (12) and DAG activates a phospholipid- and Ca^{2+}-dependent protein kinase called protein kinase C (PKC) (13). cAMP is metabolized to AMP and Ins(1,4,5)P_3 is metabolized to a variety of derivatives some of which may have messenger function of their own (14).

Importance of β-cell signal transduction studies is underscored by the widely held view that abnormalities in these mechanisms may underlie defective insulin secretion in non-insulin-dependent diabetes mellitus (NIDDM). From this point of view, studies of Ca^{2+}- and inositol polyphosphate-mediated signalling is particularly interesting. This is so not only because it is one of the most complex mode of signalling (13), but also because Ca^{2+}-dependent signalling pathways are implicated in different phases of insulin secretion (15). During recent years, many fascinating aspects of Ca^{2+} signalling in β-cells have been revealed by studies using the fura-2 technique or by monitoring of Ca^{2+}-dependent conductances (16,17). These include various forms of $[Ca^{2+}]_i$ oscillations and propagating Ca^{2+} waves. The β-cell, being electrically excitable, possesses dual oscillators: the plasma membrane oscillator and the cytoplasmic oscillator. In these cells, periodic entry of Ca^{2+} through the voltage-gated Ca^{2+} channels is one important mechanism of $[Ca^{2+}]_i$ oscillation (18). In addition, periodic Ca^{2+} release from intracellular stores also appears to be involved in the generation of $[Ca^{2+}]_i$ oscillations (16).

The precise identity and nature of intracellular Ca^{2+} stores that are mobilized on agonist-stimulation is unclear. They appear to be associated with the endoplasmic reticulum (ER) and are equipped with sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and Ca^{2+} release channels. In many cells these intracellular Ca^{2+} pools are structurally and functionally heterogenous. In β-cells, it is unclear what intracellular Ca^{2+} pools and Ca^{2+} release channels are present and how they are functionally organized. One approach to answer these questions is to study the effects of different endogenous ligands or pharmacological agents targeted against the Ca^{2+} release channels and SERCA associated with the Ca^{2+} pools. For this purpose, inhibitors of SERCA, like thapsigargin and tBuBHQ have proved particularly useful because of their ability to mobilize Ca^{2+} from the Ins(1,4,5)P_3-sensitive Ca^{2+} pool without generating Ins(1,4,5)P_3 (19,20). Caffeine and many sulfhydryl(SH)-reagents have been used to activate an intracellular Ca^{2+} release channel, the ryanodine-receptor (RyR), which mediates Ca^{2+}-induced Ca^{2+} release (CICR) in many cells (21-23). A potential difficulty in

using various pharmacological tools, however, is that they tend to have multiple actions which might complicate interpretation of results obtained with these agents. It is therefore necessary to study the effects of such agents on different components of signal-transduction systems, in any particular cell type.

In the present study I asked several questions: What intracellular non-mitochondrial Ca^{2+} pools and Ca^{2+} release channels are present in β-cells? How are they functionally organized? Which pools are mobilized by the SERCA inhibitors? What effect do the Ca^{2+}-mobilizing second messengers have on Ca^{2+} fluxes from these pools? In addition, I studied the effects of several pharmacological agents on the ion-channels of key importance in β-cell signal-transduction, to understand molecular basis of their actions.

BACKGROUND

Ca^{2+} release by Ins(1,4,5)P_3

Ins(1,4,5)P_3 level in pancreatic β-cells can be raised by: (a) activation of receptors linked to PLC (24), (b) Ca^{2+}-mediated hydrolysis of PtdIns-4,5-P_2 (25) and (c) possibly also following glucose metabolism (25,26). Stimulation by muscarinic agonists induces formation of Ins(1,4,5)P_3 and increase of $[Ca^{2+}]_i$ in β-cells (24). For the study of Ca^{2+}-release by Ins(1,4,5)P_3, most studies have used permeabilized-cells, where minute pores are made in the plasma membrane by using digitonin or intense electric shock (27). Ca^{2+} release by Ins(1,4,5)P_3 has been demonstrated in digitonin-permeabilized RINm5F cells (28), digitonin-permeabilized whole rat islets (29), electro-permeabilized dispersed mouse β-cells (24) and rat insulinoma microsomes (30). However, a recent report questions the role of Ins(1,4,5)P_3 as an intracellular Ca^{2+}-mobilizing second messenger in the β-cell on the ground that Ins(1,4,5)P_3 did not release Ca^{2+} from rat islet microsomes (31). In all studies with permeabilized cells and microsomes, Ins(1,4,5)P_3 releases only part of the Ca^{2+} sequestered into the non-mitochondrial Ca^{2+} pools, suggesting division of intracellular Ca^{2+} pools into Ins(1,4,5)P_3-sensitive and -insensitive components (28-30).

Ca^{2+} release by Ins(1,4,5)P_3 is fast. Kinetic studies using stopped-flow technique, demonstrate that a maximal dose of the trisphosphate releases Ca^{2+} with a half-time of 150-300 ms (32,33). The rate-limiting step in the channel opening is Ins(1,4,5)P_3-binding (34). Ins(1,4,5)P_3-binding to the receptor shows a Hill coefficient of one, indicating lack of cooperativity (35). But Ca^{2+} release by Ins(1,4,5)P_3 is highly cooperative with a Hill coefficient greater than three, as has been shown in permeabilized basophilic leukaemia cells (34,36). Apparently, four molecules of the trisphosphate must bind to the receptor before the channel can open (34). Cooperative Ca^{2+} release, however, is not seen in experiments where $[Ca^{2+}]$ is clamped, which may possibly be due to an inhibitory effect of Ca^{2+} chelators on IP_3R (37,38). The issue, however, is not clear since in planar lipid bilayer, IP_3R channel shows four subconductance levels suggesting that the trisphosphate opens the channel in an additive manner (39).

Ins(1,4,5)P_3 *per se*, even after prolonged exposure, does not desensitize its receptor (40,41). But high cytoplasmic $[Ca^{2+}]$ can convert the receptor to a "desensitized" form (42). Ins(1,4,5)P_3-induced Ca^{2+} release is characteristically biphasic (32,33). The initial phase is rapid and transient but then it spontaneously slows down without, however, losing responsiveness to a subsequent addition of Ins(1,4,5)P_3. Apparently, there is a spontaneous "inactivation" of the receptor shortly after Ins(1,4,5)P_3 binding and channel opening. Following Ca^{2+} release, the receptor possibly switches from a low-affinity conducting state to a high affinity poorly conducting state (32). Champeli *et al.* called it "partial desensitization", while Meyer *et al.* coined the term "increment detection" to describe this property of IP_3R

(32,33).

Another intriguing feature of Ca^{2+} release by Ins(1,4,5)P_3, is that submaximal concentrations of the trisphosphate release only a fraction of the releasable Ca^{2+} (43,44). Successive additions of increasing concentrations of Ins(1,4,5)P_3 elicit arithmetic increase in Ca^{2+} release (33,45). Muallem *et al* suggested that Ins(1,4,5)P_3-induced Ca^{2+} release is a "quantal" rather than a continuous process (43). According to this view, the Ca^{2+} stores are extensively compartmentalized and equipped with IP_3R that have different thresholds for activation by Ins(1,4,5)P_3, so that individual stores are depleted in an all-or-none fashion (43). This view is consistent with results obtained from intact cells, where Ins(1,4,5)P_3-induced Ca^{2+} release is highly localized and of almost all-or-none type (46,47). A second hypothesis holds that the Ins(1,4,5)P_3-sensitive Ca^{2+} stores are equally sensitive to the trisphosphate and that Ca^{2+}-release occurs by a steady-state mechanism from all these stores until the luminal [Ca^{2+}] is lowered to a level that reduces the affinity of the IP_3R for the trisphosphate (48,49).

Ins(1,4,5)P_3 receptors

IP_3R is a ligand-gated cation-channel, as evidenced by the ability of reconstituted receptors in lipid vesicles to mediate Ca^{2+} flux in response to Ins(1,4,5)P_3 (50). The receptor is a large protein. Cross-linking studies show that the native receptor is a noncovalently coupled homotetramer of 320 kDa subunits (39). A three domain model for IP_3R has been proposed based on analysis of deduced amino acid sequence and mutagenesis studies. According to this model, each subunit has a cytoplasmic N-terminal ligand-binding domain, a short C-terminal membrane-spanning domain and a large intervening coupling domain (51). The membrane-spanning domains of the four subunits form the Ca^{2+} channel. Diversity of IP_3R is caused by the existence of four gene products (52-55), alternative splicing of the messenger RNA (53,56), posttranslational modifications of the protein (57,58) and the tetrameric nature of the receptor. The original cerebellar IP_3R is now called IP_3R-1. Subtypes of IP_3R-1 arise from variable splicing of two segments SI and SII. SI (45 b.p.) lies in the Ins(1,4,5)P_3-binding domain and SII (120 b.p.) in the regulatory domain between the two phosphorylation sites. Accordingly, the IP_3R-1 may be SI+ or SI- and SII+ or SII- (53,56). The SII has three further splicing subsegments, A, B, and C producing SII+, SIIB-, SIIBC-, SIIABC-. Peripheral tissues express only the SII- isoform, whereas isoforms that carry any of the subsegments within the SII are expressed only in the nervous system (56,59). IP_3R-2 has 69% sequence homology with the IP_3R-1 and has a higher affinity for Ins(1,4,5)P_3 (54). IP_3R-3 has been cloned from RINm5F cells and has 62 and 64% identity between amino acid sequences with rat IP_3R-1 and IP_3R-2, respectively (60). The most conserved region among the IP_3Rs is the ligand-binding domain. The coupling domain which contains two consensus sequences for PKA phosphorylation (61) and ATP binding site, is the least conserved region and is a likely target for regulatory control (54). In contrast to IP_3R-1, IP_3R-2 has no consensus sequence for phosphorylation by PKA (54).

Distribution of IP_3R types appears to be tissue specific and more than one type may be expressed in a single cell (55,62). They may also have different binding, gating and regulatory properties as well as different subcellular localizations (54,62). In insulin-secreting cells, IP_3R-3 is the predominant type (60). Functional differences between the different types have not been studied in detail. Imaging studies in pancreatic acinar cells, suggest that high affinity IP_3Rs are localized in a small trigger zone in the granular area and that low affinity IP_3Rs are localized in the basal area (63).

Structurally similar IP_3Rs can also exist in different interconvertible states, distinguished by different rates of association and dissociation and different channel activity (64,65). Interconversion between different states of the receptor may be mediated by both $[Ca^{2+}]_i$ and luminal $[Ca^{2+}]$. High $[Ca^{2+}]_i$ transforms IP_3R into a high affinity and poorly conducting state (64). This high affinity state of the receptor is characterized by a slow rate of association and a slow rate of dissociation of the ligand-receptor complex. Interestingly, the SH-reagent thimerosal also transforms the receptor into a high affinity state. But in thimerosal-induced high affinity state, the receptor remains in its active form; there is slow rate of dissociation but the rate of association remains unaltered (65). In contrast to the high $[Ca^{2+}]_i$, high luminal $[Ca^{2+}]$ appears to favour a high-affinity conducting state of the receptor (66).

$Ins(1,4,5)P_3$-binding and Ca^{2+} mobilization is inhibited by polymeric anions like heparin and polyvinyl sulfate (67-69). The basis of structural similarity between these compounds and $Ins(1,4,5)P_3$ is that they are polyanions (69). Heparin, however, binds with many macromolecules where its actions are generally inhibitory (70). It inhibits, for instance, $Ins(1,4,5)P_3$ 3-kinase (71), $Ins(1,3,4,5)P_4$ binding and action (72) and protein phosphatase-1 (73,74). An agonist-like action of heparin on intracellular Ca^{2+} release channels is seen in platelets (75) and on L-type voltage-gated Ca^{2+} channels in muscle (76). Decavanadate also inhibits IP_3R but has many unspecific actions (77). Specific blocking of the $Ins(1,4,5)P_3$-induced Ca^{2+} release may be achieved by monoclonal antibodies that recognize an epitope near the free C-terminal tail of the IP_3R (78).

Localization of IP_3R

Localization of IP_3R varies in different cell types. Immunocytochemical studies and immunogold labelling in Purkinje cells show that IP_3Rs are located mainly on smooth-surfaced ER (79-81). But a close association of IP_3R and the $Ins(1,4,5)P_3$-sensitive organelle with the plasma membrane, has been shown in many non-neuronal cells. In liver cells, $Ins(1,4,5)P_3$-binding is associated with the plasma membrane fraction but the receptor is probably not *on* the plasma membrane (82,83). In olfactory neurones and T-lymphocytes IP_3R appears to be located *on* the plasma membrane, where it may be involved in Ca^{2+} entry (84,85). In HIT-T15 cells, the $Ins(1,4,5)P_3$-sensitive organelle is associated with a surface-derived vesicle which can be isolated by a hydrodynamic shearing technique (86). In islet

cells, localization of IP_3R on the nuclear membrane has been described (60). Finally, electron microscopy shows that IP_3R-like structures are present in the plasma membrane caveolae in some cells (87). In bovine adrenal medullary cells, the secretory vesicles also act as $Ins(1,4,5)P_3$-sensitive stores (88).

Regulation of IP_3R

Ca^{2+}, ATP, pH, phosphorylation and redox changes may affect IP_3R activity. IP_3R binds Ca^{2+} directly and the coupling domain of IP_3R contains several sequences resembling Ca^{2+}-binding domains (89). The feedback effect of Ca^{2+} on the rate of $Ins(1,4,5)P_3$-induced Ca^{2+} release is biphasic and may be immediate (90,91). In the concentration range of 0.01-0.3 μM, Ca^{2+} acts like a co-agonist of IP_3R (38,90). In the presence of a fixed concentration of $Ins(1,4,5)P_3$, the receptor is apparently able to mediate CICR (91). The molecular mechanism of the effects of Ca^{2+} on $Ins(1,4,5)P_3$-induced Ca^{2+} release appears to involve a direct alteration of the gating property of IP_3R (89,92). Ca^{2+} was reported to inhibit $Ins(1,4,5)P_3$-binding in cerebellar microsomes but not in other cells (64,67). It has now convincingly been shown that the inhibitory effect of Ca^{2+} on $Ins(1,4,5)P_3$-binding in cerebellar microsomes was an artifact due to Ca^{2+}-dependent activation of PLC, producing large amount of cold $Ins(1,4,5)P_3$ (89). Pre-receptor events, like the chelation of $Ins(1,4,5)P_3$ by Ca^{2+}, have also been postulated as possible mechanisms of inhibition of $Ins(1,4,5)P_3$ binding by Ca^{2+} (93).

In IP_3R-1, two ATP-binding consensus sequences are present in the N-terminal domain, close to the phoshorylation site (39). IP_3R-2 and IP_3R-3 have only one such consensus sequence (62) Photoaffinity labelling confirms that ATP binds to IP_3R (39). ATP is not an agonist of the channel, rather it regulates the channel allosterically (94). In the presence of the trisphosphate, low concentrations of ATP enhances IP_3R-channel activity and $Ins(1,4,5)P_3$-induced Ca^{2+} release (39,94,95). Higher concentrations of ATP inhibits IP_3R, probably by competing for the $Ins(1,4,5)P_3$-binding site (94).

Intracellular alkalinization, as occurs after glucose stimulation of β-cells (96), may increase $Ins(1,4,5)P_3$ binding and $Ins(1,4,5)P_3$-induced Ca^{2+} release (33,67). Some SH-reagents increase $Ins(1,4,5)P_3$ binding and sensitize $Ins(1,4,5)P_3$-induced Ca^{2+} release (*vide supra*), whereas reducing agents have the opposite effect (65,97,98).

Phosphorylation of the IP_3R by PKA, PKC and CAM Kinase II, may be a means for cross-talk between different messenger systems. PKA phosphorylation decreases the potency of $Ins(1,4,5)P_3$ for Ca^{2+} release in cerebellar microsomes (57). In contrast, purified IP_3R 1 reconstituted in lipid vesicles show increased Ca^{2+} fluxes upon PKA phosphorylation (99). In other cells it either does not affect (100) or markedly increases the potency of $Ins(1,4,5)P_3$ and the maximal $Ins(1,4,5)P_3$-releasable Ca^{2+} (101,102). The net effect of PKA phosphorylation on Ca^{2+} release may be determined by the effect of phosphorylation on

different types of IP_3R (99) as well as other components of the Ca^{2+} regulatory system, e.g. the ER Ca^{2+}-ATPase (57).

Prolonged treatment with muscarinic agonists, may suppress Ins(1,4,5)P_3-induced Ca^{2+} release by down regulating IP_3R (100,103).

Ca^{2+} SEQUESTRATION INTO ER

ER Ca^{2+}-ATPase

Active Ca^{2+} uptake into ER of islet cells is ATP- and Mg^{+}- dependent, stimulated by K^{+} and has a K_m of 1.5 μM for free Ca^{2+} and 26 μM for ATP (104). The uptake is mediated by SERCA, which translocates 2 mol of Ca^{2+} per mol of ATP hydrolysed (105). SERCA proteins are E_1-E_2 type ATPases with molecular mass around 110 kDa. They are encoded by three separate genes: SERCA1 is expressed only in skeletal muscle and undergoes alternative splicing to yield SERCA1a and SERCA1b (106). Differential processing of SERCA2 gene transcript yields four mRNAs (classes 1-4). Class 1 mRNA encodes the SERCA2a isoform. Class 2, 3 and 4 mRNA encodes the SERCA2b isoform (115 kDa; 1042 amino acids). The SERCA2a and SERCA2b isoforms are identical except that the C-terminal tetrapeptide of 2a is replaced by a tail of 49 amino acids in 2b (107). It is not known which isoforms of SERCA are expressed in β-cells. Non-muscle cells generally express SERCA2b and SERCA3, the former being more ubiquitous and abundant than the latter. SERCA3 has a reduced affinity for Ca^{2+} and higher affinity for vanadate (108). The three isoforms are similar in sensitivity to thapsigargin as inhibitor (*vide infra*) (109,110). However, in the platelet an unidentified isoform of SERCA (97 kDa) has lower sensitivity to thapsigargin and higher sensitivity to tBuBHQ (111,112).

The predicted secondary structure of SR Ca^{2+}-ATPase includes a large extramembraneous domain and ten helical segments, spanning the SR membrane as five hairpins (113). Four of these transmembrane helices contain residues that are involved in Ca^{2+} binding and may cluster to form a channel for Ca^{2+} translocation (114,115). The aspartyl residue that undergoes phosphorylation, is in the extramembraneous domain of the ATPase, distant from the Ca^{2+}-binding domain (114). Functional studies (116) and single channel recordings (117) suggest that SERCA pumps may behave like a Ca^{2+}-conducting channel that may account for passive permeability of the Ca^{2+} store. One study demonstrated that cells overexpressing ER Ca^{2+}-ATPase show increased frequency of Ca^{2+} waves, suggesting a role for the ATPase in the modulation of Ca^{2+} signalling as apart from its role in Ca^{2+} homeostasis (118).

In insulin-secreting cells, Ca^{2+} sequestration into ER was reported to be increased by glucose 6-phosphate (119, but see Ref. 24) and long chain acyl-CoA esters (120) but not by cAMP or calmodulin (121). The action of glucose 6-phosphate is suggested to be due to

binding of Ca^{2+} by P_i, which is formed on hydrolysis by glucose 6-phosphatase, an enzyme located within ER (119). Long chain acyl-CoA esters appear to promote Ca^{2+} sequestration by stimulating the ER-Ca^{2+} ATPase (120). In liver-cell, fatty acyl-CoA derivatives do not promote Ca^{2+} sequestration, rather mobilize Ca^{2+} from the non-mitochondrial pool (122). Colca *et al* also described an unidentified heat-stable islet cell cytoplasmic factor that stimulates Ca^{2+} uptake into ER (121).

In many cells, Ca^{2+} uptake into non-mitochondrial Ca^{2+} pools occurs also by mechanisms which are not sensitive to the inhibition by thapsigargin and do not involve ER Ca^{2+}-ATPase (123). These include H^+-dependent Ca^{2+} uptake (*vide infra*) and passive Ca^{2+} uptake from extracellular space by an ATP-dependent mechanism, where ATP possibly acts as a Ca^{2+}-binding agent rather than as a substrate for Ca^{2+}-ATPase (124).

Inhibitors of ER Ca^{2+}-ATPase

Three structurally dissimilar compounds, thapsigargin, tBuBHQ and cyclopiazonic acid, potently inhibit SERCA, without affecting other ion-motive ATPases or inositol phosphate metabolism (19,20,125,126). The mechanism of Ca^{2+} transport by Ca^{2+}ATPase is usually described by the E_1-E_2 model (127). According to this model the transition between two conformational states, E_1 and E_2, is an essential feature of Ca^{2+}-ATPase action. The two states differ in their affinity for Ca^{2+}. The dephosphorylated E_1 state has high affinity Ca^{2+} sites and the phosphorylated form E_2 has low affinity Ca^{2+} sites. Ca^{2+} binding sites are exposed to the cytoplasmic side in E_1 and to the luminal side in E_2. Binding of two Ca^{2+} to high affinity sites of E_1 is the essential first step. When these sites are occupied, ATP hydrolysis is triggered and the enzyme is phosphorylated to form the E_1PCa_2 intermediate. After a conformational change to E_2PCa_2, loss of Ca^{2+} into the lumen of ER allows dephosphorylation of the ATPase and return to the E_1 state, to repeat the cycle. The chemical specificity for phosphorylation is determined by the presence or absence of bound Ca^{2+}. The vectorial specificity for Ca^{2+} binding or dissociation on the two sides of the membrane is determined by the state of phosphorylation of the enzyme. The three inhibitors shift the E_1-E_2 equilibrium towards E_2 by reducing the rate of E_2 to E_1 transition and all of them inhibit Ca^{2+}-dependent phosphoenzyme formation (128,129).

Thapsigargin (Fig. 2) was isolated from *Thapsia gargantica*, a plant found in western Mediterranean countries (130). The compound is a hexaoxygenated tetraacylated sesquinterpene lactone and has tumor promoter activity (131). Thapsigargin inhibits SERCA with a stoichiometric ratio of 1 mol of inhibitor per mol of enzyme (110,132). Thapsigargin-binding site of SERCA molecule is probably in the N-terminal third of the molecule (133).

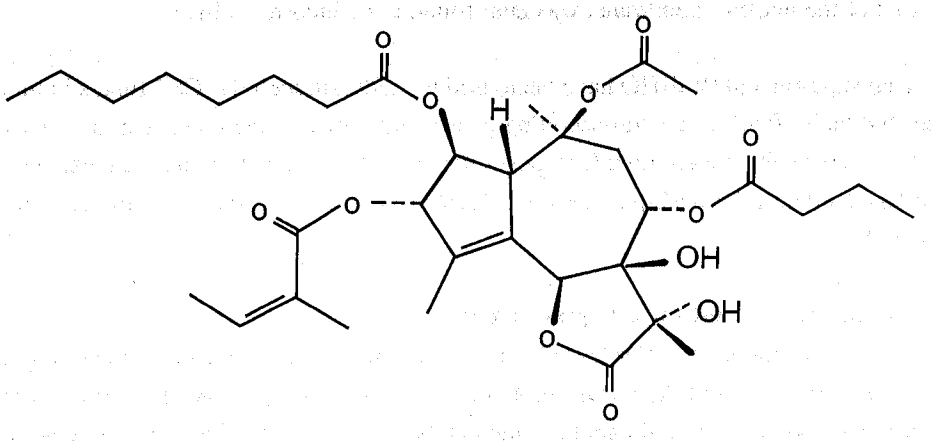

Fig. 2. Thapsigargin

Inhibition involves two partial reactions of the catalytic cycle of Ca^{2+} ATPase, *i.e.* Ca^{2+} binding in the absence of ATP and phosphorylation by P_i in the absence of Ca^{2+} (132). The interaction is extremely rapid in the absence of Ca^{2+}, almost irreversible and is unaffected by ATP concentration (110,134). The interaction of thapsigargin with ER Ca^{2+}-ATPase appears to be specific. However, a recent report demonstrated an inhibitory effect of thapsigargin on voltage-gated Ca^{2+} channels in adrenal glomerulosa cells (135). Moreover, thapsigargin and tBuBHQ have been shown to induce phosphorylation of different proteins (136,137), inhibition of DNA synthesis, protein synthesis and cell growth (138).

It was discovered by Moore *et al* that the hydroquinone tBuBHQ (Fig. 3) inhibits ER

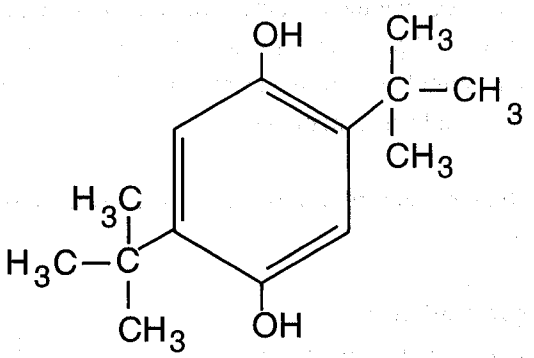

Fig. 3. tBuBHQ

Ca^{2+}-ATPase of hepatocytes, without inhibiting plasma membrane Ca^{2+}-ATPase or the mitochondrial F_1F_0-ATPase (20). It is less potent than thapsigargin and may interact with other proteins (139,140). Earlier claims that tBuBHQ would inhibit Ca^{2+}-pump of the ER and

not that of the nuclear membrane, was later found to be incorrect (141).

Thapsigargin and tBuBHQ have been used to study intracellular Ca^{2+} fluxes in many cell types but their effects in insulin-secreting cells is unknown. In the present study, I used these agents to define the intracellular Ca^{2+} pools that can be mobilized by these agents and investigated their relationship to the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in insulin-secreting cells (paper IV).

Ca^{2+}-sequestration and Inositol phosphates

Ins(1,4,5)P_3-induced Ca^{2+} release is rapid and transient. The transient nature is probably due to rapid inhibition of the release process and reuptake of Ca^{2+} (38). Inhibition of the release process can be brought about immediately by an elevated $[Ca^{2+}]_i$ and may possibly also be an intrinsic property of the Ins(1,4,5)P_3 receptor (38,91). Metabolism of Ins(1,4,5)P_3 does not seem to be a prerequisite for the reuptake of Ca^{2+}, although there is some correlation between the rate of reuptake of Ca^{2+} and the metabolism of the trisphosphate (28,142,143). However, most of the Ins(1,4,5)P_3 must be metabolized before response to a second dose of Ins(1,4,5)P_3 can be elicited (28,143).

Inositol 1,3,4,5-tetrakisphosphate, a metabolite of Ins(1,4,5)P_3, induced Ca^{2+} sequestration in a particular clone of electropermeabilized 261B neoplastic-rat-liver epithelial cells (144). In these cells, addition of Ins(1,3,4,5)P_4, under basal conditions, did not cause Ca^{2+} sequestration. Once $[Ca^{2+}]$ was raised by addition of Ca^{2+}, Ins(1,3,4,5)P_4 induced sequestration. Ca^{2+}-sequestering effect of Ins(1,3,4,5)P_4 has been described in only one other study, *i.e.* in adrenal medullary secretory vesicles and microsomes (145). The mechanism of Ins(1,3,4,5)P_4-induced Ca^{2+} sequestration is unknown, although the authors suggested that Ins(1,3,4,5) might stimulate Ca^{2+} pump or activate Ca^{2+} exchange mechanisms (144,145). Alternatively, the apparent stimulation of sequestration could be due to switching the Ins(1,4,5)P_3 receptor to a closed conformation (48).

Whether inositol polyphosphates play a role in the sequestration of Ca^{2+} in insulin-secreting cells is unknown.

H^+-dependent Ca^{2+} sequestration

An inside-to-outside directed H^+-gradient generated by V-type H^+ ATPase (V, for vesicular), can mediate Ca^{2+} uptake into non-mitochondrial Ca^{2+} pools in exchange for H^+ (146,147). In the presence of a preformed H^+-gradient, Ca^{2+} uptake can occur without ATP and in the presence of ER Ca^{2+} ATPase-inhibitors like vanadate (146). Proton translocating ATPases operate in secretory granules, which have acidic pH and high Ca^{2+} content (148,149). However the membrane potential of the insulin secretory granule is positive inside, which may make inward Ca^{2+} movement unfavourable. Ca^{2+} uptake into

adrenal medullary secretory vesicles is ATP-independent, the driving force for Ca^{2+} uptake being the transmembrane proton gradient (88). Low concentrations of NEM (1-2 μM) inhibit V-type ATPases, (P-type ATPases are inhibited only by high concentrations of NEM *e.g.* 0.1-1 mM and F_1F_0-type ATPases are virtually resistant to the inhibition by NEM). V-type H^+ ATPases are also inhibited by NBD-Cl and specifically by bafilomycin A_1, but are insensitive to vanadate (150). Oligomycin, an inhibitor of mitochondrial H^+-ATPase may inhibit V-type ATPase, when used at high concentrations (146). A H^+-dependent Ca^{2+} uptake has not been demonstrated in β-cells.

Cyclic ADP-ribose

While testing for Ca^{2+}-release by pyridine nucleotides, it was found that incubation of NAD^+ with sea urchin egg-extracts, yields a metabolite which releases Ca^{2+} from the egg microsomes (151). The active metabolite is cyclic adenosine diphosphate-ribose (cADPR) (Fig. 4).

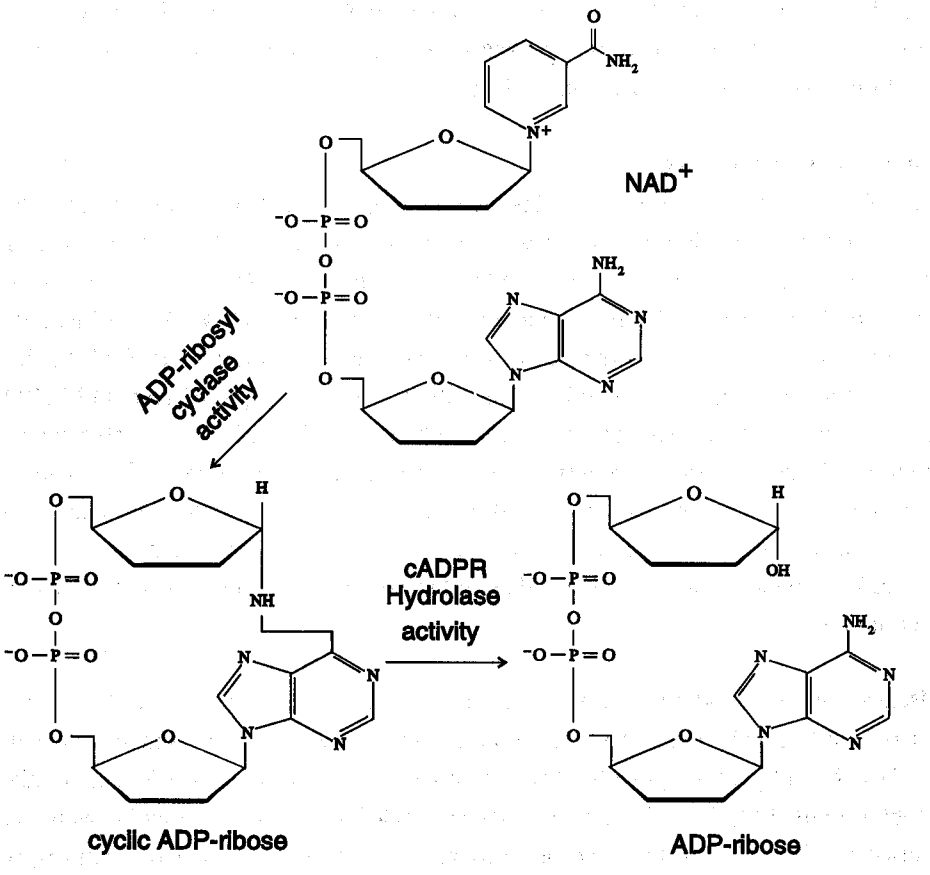

Fig. 4. Synthesis and degradation of cADPR

Formation of cADPR from NAD^+ involves cleavage of the nicotinamide-ribose bond and

cyclization of ribose to the adenine ring. Cyclization occurs by *N*-glycosyl linkage between the anomeric carbon of the terminal ribose and either the N^6-amino group (152) or position 1 of the adenine ring (153). In mammalian tissues, synthesis and degradation of cADPR is probably mediated by NAD glycohydrolases, a class of bifunctional enzymes exhibiting both ADP ribosyl cyclase and cADPR hydrolase activity (Fig. 4)(154,155). Intriguingly, the lymphocyte surface antigen CD38 also shows similar activity and can catalyze formation and hydrolysis of cADPR (156). In sea urchin egg, ADP-ribosyl cyclase activity can be stimulated by cyclic GMP and consequently it appears that ligands like NO, that activates guanyl cyclase, may act through the formation of cADPR (157). cADPR is a potent mediator of Ca^{2+} release in sea urchin eggs and also in some mammalian cells (31,151,158,159). In sea-urchin egg microsomes, specific binding of cADPR has been demonstrated and two binding proteins of 140 kDa and 100 kDa have been identified by photoaffinity labelling (154,160). Single-channel recording and pharmacological data suggest that cADPR is an endogenous regulator of type 2 RyR (161,162). According to Takasawa *et al*, in β-cells, cADPR and not $Ins(1,4,5)P_3$ is the intracellular Ca^{2+}-mobilizing second messenger (31). It was therefore, necessary to examine the role of the two messengers in the mobilization of Ca^{2+} from intracellular stores in insulin-secreting cells (paper V).

The Ryanodine Receptors

The Ca^{2+} release channel of sarcoplasmic reticulum is called ryanodine receptor, because the plant alkaloid ryanodine binds to it with nanomolar affinity (163). RyRs are present in most excitable cells e.g. cardiac muscle cells, smooth muscle cells, neurons and electrically excitable endocrine cells (164-169). In many cells the RyR coexists with the IP_3R, but the distribution of RyR is much more restricted compared to the ubiquitous IP_3R (165,170-172). RyR has been purified, cloned, expressed and studied in planar lipid bilayers (173,174). The functional RyR is a homotetramer of 560 kDa subunits. IP_3R and RyR, resemble each other in possessing a large cytoplasmic N-terminal domain and short C-terminal Ca^{2+} channel domain, which show some sequence homology (13). Purified RyRs, incorporated into planar lipid bilayers, show high conductance cation-permeable channels, which are activated by Ca^{2+}, ATP, caffeine, nanomolar ryanodine and are inhibited by Mg^+ and the polycationic dye ruthenium red.

Heterogeneity of RyRs is just being revealed (175). The skeletal muscle RyR (type 1 RyR) and cardiac RyR (type 2 RyR) are products of two genes on different chromosomes (174,176). Recently a third gene has been identified in TGF-β-treated mink lung epithelium and rabbit brain (177,178). The new RyR (type 3 RyR) is expressed at low levels in many non-muscle cells (177). Further heterogeneity may arise from tissue specific transcripts of RyR genes (179). Because of the tetrameric nature of the native RyR, it is also possible that heterotetrameric or multiple homotetrameric forms of RyRs exist (175). RyRs vary strikingly in their putative physiological mechanisms of activation. Type 1 RyR is activated in response

to depolarization, probably through its direct interaction with the dihydropyridine receptor (163). Type 2 RyR is activated by Ca^{2+} (166). The type 2 RyR is more sensitive to activation by Ca^{2+} and less sensitive to inhibition by Mg^{+} and ruthenium red than the type 1 RyR (173). Surprisingly, type 3 RyR is insensitive to caffeine, the usual probe for the RyRs (177). Cyclic ADP-ribose activates type 2 but not type 1 RyR (152,162), whereas its effect on type 3 RyR is unknown. Different RyRs may be expressed in a single cell and may possibly have different binding, gating, regulatory properties and subcellular localization. A 106 kDa Ca^{2+} release channel, which has pharmacological characteristics of RyR, has been isolated from skeletal muscle (23). The relationship of this channel to the RyR remains intriguing, but it may represent a fragment of the RyR in the transmembrane channel region of the receptor (180).

RyR mediates CICR, a process best illustrated in cardiac muscle where it is crucial for excitation-contraction coupling (166). The Ca^{2+}-sensing domain of RyR is thought to be located inside the channel, as suggested by the presence of some charged amino acids in this region of the type 1 RyR (114,181).

It is unknown whether β-cells contain a RyR-like channel.

Pharmacological tools for activation of RyR

A major drawback in studying CICR is the lack of suitable pharmacological agents for activation of the RyR. Caffeine (Fig. 5), the usual tool, is required to be used in high concentrations and has several actions unrelated to its action on the RyR. Single channel

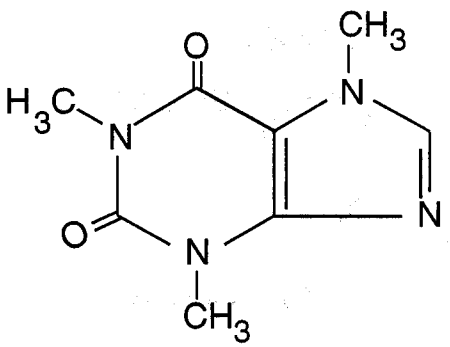

Fig. 5. Caffeine

recordings in cardiac SR show that caffeine increases channel open-time by increasing frequency and duration of open-events, without changing the unitary conductance (182). In contrast to its effects in muscle, high concentrations of caffeine (>20 mM) inhibit Ca^{2+} release

and ryanodine-binding in some non-muscle cells (183,184). As already mentioned, type 3 RyR is peculiar in being caffeine-insensitive (177).

Ryanodine, a plant alkaloid isolated from *Ryania speciosa Val* (185,186), is more potent and specific but is poorly permeable and has extremely slow association and dissociation kinetics (187). In muscle the $t_{1/2}$ for association and dissociation are 23 min and 14 h, respectively (188). But in liver ER, where ryanodine binds with high affinity, the association and dissociation kinetics are 100-1000 fold faster (184). Ryanodine binds preferentially to the open state of the channel (use dependence) and the effects are practically irreversible (167,189,190). It can act as both agonist and antagonist, depending on dose and experimental conditions (186). At a low concentration it locks the channel in a long-lasting low conductance open state (190), leading to depletion of the Ca^{2+} store (167). After binding with ryanodine, the channel also becomes unresponsive to Ca^{2+}, Mg^{+} and ATP (190). High concentrations of ryanodine (>50 μM) inhibits not only the RyR (189), but also the dihydropyridine-sensitive Ca^{2+} channel (191).

Other agents that have been used for activating the RyR include SH-reagents (23), scorpion venoms (192) and analogs of 9-methyl-7-bromoeudistomin D (193). Many SH-reagents release Ca^{2+} from sarcoplasmic reticulum by oxidizing critical SH-groups of the RyR (194,195). Two highly conserved cysteines in the C-terminal region of both the RyR and IP_3R are possible targets for the SH-reagents (13). Many proteins including the ER Ca^{2+}-ATPase, also contain critical SH-groups and it may be difficult to obtain specific effect with SH-reagents. Thimerosal (sodium ethylmercurithiosalicylate) (Fig. 6), for instance,

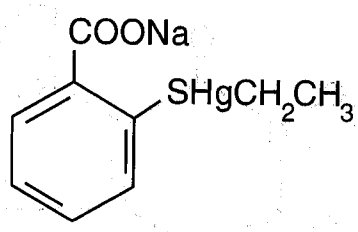

Fig. 6. Thimerosal

affects both RyR (196,197) and IP_3R (198) and may also inhibit the ER Ca^{2+} ATPase. However, higher concentrations of SH-reagents are generally needed for pump inhibition than for Ca^{2+} release (194). Some SH-reagents, *e.g.* the reactive disulfides react with SH-groups of RyR and not with those of Ca^{2+}-ATPase (199). Some other SH-reagents, specially in the presence of Ca^{2+}, may permeabilize the inner mitochondrial membrane and release Ca^{2+} taken up by mitochondria (200).

In intact β-cells caffeine increases $[Ca^{2+}]_i$ (272,273). Whether this is mediated by activation of RyR in these cells, is unclear. Therefore, in the present study, I examined the molecular mechanisms of action of caffeine and used alternative tools to obtain evidence for the presence of a RyR-like channel (paper II and VII).

Intracellular Ca^{2+} Pools

The relationship of rapidly exchangeable intracellular Ca^{2+} stores with the ER have been repeatedly discussed. The emerging consensus is that ER, as a whole, is not a specialized Ca^{2+} store, rather that Ca^{2+} stores are specialized subcompartments of ER (201,202). Both ER and the intracellular Ca^{2+} stores appear to be heterogenous (203). Ins(1,4,5)P_3 usually releases only a fraction of Ca^{2+} from the non-mitochondrial Ca^{2+} pools, indicating the presence of Ins(1,4,5)P_3-sensitive as well as -insensitive Ca^{2+} pools. This is seen particularly in experiments with permeabilized cells (12,24,204). To what extent this is an artifact of permeabilization, is an open question. However, also in experiments with intact cell-suspensions, such a division is seen (24). Presumably Ins(1,4,5)P_3-insensitive pools are the ones that do not posses the IP_3R. Transfection of IP_3R cDNA into an L-fibroblast cell line converts some Ins(1,4,5)P_3-*in*sensitive pools into Ins(1,4,5)P_3-sensitive ones, resulting in a larger Ins(1,4,5)P_3-sensitive pool in the transfected cells (205). The relationship between the Ins(1,4,5)P_3-sensitive pool and pools sensitive to SERCA inhibitors, like thapsigargin, has been the subject of study in many cells. In some clones of PC12 cells for instance, there is a pool that is sensitive to both Ins(1,4,5)P_3 and caffeine-ryanodine and is loaded by a thapsigargin-sensitive pump (164). In some other cells there seems to be two distinct pools, one of which is sensitive to Ins(1,4,5)P_3 and the other to caffeine-ryanodine (206,207). The caffeine-sensitive pool is generally known to mediate CICR.

The uptake and release sites of intracellular Ca^{2+} pools appear to be spatially separated. In sarcoplasmic reticulum, Ca^{2+} is taken up mainly in the tubular portion of the reticulum and is slowly translocated to the terminal cisternae, from where it is released (208). Furthermore, IP_3R and Ca^{2+} pump usually do not co-fractionate or co-localize (83,209).

In insulin-secreting cells, Ins(1,4,5)P_3-sensitive and -insensitive intracellular Ca^{2+} pools have been described (24,28). How these pools are functionally organized and in particular, the nature of the Ins(1,4,5)P_3-insensitive pools is unclear. In the present work, therefore, I aimed to answer some of these questions (papers II, IV and V).

The K_{ATP} Channel (K_{ATP})

A class of K^+-selective channels that can be blocked by intracellular ATP was first discovered in cardiac cells (210) and later in β-cells, where they are thought to couple nutrient metabolism to membrane depolarization (4). Available evidence suggests that different tissues express different subtypes of K_{ATP} channels, that may differ functionally. In

β-cells the channel provides K^+ conductance for the resting membrane potential. Furthermore, substances that close the channel depolarize the β-cell (3). In cell-free patches, channel activity is extremely sensitive to ATP, K_i being 0.015 mM (2). In resting cells, however, the channels remain open in spite of high intracellular ATP concentration (211). Possible explanations for this discrepancy are that intracellular ATP may be compartmentalized, free ATP may be more important than total ATP and that other molecules oppose the inhibitory effect of ATP. In fact, ADP markedly activates K_{ATP} channels that are inhibited by ATP and it is suggested that the physiological control of the channel is mediated by the ATP/ADP ratio, rather than by ATP alone (212).

K_{ATP} channel activity rapidly declines after patch-excision or cell-dialysis in whole-cell recordings. This phenomenon is called "run-down" and can be reversed by MgATP (213). This action of ATP is believed to be mediated by phosphorylation since non-hydrolyzable analogs of ATP can not activate the channel although they inhibit it (213). However, no kinase is known to mediate phosphorylation of the channel and an alternative hypothesis is that "rundown" is mediated by Mg^+. Changes in membrane potential and $[Ca^{2+}]_i$ do not have important effects on channel activity (2). Sulfonylurea drugs, that are used in the treatment of diabetes, and the hyperglycemic sulfonamide diazoxide act by closing and opening the K_{ATP} channel, respectively (5). The relationship of sulfonylurea receptor to the K_{ATP} channel is unclear, but the emerging consensus is that the receptor is a constitutive part of the channel (214,215).

The K_{ATP} channel is a common site of action of various pharmacological agents that affect insulin secretion. In mouse skeletal muscle, the K_{ATP} channel contains reactive SH-groups (216). Since some SH-reagents stimulate insulin-secretion, I hypothesized that functionally important SH-groups may also be present in the K_{ATP} channel of β-cells (paper III).

SH-groups and SH-reagents

Cysteine residues with their SH-groups are present in most proteins. Substances that react with SH-groups are called SH-reagents. Protein SH-groups vary in their reactivity with SH-reagents; some are readily reacting, some are slowly reacting while others are unreactive or masked (217). Differential reactivity of any particular SH-group with any particular SH-reagent may be due to steric and electrostatic factors, hydrophobicity, pH and ionization state of the SH-groups. The pKa-value of cysteine residues is one determinant of its reactivity, since in most reactions SH-groups take part as a mercaptide or a thiolate anion RS^-. pKa of cysteine within proteins can vary due to many factors, including presence or absence of charged neighboring groups and differences in hydrophobicity of the microenvironment. The reactivity of protein SH-groups can also be altered by conformational changes induced by agonist or antagonist binding (218).

There are numerous SH-reagents which may be broadly grouped into: 1. oxidizing agents e.g. GSSG, *o*-iodosobenzoate and alloxan; 2. mercaptide-forming agents e.g. metal ions and organic mercurials; 3. alkylating/arylating agents e.g. iodoacetate, NEM and chlorodinitrobenzene. Among the SH-reagents, the disulfides (Fig. 7) are known to be

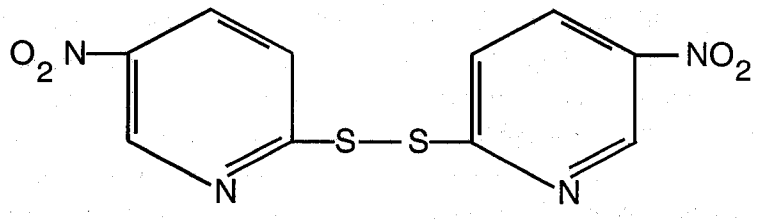

Fig. 7. DTBNP

absolutely specific for the oxidation of free SH-groups (199,219). Thiol-disulfide exchange reactions occur in two steps with the formation of a mixed disulfide as an intermediate. If there is only one reactive SH-group in a protein, the reaction ends with the formation of a mixed disulfide. Reaction of organic mercurials of the type R-Hg-X, occurs with only one SH-group. DTT and cysteine are used as disulfide reducing agents but the latter can also act as SH-oxidizing agent (220). The SH-group of cysteine, specially in the presence of micromolar Cu^{2+} or Hg^{2+}, can form mixed disulfide bond with protein SH-groups (195). The reaction with SH-group of metal ions and organic mercury compounds is reversible, with the equilibrium shifted to the weakly dissociating mercaptides. Alkylation reactions are irreversible.

Role of SH-groups in proteins

In different proteins, SH-groups have been shown to be involved in determining protein structure, binding of ligands to receptors (221), substrate and cofactor binding, enzyme catalysis, protein-DNA interactions (222) and ion-channel regulation (223). In some proteins, SH-groups are also the sites for covalent modification e.g. S-nitrosylation by NO and ADP-ribosylation (224). Information on the role of cysteine residues in structure and function of proteins are obtained from kinetic studies, chemical modifications, X-ray crystallography, analysis of evolutionarily conserved residues and site-directed mutagenesis. However, inhibition of the function of a protein by SH-reagents alone, may not always provide much useful information on the functional role of the SH-group.

Voltage-gated Ca^{2+} channels

Four types of voltage-gated Ca^{2+} channels namely the L, T, N, and P type channels have been described in different cells (225). In the β-cell, the L-type Ca^{2+} channel plays a key role in the stimulus-secretion coupling. These channels are activated by membrane depolarization to >-40 mV, inactivate slowly ($t_{1/2}$, 300-700 msec) and are blocked by three classes of organic Ca^{2+} channel blockers: 1,4 dihydropyridines e.g. nifedipine, phenylalkylamines e.g verapamil, and benzothiazepins e.g. diltiazem (226). The DHP-sensitive channel of skeletal muscle has five subunits: α_1, α_2, β, γ, and δ, all of which have been cloned (227). The α_1 subunit has transmembrane orientation and contains the binding sites for the antagonists (228,229). Tissue-specific isoforms of the α_1 subunit have been cloned from various cells including the β-cell (229,230). Transfection studies demonstrate that the α_1 and β subunits are the most important ones for channel function (231). Both of these subunits are substrates for PKA *in vitro* (228). Studies of purified L-type Ca^{2+} channels of skeletal muscle, show that cAMP-dependent phosphorylation increases the number of active Ca^{2+} channels and their probability of opening (232,233). There is also evidence that a voltage-dependent change in the conformation of the subunits favors phosphorylation by PKA (234). In the β-cell, however, the effect and physiological role of PKA phosphorylation of L-type voltage-gated Ca^{2+} channel remain less clear (8).

At the resting membrane potential of -70 mV, most of the channels are closed most of the time. Only about 10% of the channels are activated at -25 mV. As the β-cell membrane is depolarized the number of open Ca^{2+} channels increases, but the current flowing through each channel is reduced due to reduction in electrical driving force. It is estimated that a single β-cell has about a 1000 Ca^{2+} channels and that only about 2% of them are open during an action potential.

T-type Ca^{2+} channels have been described in rat and human β-cells and RINm5F cells, but their role in insulin secretion is unclear (235,236).

AIMS OF THE PRESENT STUDY

The aims of this thesis were to:

1. test the hypothesis that Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 affect Ca^{2+} sequestration in permeabilized insulin-secreting cells;
2. characterize different pharmacological agents namely thapsigargin, tBuBHQ and thimerosal for the study of intracellular Ca^{2+} pools and signal-transduction in β-cells;
3. test if insulin-secreting cells possess a RyR-like channel and a CICR pool;
4. study the effects of inhibitors of SERCA on Ca^{2+} fluxes and determine the relationship of inhibitor-sensitive pools to the Ins(1,4,5)P_3-sensitive pool in permeabilized insulin-secreting cells;
5. compare the Ca^{2+}-mobilizing effects of cADPR and Ins(1,4,5)P_3 in insulin-secreting cells;
6. investigate the effects of SH-reagents on the K_{ATP} channel activity and thereby test the hypothesis that functionally important SH-groups are associated with the channel;
7. study the mechanisms of actions of caffeine on $[Ca^{2+}]_i$ in β-cells;

METHODS

Cells

Clonal insulin-secreting RINm5F cells were used for studies described in papers I, II, IV, and V. This cell line, derived from a radiation-induced rat insulinoma (237), is the most commonly used insulin-secreting cell line for many studies including study of intracellular Ca^{2+} pools (28). These cells have low insulin content and do not respond to glucose but there is relatively high basal insulin secretion (238). The cells do respond to nutrients like glyceraldehyde, leucine and alanine (239). The cause of glucose-unresponsiveness is not clear, but a lack of glucokinase activity and increased hexokinase activity have been demonstrated in these cells (240,241).

β-cells from *ob/ob* mice were used for studies described in papers III, V and VII. The *ob/ob* mice are homozygous for the autosomal recessive obese (*ob*) gene and have been extensively used for signal transduction studies in β-cells. The mice used in this study were taken from a local non-inbred colony. These mice are characterized by extreme obesity, and marked inactivity (242). The islets are large and more than 90% of the cells are β-cells (243). From functional point of view these β-cells can be considered normal and if anything there is usually marked insulin secretion in response to glucose and a variety of other insulin secretagogues (5,244).

It must be emphasized that the phenotypic expression of the *ob/ob* syndrome depends on the background strain and accounts for the marked differences in different colonies. For

instance, the Aston *ob/ob* mice are characterized by a more severe form of diabetes and the β-cells from these mice appear to be insensitive to glucose and glibenclamide (245,246).

Permeabilization of cells

I used the technique of electropermeabilization (27) (papers I, II, IV and V). The apparatus consists of a plexiglass chamber with platinum electrodes placed 0.5 cm apart, a capacitor and a switch designed to discharge the capacitor in a single event. The voltage and capacitance were set at 1.6 kV (3.2 kV/cm) and 2 μF respectively. The capacitor was discharged six times through the cell-suspension in the permeabilization chamber. This treatment resulted in more than 90% permeabilized cells, as verified by Trypan blue uptake. The principle of electropermeabilization is that when cells are exposed to an intense electric field for short duration, there is development of voltage across the membrane which causes the membrane to break down and form pores. The magnitude of potential difference across a point in the spherical membrane depends on the intensity of the field (E), the radius of the membrane-bound vesicle (r) and the position of the point (P) in the circumference. This can be given by the equation:

$V_p = C \times r \times E \cos\theta$,

where C is a constant derived from relative electrical conductivities of the extracellular fluid, the cytosol, the membrane and the size of the cell. From the equation it is apparent that maximum transmembrane potential will be generated at opposite ends of the cell in line with the electric field. Intracellular organelles are of such size that the voltage developed across their limiting membrane is very small and they remain unaffected. One discharge is expected to produce two holes, which have a functional diameter of 2-4 nM (27,247,248). If the intensity of the electric field is increased, the apparent diameter of the pore is expected to increase (249). Increasing the number and duration of the electrical pulses increases the number of pores (249). This method of permeabilization is clean and quick, leaves large areas of plasma membrane unimpaired and can be used also for secretion studies (248).

Most studies with permeabilized cells have used detergent permeabilization. With this method, usually large areas of plasma membrane are lost and even the entire plasma membrane may be stripped off (247). It is difficult to ensure that the intracellular membranes are not damaged. This may cause leakage of macromolecules. In some studies, even the Ca^{2+} storage vesicles leaked out from detergent permeabilized cells (32). In most cases it probably does not matter which method of permeabilization is used. Nevertheless, it is possible that in some cases the results can be different if detergents are used instead of electric shock for permeabilization. Some cells can be permeabilized simply by washing with calcium-free solution, a method which should not damage the intracellular membranes (12).

Measurements of $[Ca^{2+}]$ by Ca^{2+}-selective minielectrodes

I used Ca^{2+}-selective mini-electrodes for measuring $[Ca^{2+}]$ in small volumes of cell

suspensions (papers I, II, IV and V). The electrodes were constructed and calibrated by modification of a method originally described by Tsien and Rink (250). I used borosilicate capillary tubing, 5 cm long and 0.6 mm inner diameter, with an "omega dot" for rapid filling. The membrane solution was prepared by dissolving Calcium Cocktail I (Fluka) and approximately 30 % (w/v) PVC in tetrahydrofuran to obtain a thin liquid of appropriate consistency (250). Calcium Cocktail I is a mixture of a neutral Ca^{2+} carrier (ETH 1001), a membrane solvent *o*-nitrophenyl-octyl ether and other membrane additives. The tip of the tubing was filled by briefly dipping it into the membrane solution. The internal filling solution for the Ca^{2+}-electrode had a $[Ca^{2+}]$ of 100 nM. The reference electrodes were made from similar tubing by using a horizontal puller and were filled with 1 M KCl. The principle of $[Ca^{2+}]$ measurements by Ca^{2+}-selective electrodes, is that Ca^{2+} from the sample solution is selectively transferred to the membrane phase of the electrode by a complex formation with the carrier. This generates a potential difference between the internal filling solution and the sample solution, which is ideally a linear function of the logarithm of the Ca^{2+} ion concentration (Nernst equation). The potential difference was measured by a purpose-built high-impedance electrometer, with respect to a reference level established by a salt bridge in contact with the sample solution. The electrodes had a response time in the order of seconds and a detection limit of between 10^{-8} to 10^{-7} M $[Ca^{2+}]$.

Measurements of $[Ca^{2+}]_i$ by microfluorimetry

$[Ca^{2+}]_i$ in intact β-cells was measured by microfluorimetry (paper VII). Cells attached to coverslips were loaded with fura-2 by incubating in an extracellular medium containing 2 μM fura-2-acetoxymethylester, for 20 min at 37°C. Coverslips were mounted as the bottom of an open chamber placed on the stage of an inverted epifluorescence microscope (Zeiss, Axiovert 35M). The microscope was connected to a SPEX fluorolog-2 CM1T11I system for dual wavelength excitation fluorimetry. The excitation wavelengths generated by two monochromators were directed to the cell by a dichroic mirror. The emitted light, selected by a 510 nm filter, was monitored by a photomultiplier attached to the microscope. The excitation wavelengths were alternated at a frequency of 1Hz and the length of time for data collection at each wavelength was 0.33 s. The emissions at the excitation wavelength of 340 nm (F_{340}) and that of 380 nm (F_{380}) were used to calculate the fluorescence ratio ($R_{340/380}$). Small clusters of cells (usually 3-4), isolated optically by means of the diaphragm of the microscope, were studied by using a 100x, 1.3 NA oil-immersion objective (Zeiss, Plan Neofluar). Background fluorescence was measured after quenching the fura-2 fluorescence with manganese and was subtracted from the traces before calculation of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was calculated from the $R_{340/380}$, according to Grynkiewicz *et al* (251). Maximum and minimum fluorescence ratios were determined, using 1 μl drops of an intracellular-like buffer containing 10 μM fura-2 free acid and either 2 mM Ca^{2+} or no Ca^{2+} in the presence of 2 mM EGTA. The K_d for the Ca^{2+}-fura-2 complex was taken as 225 nM. In order to compensate for variations in output light intensity from the two monochromators, all experiments were

corrected for by the inclusion of a fluorescence ratio where both monochromators were set at 360 nm.

Fluorescent Ca^{2+} indicators are extensively used for measuring $[Ca^{2+}]_i$. Indicators like fura-2 and indo-1, signal $[Ca^{2+}]_i$ not only by altering the intensity of the fluorescence but also by shifting the excitation or emission wavelengths. $[Ca^{2+}]_i$ can be measured from the ratio of intensities at two wavelengths independent of variations in parameters such as, dye concentration, cell thickness, absolute intensities of illumination and absolute sensitivity of detection (252).

Electrophysiological measurements

The patch-clamp technique is the only electrophysiological technique by which it is possible to resolve single ion-channel currents in relatively small cells such as the β-cell. This requires the formation of a very good contact between a recording pipette and the cell membrane, a so called gigaseal (253). This is accomplished by placing a glass-pipette on to the cell membrane and applying a light suction to the pipette interior. When the cell membrane and the pipette glass interacts, it produces a high shunt resistance (>1 gigaohm). The patch-clamp technique has different recording configurations of which I used the whole-cell, inside-out and outside-out modes (253). In the whole-cell configuration (used in papers III, V and VII), a pulse of negative pressure is applied after the formation of a gigaseal; the membrane ruptures and physical contact with the cell interior is established. A problem with the whole-cell technique is the wash-out of intracellular components. This can however be taken advantage of as in paper III. In this study, the cell was perfused with an intracellular-like solution lacking ATP, allowing K_{ATP} channels to open. This made it possible to study the blocking effect of SH-reagents on K_{ATP} channel activity resulting in membrane depolarization. Another advantage with this configuration, is the possibility to introduce substances into the cell. This is exemplified in paper V, where cADPR was introduced into the cell via the pipette solution.

If the pipette is withdrawn from the cell after the establishment of a gigaseal, an inside-out patch is formed. This configuration was used in papers III and VII to monitor single K_{ATP} channel activity. In this configuration, the cytosolic face of the cell membrane is in contact with the bath solution, allowing alterations of the intracellular-like solution. It follows that only proteins attached to the cell membrane will still be present in this configuration. Withdrawing the pipette after establishment of the whole-cell mode, will result in an outside-out patch. The outside-out configuration (used in paper III), actually results in a micro whole-cell or small vesicle, with the same orientation of the membrane as in the whole-cell mode. This configuration also allows recording of single ion channels, and was used to determine the effects of SH-reagents applied to the outside of the cell membrane.

The pipettes were pulled from borosilicate or aluminosilicate glass, coated with Sylgard,

fire-polished and had resistances between 3-6 MΩ. Data were stored on video tape or in a computer, pending analysis. All patch-clamp experiments were performed at room temperature.

Reagents

The reagents used in this study are listed in table I.

Table I. List of reagents used in this work and their typical actions

Reagents	Actions(reference)
Antimycin A	Inhibits respiratory chain at site II (254)
Bafilomycin A_1	Inhibits V-type H^+-ATPase (150)
cADPR	Activates RyR in some cells (152)
Caffeine	Activates RyR (22), inhibits cyclic nucleotide phosphodiesterases (255), blocks adenosine receptor (256)
CCCP	Uncouples oxidative phosphorylation (254)
Cysteine	Disulfide reducing/SH-oxidizing agent (195)
D-600	Blocks L-type Ca^{2+}-channel (5)
Diazoxide	Activates K_{ATP} channel (5)
DTBNP	SH-reagent (219)
DTT	Disulfide reducing agent (220)
Forskolin	Activates adenylyl cyclase (257)
Fura-2/AM	A membrane-permeable, Ca^{2+}-indicator (252)
Heparin	Blocks IP_3R (68)
IBMX	Inhibits phosphodiesterase (255)
$Ins(1,3,4,5)P_4$	Metabolite of $Ins(1,4,5)P_3$ (14)
$Ins(1,4,5)P_3$	Releases Ca^{2+} through IP_3R (24)
$Ins(2,4,5)P_3$	Poorly metabolizable $Ins(1,4,5)P_3$ analog (258)
Manoalide	Non-specific Ca^{2+} channel-inhibitor (259)
Oligomycin	Inhibits mitochondrial H^+-ATPase (254)
*R*p-cAMPS	PKA antagonist (260)
Ruthenium Red	Blocks RyR (22)
Ryanodine	Activates/inactivates RyR (189)
tBuBHQ	SERCA inhibitor (20)
Thapsigargin	SERCA inhibitor (110)
Thimerosal	SH-reagent, activates RyR and IP_3R (21,198)

RESULTS AND DISCUSSION

Increased Ca^{2+} sequestration after Ins(1,4,5)P_3 (paper I)

In electropermeabilized RINm5F cells, a supramaximal concentration of Ins(1,4,5)P_3 released Ca^{2+} from non-mitochondrial stores and following re-sequestration of Ca^{2+}, steady state [Ca^{2+}] was lower than that before addition of the trisphosphate. It was a possibility that experimental artifacts like a down-ward "drift" of the Ca^{2+}-electrode might account for apparent lowered steady state [Ca^{2+}]. To exclude this possibility the electrodes were calibrated both at the beginning and at the end of the experiments. Increased Ca^{2+} sequestration could not be explained by any down-ward "drift" of the electrode. When a pulse of $CaCl_2$ was added to the suspension, Ca^{2+} was taken up resulting in an elevated [Ca^{2+}]. Addition of Ins(1,4,5)P_3 at this stage, also caused increased sequestration of the ion. Increased Ca^{2+} sequestration also occurred after addition of the IP_3R-channel-blocker heparin. Heparin, a charged polyanion, did not interfere with electrode function, as tested in a cell-free system. When Ins(1,4,5)P_3 or its poorly-metabolizable analog Ins(2,4,5)P_3 was added after heparin, there was no Ca^{2+} release by the trisphosphates; instead Ca^{2+} sequestration, as occurred after heparin alone, was observed. This suggests that increased Ca^{2+} sequestration observed after Ins(1,4,5)P_3 in the absence of heparin and that caused by heparin alone, occurred by similar mechanisms. Ins(1,3,4,5)P_4, which was reported to induce Ca^{2+} sequestration in a liver cell line (144), did not promote sequestration in RINm5F cells.

The mechanism by which Ins(1,4,5)P_3 promoted Ca^{2+} sequestration is unclear. It could be argued that Ca^{2+} released by Ins(1,4,5)P_3, in the continued presence of the trisphosphate, was sequestered in Ins(1,4,5)P_3-insensitive pools. This seems unlikely since in permeabilized RINm5F cells and β-cells, Ca^{2+} released from the Ins(1,4,5)P_3-sensitive pool is sequestered back into the same pool (24,28). Furthermore, any postulated mechanism should be able to explain the Ca^{2+}-sequestering effect of both Ins(1,4,5)P_3 and heparin. Ca^{2+} sequestration into ER can be increased by stimulation of the Ca^{2+} pump (120), by increasing Ca^{2+} binding within the lumen of ER (119) or by reducing permeability of the Ca^{2+} storing organelle. Inhibition of Ca^{2+} leakage through the IP_3R channels could be a mechanism of Ca^{2+}-sequestering action of both heparin and Ins(1,4,5)P_3. It appears that under the influence of a low basal concentration of Ins(1,4,5)P_3, some IP_3Rs remain open. These IP_3Rs may have higher affinity for the trisphosphate and may possibly be structurally different (54). Closure of these IP_3R channels by heparin leads to increased Ca^{2+} sequestration. In this respect, Ca^{2+}-sequestering effect of heparin is comparable to that of ruthenium red (261) or high concentration of ryanodine (262)

which promote Ca^{2+} sequestration by blocking the RyR channel in sarcoplasmic reticulum.

Following Ca^{2+} release by Ins(1,4,5)P_3, Ca^{2+} leakage through the basally active IP_3Rs may be reduced due to transformation of the receptor to a low-conductance state. Previous studies in different cell types described that IP_3R can be down-regulated (100,103) and in paper I it was mentioned as a possible mechanism for the increased Ca^{2+} sequestration observed in my experiments. However, down regulation of IP_3R apparently takes place over a longer time scale (100,103). Hence, a more plausible mechanism may be the switching of the receptor to a low-conductance state. As mentioned before, it is now amply documented that IP_3Rs can exist in different interconvertible states of conductivity and affinity (65). Mechanisms determining such an interconversion, under our experimental conditions, remain unclear. Since Ins(1,3,4,5)P_4 was not effective, metabolism of Ins(1,4,5)P_3 and thereby generation of some active factor is not likely to be involved.

Ca^{2+}-sequestering action of Ins(1,4,5)P_3 in permeabilized cells was not reported in previous studies (24,28,204). The effect might have been missed in those studies because Ins(1,4,5)P_3 was added in the face of continuing uptake of Ca^{2+} and sufficient time was not allowed after addition of the trisphosphate to see a lowered steady state [Ca^{2+}]. The physiological relevance of increased Ca^{2+} sequestration after Ins(1,4,5)P_3-induced Ca^{2+} release as observed in permeabilized cells is unclear. It may be a crude *in vitro* demonstration of what happens in intact cells. In intact cells, inhibition of the Ins(1,4,5)P_3-induced Ca^{2+} release by Ca^{2+}, promotes re-sequestration of Ca^{2+} and pool refilling in different cells (47,263).

RyR-like channel in insulin-secreting cells (papers II, V, VI and VII)

The RyR mediates CICR in sarcoplasmic reticulum and in many non-muscle cells. As mentioned before, it is now known that the IP_3R can also mediate a form of CICR in the presence of the trisphosphate (91). In this work, I restrict the term CICR to describe the CICR mediated by RyR, unless otherwise mentioned. I studied whether insulin-secreting cells contain a RyR-like channel and an intracellular Ca^{2+} pool that mediates CICR. The usual pharmacological tool for the study of RyR is caffeine and Ca^{2+} release by caffeine is generally taken as an evidence for the presence of a CICR pool in the cell. In permeabilized insulin-secreting RINm5F cells and in β-cells obtained from *ob/ob* mice, caffeine either did not release Ca^{2+} or was only marginally effective. In the experiments with permeabilized cells, I could not use caffeine in maximal concentrations because of difficulty in obtaining a concentrated enough stock-solution of the substance. However, in fura-2 loaded intact β-cells, even high concentrations of caffeine (up to 50 mM) did not increase $[Ca^{2+}]_i$ in the presence of

low extracellular Ca^{2+} (100 nM). Together, these data suggest that if a RyR-like channel is present in insulin-secreting cells it is largely insensitive to caffeine. Caffeine-insensitive RyR is now known to be present in different cells (175). It was therefore necessary to try other substances that could activate RyR.

Numerous studies showed that SH-reagents release Ca^{2+} from sarcoplasmic reticulum by oxidizing critical SH-groups associated with the RyR. From the study of Swann, it appeared that thimerosal might act exclusively on RyR in hamster eggs (21). Thimerosal also releases Ca^{2+} from sarcoplasmic reticulum, presumably by activating the RyR (Guy Salama, personal communication). Subsequently, however, a number of studies showed that thimerosal also sensitizes the IP_3R (65,264). This reagent can thus activate both of these Ca^{2+} release channels. The situation is best illustrated in sea-urchin egg where thimerosal releases Ca^{2+} by activating both RyR and IP_3R (265).

In papers II and V, thimerosal released Ca^{2+} from non-mitochondrial intracellular Ca^{2+}pools in both RINm5F cells and β-cells. Insulin-secreting cells, have IP_3Rs which like the related RyR channel, also contain critical SH-groups (13). However, several lines of evidence suggests that, under the conditions of my experiments, thimerosal released Ca^{2+} from an $Ins(1,4,5)P_3$-insensitive pool. First, after releasing Ca^{2+} from the $Ins(1,4,5)P_3$-sensitive pool by a maximal dose of the trisphosphate, further Ca^{2+} was released by thimerosal. Second, Ca^{2+} release by thimerosal was not blocked by heparin. In cells where thimerosal releases Ca^{2+} through the $Ins(1,4,5)P_3$-sensitive channel, the release can be blocked by heparin, although a higher concentration of heparin might be needed (264,266,267). In permeabilized insulin-secreting cells, heparin, at a concentration as low as 50-100 μg/ml, completely inhibits Ca^{2+} release by a maximal dose of $Ins(1,4,5)P_3$ (68). But even high concentrations of heparin (1000 μg/ml) did not block Ca^{2+} release by thimerosal. Third, Ca^{2+} release by thimerosal was potentiated by caffeine, which sensitizes CICR. One previous study have shown that $Ins(1,4,5)P_3$-induced Ca^{2+} release is not potentiated, rather it is inhibited by caffeine (268). Thus, the results suggest that the RyR-like channel in the β-cell, which is largely insensitive to caffeine, can be activated by SH-reagents like thimerosal. It is noteworthy that the type 3 RyR, which has recently been cloned from mink lung epithelium and rabbit brain, can not be activated by caffeine, even at concentrations as high as 90 mM (177).

cADPR activates RyR in some cells (159,162). In β-cells, one study claimed that cADPR and not $Ins(1,4,5)P_3$ is the intracellular Ca^{2+}-mobilizing second messenger (31). In paper V, I examined the quantitative importance of the two intracellular Ca^{2+}-mobilizing second messengers in insulin-secreting cells. In my experiments, under conditions where pronounced Ca^{2+} release was evoked by $Ins(1,4,5)P_3$, no release was obtained by cADPR in permeabilized insulin-secreting cells. These results establish $Ins(1,4,5)P_3$ as the key Ca^{2+}-mobilizing second messenger in both insulin-secreting tumor cells and normal β-cells. Lack of Ca^{2+} release from rat islet microsomes by $Ins(1,4,5)P_3$, as reported by Takasawa *et al*, probably indicates that

the Ins(1,4,5)P_3-sensitive organelle can be damaged during microsome preparation (83,154). Whether the inability of cADPR to release Ca^{2+} in my experiments, was due to experimental conditions or due to the fact that the putative RyR-like channel of insulin-secreting cells is insensitive to cADPR, is at present unclear. It is noteworthy that the type 1 RyR is insensitive to cADPR (162). Measurements of $[Ca^{2+}]$ by electrodes is a relatively insensitive method and might have missed a small Ca^{2+} release by cADPR. However, no Ca^{2+} release could be detected even by measuring K_{Ca} current, which is a highly sensitive method for measuring $[Ca^{2+}]_i$ (16). As a Ca^{2+}-mobilizing second messenger, cADPR is relatively new. It is possible that different experimental conditions were required to demonstrate Ca^{2+} release by cADPR. For instance, the magnitude of cADPR-induced Ca^{2+} release may depend on the composition of the buffer (151). A high Cl^- concentration of the buffer may be inhibitory to Ca^{2+} release, although Ca^{2+} release by cADPR has been demonstrated in studies where Cl^- was the sole anion (162).

The lack of response to cADPR in my experiments, can not be explained by presumed glucose-insensitivity of the cell types that I have used (269). As pointed out (paper VI), the *ob/ob* β-cells used in my studies are highly sensitive to glucose. Over the past 30 years, this type of cells has been extensively used for basic studies in stimulus-secretion coupling. From this point of view, the normality of these cells is well established by numerous electrophysiological investigations as well as ionic and secretion studies (5,16,24,96,270). The effect of cADPR on insulin-secretion has not been studied in the present study. Takasawa *et al* demonstrated Ca^{2+}-mediated stimulation of insulin-secretion by cADPR and proposed a model of β-cell stimulus-secretion coupling (269). I find this intriguing because the model does not take into account the established roles of K_{ATP} channel or voltage-gated L-type Ca^{2+} channel and postulates that Ca^{2+} released by cADPR alone is a sufficient trigger for exocytosis. It may be pointed out that in the β-cell, $[Ca^{2+}]_i$- increase through mobilization of intracellular stores alone is only a poor trigger for exocytosis (271).

Effects of caffeine (papers II, V and VII)

Caffeine is the most commonly used pharmacological agent to prove the existence of a CICR pool in any cell. The most well-known mechanism by which caffeine increases $[Ca^{2+}]_i$ is activation of the RyR (22,182). Some studies showed that in pancreatic β-cells, caffeine increases $[Ca^{2+}]_i$, an action attributed to caffeine´s intracellular Ca^{2+}-mobilizing action (272,273). Moreover, one previous study showed that in glucose-stimulated islets, caffeine

can also decrease $[Ca^{2+}]_i$, an action attributed to the inhibitory action of caffeine on the RyR (273). However, as I have demonstrated, in permeabilized insulin-secreting cells, Ca^{2+} release by caffeine is at best marginal (papers II and V). I therefore investigated whether caffeine could affect $[Ca^{2+}]_i$ by mechanisms unrelated to its action on the RyR. In paper VII, I studied the effects of caffeine on $[Ca^{2+}]_i$ and the mechanisms by which these changes were brought about. In fura-2 loaded β-cells, caffeine consistently increased $[Ca^{2+}]_i$. Several lines of evidence indicated that this increase was not due to Ca^{2+} mobilization from intracellular stores. First, $[Ca^{2+}]_i$-increase occurred only when extracellular Ca^{2+} was present. Second, when the agonist-sensitive intracellular Ca^{2+} pool was depleted by thapsigargin, caffeine still increased $[Ca^{2+}]_i$. Third, increase in $[Ca^{2+}]_i$ was blocked by the L-type Ca^{2+} channel blocker D-600 or nifedipine. Fourth, in the presence of low extracellular $[Ca^{2+}]$, caffeine did not increase $[Ca^{2+}]_i$.

In β-cells, the K_{ATP} channel couples stimulation by glucose and antidiabetic sulfonylureas to an increase in $[Ca^{2+}]_i$. Closure of the channel leads to cell depolarization and opening of L-type voltage-gated Ca^{2+} channels. I examined the effects of caffeine on K_{ATP} channel activity. In excised inside-out patches, caffeine inhibited the K_{ATP} channel in a dose-dependent manner. At a concentration of 10 mM, caffeine completely blocked K_{ATP} channel activity. Hence, in β-cells caffeine increased $[Ca^{2+}]_i$ primarily by inhibiting the K_{ATP} channel, leading to cell depolarization and opening of L-type voltage-gated Ca^{2+} channels.

The effect of caffeine on $[Ca^{2+}]_i$ was complex in that it not only increased but in glucose-stimulated β-cells, it also reduced $[Ca^{2+}]_i$. When caffeine (10 mM) was added after glucose-induced increase in $[Ca^{2+}]_i$, there was a decrease in the cytoplasmic free concentration of the ion. Since the main mechanism by which $[Ca^{2+}]_i$ is increased after glucose-stimulation is Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channels, a reduction of Ca^{2+} entry through these channels could explain the lowering effect of caffeine on $[Ca^{2+}]_i$. Patch-clamp recordings of whole-cell Ca^{2+} currents showed that caffeine indeed reduced Ca^{2+} current through the L-type voltage-gated Ca^{2+} channels. The inhibitory action of caffeine on ion channels appear to be a widely occurring phenomenon. A direct inhibitory effect of caffeine on L-type Ca^{2+} channels has been described in cardiac myocytes (274,275). Caffeine also inhibits the $Ins(1,4,5)P_3$ channel (268) and perhaps even the RyR-like channel in non-muscle cells (183).

A third type of effect on $[Ca^{2+}]_i$ was observed in glucose-stimulated β-cells, when caffeine was used at relatively low concentrations (2.5-5 mM). Under these conditions, caffeine induced fast $[Ca^{2+}]_i$ oscillations usually with $[Ca^{2+}]_i$ spikes superimposed on an elevated $[Ca^{2+}]_i$ or slower $[Ca^{2+}]_i$ oscillations. Earlier studies in *ob/ob* β-cells, demonstrated that similar $[Ca^{2+}]_i$ oscillations and spiking can be induced by cAMP-forming agonists or cAMP analogs (270). I have further demonstrated that these oscillations can be largely prevented by

*R*pcAMPS, a PKA-antagonist (260), indicating that the phenomenon may be mediated by PKA-induced phosphorylation of the L-type voltage-gated Ca^{2+} channel. Ca^{2+} spiking induced by caffeine in a vascular smooth muscle cell line is also mediated by cAMP (276). Studies in muscle and some endocrine cells, demonstrate pronounced effects of PKA phosphorylation on L-type Ca^{2+} channel activity (233,234). cAMP-mediated modulation of L-type Ca^{2+} channel has been demonstrated also in β-cells (277,278).

My study thus demonstrates that in β-cells, caffeine has inhibitory actions on the K_{ATP} channel and L-type voltage-gated Ca^{2+} channel, in addition to its well known inhibitory effect on the phosphodiesterases. These effects are unrelated to the interaction of caffeine with the RyR, but still affects $[Ca^{2+}]_i$ in distinct ways depending on the prevailing conditions. Moreover, both the cAMP-elevating action and the inhibitory action of caffeine on the K_{ATP} channel may determine the insulin-secretagogue-like action of the compound. Interestingly, at least some studies have shown that caffeine may stimulate modest insulin secretion even in the absence of glucose, an action consistent with the inhibitory effect of this xanthine drug on K_{ATP} channel activity (279,280). Most studies however demonstrated a potentiation of glucose-stimulated insulin-secretion by caffeine, an action mediated by cAMP. It may be noted that cAMP-elevating agents potentiate glucose-stimulated insulin secretion but do not stimulate insulin-secretion in the absence of glucose.

Effects of SERCA inhibitors (papers II, IV and VII)

Inhibition of SERCA results in release of Ca^{2+} from intracellular stores, as has been demonstrated in many cells. In a few studies, difference between the actions of the SERCA inhibitors has also been compared. But, little information is available on the action of SERCA inhibitors in insulin-secreting cells. In my study, thapsigargin and tBuBHQ released Ca^{2+} from non-mitochondrial Ca^{2+} pools in electropermeabilized RINm5F cells. Small differences in the Ca^{2+}-mobilizing action of the two inhibitors were observed. Thapsigargin was about 60 times more potent than tBuBHQ and it mobilized Ca^{2+} almost exclusively from the $Ins(1,4,5)P_3$-sensitive pool. Maximal Ca^{2+} release by tBuBHQ was more than that by thapsigargin, but the difference was not statistically significant. In platelets, thapsigargin mobilizes slightly more Ca^{2+} than tBuBHQ (112,136). After Ca^{2+} release by the inhibitors, further Ca^{2+} was released by a Ca^{2+}-ionophore, indicating the presence of Ca^{2+} pools that were not mobilized by these inhibitors. Also, after release by thapsigargin and tBuBHQ, Ca^{2+} was partially and slowly taken up into Ca^{2+} pools insensitive to the inhibitors. Ca^{2+} release by thapsigargin and tBuBHQ was not blocked by heparin or a non-specific Ca^{2+} channel blocker manoalide.

The relationship between the inhibitor-sensitive Ca^{2+} pools and the $Ins(1,4,5)P_3$-sensitive pool has previously been studied in many cells. In RINm5F cells, both the inhibitors released Ca^{2+} predominantly from the $Ins(1,4,5)P_3$-sensitive pool. In this respect, thapsigargin was more specific than tBuBHQ. Increase in $[Ca^{2+}]$ after thapsigargin plus $Ins(1,4,5)P_3$ was not

significantly greater than that after Ins(1,4,5)P_3 alone. This is in agreement with studies in other cells, where thapsigargin mobilizes Ca^{2+} specifically from the Ins(1,4,5)P_3-sensitive pool. On the other hand, increase in [Ca^{2+}] after tBuBHQ plus Ins(1,4,5)P_3 was 33% greater than that after Ins(1,4,5)P_3 alone, indicating that tBuBHQ released Ca^{2+} also from the Ins(1,4,5)P_3-insensitive pools.

In RINm5F cells, following Ca^{2+} release by thapsigargin and tBuBHQ, further Ca^{2+} was released by Ins(1,4,5)P_3, even after prolonged treatment with the inhibitors. Ca^{2+} release by thapsigargin and Ins(1,4,5)P_3 has been compared in different cells in many previous studies. In studies that used microsomes instead of cells, thapsigargin released substantially more Ca^{2+} than did Ins(1,4,5)P_3 (19,281). In other studies thapsigargin or tBuBHQ depleted the Ins(1,4,5)P_3-sensitive pool within short time and no additional Ca^{2+} release was obtained by Ins(1,4,5)P_3 (123,126,164). On the other hand, some studies in different cell types showed that thapsigargin did not completely deplete the Ins(1,4,5)P_3-sensitive pool in short time and further Ca^{2+} could be released by Ins(1,4,5)P_3 or Ins(1,4,5)P_3-forming agonists (136,281,282).

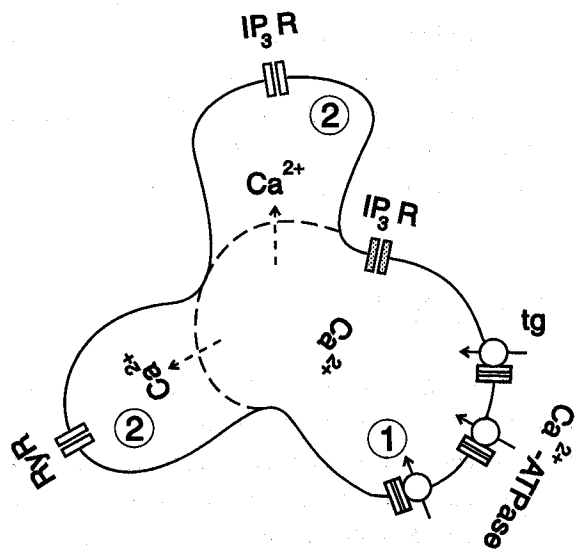

Fig. 8. A model of functional organization of rapidly exchanging intracellular Ca^{2+} pools in insulin-secreting cells. The Ca^{2+}-storing organelle has spatially separated uptake-compartment (1) and release-compartments (2). Ca^{2+}-uptake occurs by Ca^{2+}-ATPase located predominantly on the uptake-compartment and is subsequently translocated to the release compartments (arrows). Inhibition of Ca^{2+}-ATPase by agents like thapsigargin (tg) causes releases of Ca^{2+} predominantly from the uptake-compartment. The release compartments contain predominantly the Ins(1,4,5)P_3-receptor (IP_3R) or ryanodine receptor (RyR). For details see text.

Ca^{2+} fluxes measured by the $^{45}Ca^{2+}$ method in electropermeabilized SH-SY5Y neuroblastoma cells, showed that prolonged treatment by thapsigargin, up to 45 min, fails to completely

deplete the Ins(1,4,5)P_3-sensitive pool and further Ca^{2+} can be released by Ins(1,4,5)P_3 (100). In RINm5F cells, since increase in [Ca^{2+}] by thapsigargin plus Ins(1,4,5)P_3 was not significantly greater than that by Ins(1,4,5)P_3 alone, it could be concluded that thapsigargin released Ca^{2+} from only part of the Ins(1,4,5)P_3-sensitive pool. The results can be interpreted in the light of observations on organization of Ins(1,4,5)P_3-sensitive organelle in different cells types. Such studies suggest that the Ins(1,4,5)P_3-sensitive Ca^{2+} pool is divided into an uptake- and a release-compartment (83,143). It appears that the Ins(1,4,5)P_3-sensitive pool in RINm5F cells also, may have separate uptake- and release-compartments. Apparently, thapsigargin mobilizes Ca^{2+} readily from the uptake-compartment but it can not easily mobilize Ca^{2+} from the release compartment, in the absence of Ins(1,4,5)P_3.

In figure 8, I propose a model of functional organization of intracellular Ca^{2+} pools, based on findings in permeabilized insulin-secreting cells as described in papers II and IV. According to this model, the intracellular Ca^{2+} pools are divided into an uptake-compartment and two release-compartments. The uptake-compartment contains mainly the SERCA pumps and Ca^{2+} in this compartment can be easily mobilized by the pump inhibitors. The release-compartments are equipped with IP_3R or RyR. Ca^{2+} from the release-compartments can be mobilized by Ins(1,4,5)P_3, and thimerosal, or by caffeine in caffeine-sensitive cells. The model is not unique. In fact, data obtained from other cell-types are compatible with such a model. Bovine adrenal chromaffin cells appear to have three distinct pools that can be mobilized by Ins(1,4,5)P_3, caffeine or tBuBHQ respectively (283). Pancreatic acinar cells also contain three different pools releasable by Ins(1,4,5)P_3, caffeine and vanadate respectively (171). A three-pool model has recently also been described in GH_4C_1 cells (284).

SH-group in the K_{ATP} channel (paper III)

The roles of SH-groups in biologically important proteins like enzymes and transport proteins, have been studied by using various SH-reagents (285). Fewer studies have looked at the roles of SH-groups in ion-channels. Effects of SH-reagents on the intracellular Ca^{2+} release channels have been studied in different cell types and in paper II, I showed that the SH-reagent thimerosal can activate a RyR-like channel in insulin-secreting cells.

Since the K_{ATP} channel plays a central role in the stimulus-secretion coupling in the β-cell and since some SH-reagents stimulate insulin secretion, I examined whether this channel contains SH-groups, modification of which may affect channel activity. Using the patch-clamp technique, I studied effects of SH-reagents on K_{ATP} channel activity and by using membrane permeable and impermeable SH-reagents, I obtained information on the localization of reactive SH-groups in the K_{ATP} channel. In excised inside-out patches, thimerosal and DTBNP inhibited K_{ATP} channel activity and the effects were reversed by the reducing agents DTT and cysteine. Thimerosal, which is poorly membrane-permeable, did

not inhibit channel activity when applied to the extracellular side of the membrane in excised out-side out patches. DTBNP which is membrane permeable, inhibited channel activity also when applied to the extracellular side of the membrane.

Inhibition of K_{ATP} channel activity should cause depolarization of the cell. This was confirmed by recording β-cell membrane potential in the whole-cell patch-clamp configuration, where SH-reagents were added to the outside of the cell. As expected, in these experiments thimerosal did not depolarize the cell whereas DTBNP did. These results indicate the presence of reactive SH-groups on the cytoplasmic side of the K_{ATP} channel or a closely related protein. The results further show, that modifications of the SH-groups of the K_{ATP} channel inhibit the activity of the channel, leading to cell depolarization. These results are in agreement with those of Weik *et al*, who demonstrated functionally important SH-groups in the K_{ATP} channel of mouse skeletal muscle by using some other SH-reagents (216). The specificity of thimerosal for the K_{ATP} channel was not studied in detail in the present study. Thimerosal has been reported to inhibit another plasma membrane ion channel activity

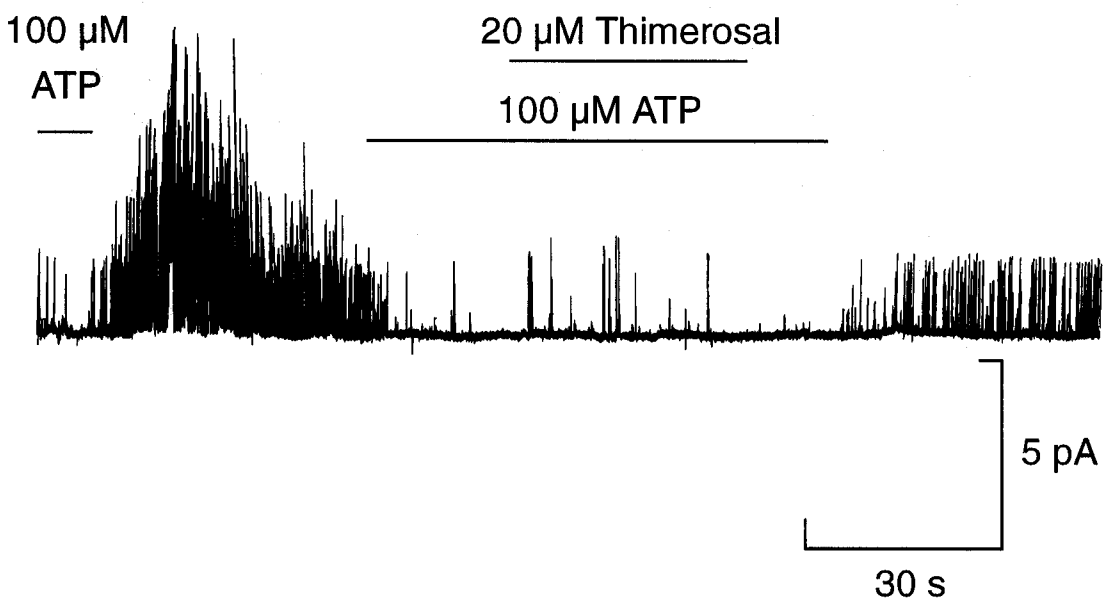

Fig. 9. ATP protects against the inhibitory effect of thimerosal on K_{ATP} channel activity. Single-channel recording from excised inside-out membrane patches of mouse β-cell. Thimerosal (20 µM) was added after prior addition of ATP (100 µM). Channel activity returned promptly after withdrawal of thimerosal and ATP

in a different cell type (286). However, large-conductance K_{Ca} channel activity was not inhibited by thimerosal in the β-cell.

The location of SH-groups in relation to the ATP-binding site in K_{ATP} channel was also examined. In the experiments, shown in fig. 9, K_{ATP} channel activity was first inhibited by ATP (100 μM). Thimerosal (20 μM) was added in the continued presence of ATP and then washed out while ATP was still present. After withdrawal of ATP, channel activity promptly returned. This was in contrast to the effect of thimerosal added in the absence of ATP, in which case K_{ATP} channel activity does not return spontaneously on withdrawl of the agent (c.f. fig. 1, paper III). This indicates that binding of ATP to the K_{ATP} channel may occlude the SH-groups thereby preventing their interaction with thimerosal suggesting further that the SH-groups may be located near the ATP-binding site of the channel. GSSG and alloxan did not inhibit K_{ATP} channel activity.

Previous studies showed that some SH-reagents markedly stimulate insulin secretion (287,288). This effect is observed characteristically with SH-reagents that are poorly membrane permeable. Insulin-secretion by these agents is immediate, occurs even in the presence of low glucose and can be inhibited by diazoxide (287,288). The mechanism of action of these SH-reagents is unclear, but it was demonstrated that they bind to SH-groups associated with the plasma membrane (288). Inhibition of K_{ATP}-channel activity by SH-reagents may be an underlying mechanism whereby SH-reagents stimulate insulin-secretion.

The presence of functionally important SH-groups in the K_{ATP} channel is of interest from several points of view. SH-reagents may be used to label and purify the channel protein from the plasma membrane of insulin-secreting cells. A similar approach has been used to label and purify a 106 kDa Ca^{2+} release channel from sarcoplasmic reticulum (23). It may be possible to design specific agents that would interact with SH-groups of the K_{ATP} channel and thereby act as insulin secretagogue.

CONCLUSIONS

1. In electropermeabilized RINm5F cells, following Ca^{2+} release by $Ins(1,4,5)P_3$, the steady state $[Ca^{2+}]$ attained after sequestration of the ion was lower than that before addition of the trisphosphate. Heparin, a blocker of IP_3R-channels, also caused increased Ca^{2+} sequestration. A possible explanation of these phenomena is that following Ca^{2+} release by $Ins(1,4,5)P_3$, Ca^{2+} leakage through IP_3Rs that are active under basal conditions is reduced, possibly due to conversion of these receptors to a poorly conducting state, thus promoting Ca^{2+} sequestration.

2. Insulin-secreting cells contain a ryanodine-receptor like intracellular Ca^{2+} release channel, which is largely insensitive to caffeine but can be activated by SH-reagents like thimerosal.

3. SERCA inhibitors thapsigargin and tBuBHQ release Ca^{2+} from intracellular non-mitochondrial Ca^{2+} pools in insulin-secreting cells. The two inhibitors mobilize Ca^{2+} mainly from the $Ins(1,4,5)P_3$-sensitive pool, and in this respect thapsigargin is more specific than tBuBHQ. $Ins(1,4,5)P_3$ can release further Ca^{2+} even after Ca^{2+} release by thapsigargin or tBuBHQ, suggesting a possibility that $Ins(1,4,5)P_3$-sensitive pool may be divided into uptake- and release-compartments. The inhibitors apparently release Ca^{2+} from the uptake-compartment, whereas Ca^{2+} from the release-compartment can not be released easily by the inhibitors in the absence of $Ins(1,4,5)P_3$.

4. Of the two intracellular Ca^{2+}-mobilizing second messengers, $Ins(1,4,5)P_3$ and cADPR, the effect of the former is consistently demonstrable in both insulin-secreting cell lines and β-cells. cADPR however, does not release Ca^{2+} from permeabilized RINm5F cells nor from normal β-cells.

5. Caffeine increases $[Ca^{2+}]_i$ by inhibiting K_{ATP} channel activity, leading to cell depolarization and Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels. The xanthine drug can also inhibit Ca^{2+} entry through L-type voltage-gated Ca^{2+} channels. In glucose-stimulated β-cells, a low dose of caffeine induces fast $[Ca^{2+}]_i$ oscillations and spikes, probably by a cAMP-dependent phosphorylation of the L-type voltage-gated Ca^{2+} channel.

6. Functionally important SH-group(s) are present on the cytoplasmic side of the K_{ATP} channel or on a protein closely associated with it. Inhibition of K_{ATP} channel activity may be a mechanism by which some SH-reagents induce insulin secretion.

FUTURE PERSPECTIVES

Further characterization of intracellular Ca^{2+} stores, particularly the study of Ins(1,4,5)P_3-insensitive pools and mechanisms of thapsigargin-insensitive Ca^{2+} uptake are required. In such studies, the pharmacological tools characterized in the present work may be used with better understanding of their actions. Studies are underway towards identification and purification of the ryanodine-receptor-like channel in insulin-secreting cells. The methods that may be used for purification of the channel are: 1. biotinylation of the channel-protein followed by biotin-avidin chromatography 2. ryanodine- and 3. antibody-affinity chromatography. Studies are aimed to answer the question whether glucose metabolism increases cADPR and to find its significance.

Future studies need to elucidate different types of IP_3Rs and RyRs in the β-cell and the way they may vary in terms of their functions and regulations. The L-type voltage-gated Ca^{2+} channels may have distinctive pharmacological properties and mode of regulation that may be β-cell-specific. Studies are also needed to understand the interactions between Ca^{2+} entry through the voltage-gated Ca^{2+} channels and intracellular Ca^{2+} pools.

The fact that the K_{ATP} channel contains reactive SH-groups on the ATP-binding site, may be useful for labelling the channel protein with reporter groups based on SH-reagents. Studies are needed to understand whether modifications of SH-groups may have a role in the physiological regulation of ion-channels.

Finally, future studies need to concentrate on experiments looking at regulation of ion-channels and signal-transduction mechanisms in the β-cell, from a more physiological point of view and finding their possible significance in the pathogenesis and treatment of diabetes mellitus.

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Interaction with the inositol 1,4,5-trisphosphate receptor promotes Ca^{2+} sequestration in permeabilised insulin-secreting cells

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Electropermeabilised insulin-secreting RINm5F cells sequestered Ca^{2+}, resulting in a steady-state level of the ambient free Ca^{2+} concentration corresponding to 723 ± 127 nM (mean ± SEM, $n = 10$), as monitored by a Ca^{2+}-selective minielectrode. Inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) promoted a rapid and pronounced release of Ca^{2+}. This Ca^{2+} was resequestered and a new steady-state Ca^{2+} level was attained, which was always lower (460 ± 102 nM, $n = 10$, $P < 0.001$) than the steady-state Ca^{2+} level maintained before the addition of $Ins(1,4,5)P_3$. Whereas the initial reuptake of Ca^{2+} subsequent to $Ins(1,4,5,)P_3$ stimulation was relatively slow, the later part of reuptake was fast as compared to the reuptake phases of a pulse addition of extraneous Ca^{2+}. In the latter case the uptake of Ca^{2+} resulted in a steady-state level similar to that found in the absence of $Ins(1,4,5)P_3$. Addition of $Ins(1,4,5)P_3$ under this condition resulted in a further Ca^{2+} uptake and thus a lower steady-state Ca^{2+} level. Heparin, which binds to the $Ins(1,4,5)P_3$ receptor, also lowered the steady-state free Ca^{2+} concentration. In contrast to $Ins(1,4,5)P_3$, inositol 1,3,4,5-tetrakisphosphate was without effect on Ca^{2+} sequestration. These findings are consistent with the presence of a high-affinity $Ins(1,4,5)P_3$ receptor promoting continuous release of Ca^{2+} under basal conditions and/or the $Ins(1,4,5)P_3$ receptor being actively involved in Ca^{2+} sequestration.

Inositol 1,4,5-trisphospate; Inositol 1,4,5-trisphosphate receptor; Intracellular Ca^{2+}-transport; Insulin-secreting cell

1. INTRODUCTION

Not only inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) but also inositol 1,3,4,5-tetrakisphosphate ($Ins(1,3,4,5)P_4$), one of its metabolites, is known to be involved in the generation of intracellular Ca^{2+} signals [1–3]. $Ins(1,4,5)P_3$ rapidly mobilizes intracellular Ca^{2+} stores in a wide variety of permeabilized cells, including insulin secreting RINm5F cells [4] and normal pancreatic β-cells [5]. The resulting rise in Ca^{2+} is short-lived and the ambient free Ca^{2+} concentration is eventually returned to basal levels reflecting reuptake into intracellular Ca^{2+} pools. Regulation of Ca^{2+} reuptake into intracellular Ca^{2+} pools following $Ins(1,4,5)P_3$-induced Ca^{2+} release is poorly understood. $Ins(1,3,4,5)P_4$ has been suggested to act in concert with $Ins(1,4,5)P_3$ in the mobilization of intracellular Ca^{2+} [2,3] and it has also been suggested that the tetrakisphosphate induces Ca^{2+} sequestration [6].

Using electropermeabilised insulin-secreting RINm5F cells and Ca^{2+} selective minielectrodes, we have investigated the reuptake of Ca^{2+} following its release by $Ins(1,4,5)P_3$. We now demonstrate that interaction with the $Ins(1,4,5)P_3$ receptor, in addition to releasing Ca^{2+}, also promotes the reuptake of this ion, resulting in a lowered ambient steady-state Ca^{2+} concentration.

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2. MATERIALS AND METHODS

Clonal insulin-secreting RINm5F cells were maintained in culture in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), all from Flow Laboratories (Scotland).

$Ins(1,4,5)P_3$ (potassium salt) was purchased from Sigma (St. Louis, USA). HPLC pure $Ins(1,3,4,5)P_4$ and $Ins(2,4,5)P_3$ were generous gifts from Dr R.F. Irvine, Cambridge, UK. Calcium ionophore cocktail, containing neutral carrier ETH 1001 was from Fluka. All other chemicals were of highly purified grade and were either from Sigma or Merck.

Cells were detached from culture flasks using Trypsin-EDTA. They were then washed twice with culture medium and twice with a cold nominally Ca^{2+} free buffer, containing 110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin and 25 mM HEPES, pH 7.0 (adjusted with KOH). After washing the cells were permeabilised by exposure to high-voltage discharges (six pulses of 3.2 kV/cm). This treatment resulted in more than 90% permeabilised cells, as verified by Trypan blue uptake. After permeabilisation, cells were centrifuged and the pellet was kept on ice until use.

8 μl of cell pellet was added to a plexiglass chamber containing 52 μl of incubation buffer. The incubation buffer was supplemented with 2 mM MgATP and an ATP regenerating system, consisting of 10 mM phosphocreatine and 20 U/ml of creatine kinase. The incubation buffer also contained mitochondrial inhibitors consisting of 0.2 μM antimycin and 1 μg/ml of oligomycin. Experiments were carried out at room temperature and the cell suspension was stirred continuously, using a small magnetic bar. Additions were made from 100–200 times concentrated solutions. Changes in the ambient free Ca^{2+} concentra-

tion were recorded using a Ca^{2+}-selective minielectrode constructed and calibrated essentially as described by Tsien and Rink [7]. Calibration of the electrode was done at the beginning and at the end of each experiment. All data on Ca^{2+} concentrations are given as mean values ± SEM and statistical significances were judged by Student's *t*-test for paired data.

3. RESULTS AND DISCUSSION

Addition of permeabilised RINm5F cells (4.7×10^7 cells/ml) resulted in a lowering of the ambient free Ca^{2+}-concentration to a steady-state level of 723 ± 127 nM, from the initial Ca^{2+} level of 4.5 ± 0.18 μM ($n = 10$). Stimulation with 5 μM $Ins(1,4,5)P_3$ induced a prompt release of Ca^{2+} reaching a value of 2.72 ± 0.16 μM ($n = 10$), which was slowly taken up again (Fig. 1A). The new steady-state Ca^{2+} level obtained was lower ($P < 0.001$) than prior to stimulation with $Ins(1,4,5)P_3$ and corresponded to 460 ± 102 nM ($n = 10$).

When a pulse of $CaCl_2$ (0.6 nmol) was added to the cells, the initial uptake of the ion was rapid but the terminal part of the uptake phase was slow and the Ca^{2+} concentration was maintained at a slightly elevated level, during the period of observation (Fig. 1B). $Ins(1,4,5)P_3$ added under these conditions induced a normal release of Ca^{2+}. In this case the initial phase of reuptake of Ca^{2+} was relatively slow, as compared to the initial rapid uptake following an extraneous Ca^{2+} pulse. However, the later part of the reuptake phase was rapid, reaching a steady-state level that was lower than that prior to the addition of $CaCl_2$ or $Ins(1,4,5)P_3$. Previous studies that have addressed the effect of $Ins(1,4,5)P_3$ on Ca^{2+} handling in permeabilised cells have not discussed the lowering effect of the trisphosphate on the steady-state Ca^{2+} level [8–13]. This effect is easy to miss if $Ins(1,4,5)P_3$ is added during continuing Ca^{2+}-uptake and if enough time is not allowed before and after the actual additions [8–12]. In neoplastic rat liver epithelial cells $Ins(1,3,4,5)P_4$ has been found to promote sequestration of pulse additions of Ca^{2+} or Ca^{2+} released by $Ins(2,4,5)P_3$ [6]. However, under these conditions 2 μM $Ins(1,3,4,5)P_4$ did not lower the steady-state free Ca^{2+} concentration and the intracellular Ca^{2+} pools had to be saturated prior to the addition of $Ins(1,3,4,5)P_4$. We were unable to demonstrate any lowering effect of 2.5 μM $Ins(1,3,4,5)P_4$ on the steady-state Ca^{2+} level (Fig. 2). Rather, at a concentration of 5 μM, $Ins(1,3,4,5)P_4$ induced a small increase in Ca^{2+} (data not shown). It is therefore unlikely, that the increased Ca^{2+} sequestration following $Ins(1,4,5)P_3$-induced Ca^{2+} release is mediated through metabolism of $Ins(1,4,5)P_3$ to $Ins(1,3,4,5)P_4$.

In the presence of heparin (200 μg/ml), which binds to the $Ins(1,4,5)P_3$ receptor [14] and blocks the trisphosphate-mediated release of Ca^{2+} [15–17], $Ins(1,4,5)P_3$ failed to induce a rise in the Ca^{2+} concentration, but the lowering effect on the steady-state Ca^{2+} concentration was still evident, starting approximately a minute after addition of $Ins(1,4,5)P_3$ (Fig. 3A). As evident from Fig. 3B, heparin also blocked the Ca^{2+} release evoked by $Ins(2,4,5)P_3$, a non-metabolisable analogue of $Ins(1,4,5)P_3$, resulting in a similar lowering in Ca^{2+} as that obtained in the presence of heparin plus $Ins(1,4,5)P_3$. Interestingly, it was observed that heparin alone also lowered the steady-state Ca^{2+} level (Fig. 4). That heparin by itself may cause increased sequestration of Ca^{2+} is apparent from at least one other study [17]. The same study also demonstrated that heparin-induced reuptake of $Ins(1,4,5)P_3$-released Ca^{2+} was extremely rapid. Noteworthy is that the effects of $Ins(1,4,5)P_3$ and heparin on Ca^{2+} sequestration were not additive (cf. Figs. 1, 3 and 4), suggesting that these agonists act through the same mechanism.

The mechanism(s) by which $Ins(1,4,5)P_3$ and heparin induce sequestration of Ca^{2+} can only be speculated upon at this stage. One possibility is the existence of high-affinity $Ins(1,4,5)P_3$ receptors. Under basal condi-

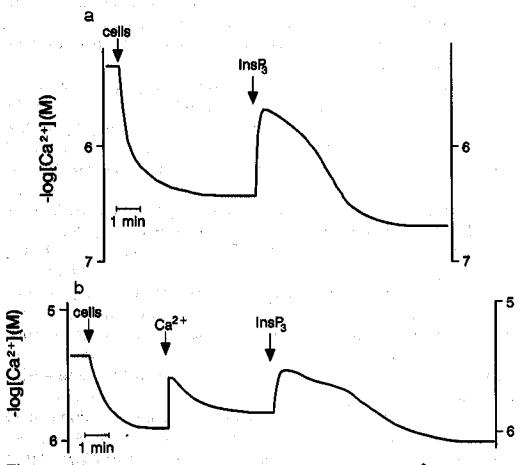

Fig. 1. Effect of $Ins(1,4,5)P_3$ on the steady-state free Ca^{2+} concentration. The figure shows Ca^{2+}-electrode traces obtained under conditions described in materials and methods section. A. At the point indicated $Ins(1,4,5)P_3$ (5 μM, final concentration) was added. The trace is typical of 6 independent experiments. B. At points indicated $CaCl_2$ (0.6 nmol) or $Ins(1,4,5)P_3$ (5 μM, final concentration) was added. The trace is representative of 4 different experiments.

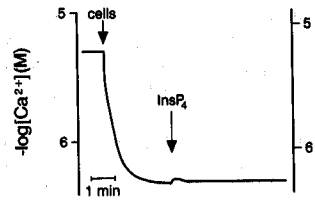

Fig. 2. Effect of $Ins(1,3,4,5)P_4$ on the steady-state free Ca^{2+} concentration. At the point indicated $Ins(1,3,4,5)P_4$ (2.5 μM, final concentration) was added. The trace is typical of three different experiments.

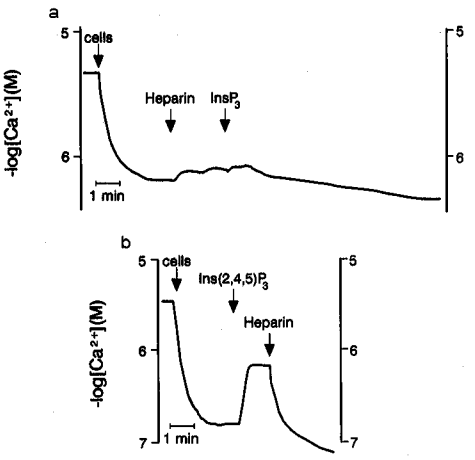

Fig. 3. Effect of Ins(1,4,5)P_3, in the presence of heparin, on the steady-state free Ca^{2+} concentration (A). Heparin (200 μg/ml) and Ins(1,4,5)P_3 (5 μM) were added as indicated. The trace is representative of three different experiments. B. Effect of adding heparin after Ins(2,4,5)P_3-induced Ca^{2+} release. As indicated Ins(2,4,5)P_3 (5 μM) or heparin (200 μg/ml) were added. The trace is representative of three different experiments.

tions low levels of Ins(1,4,5)P_3 [11] mediate continuous mobilisation of Ca^{2+} from endoplasmic reticulum, thus balancing the uptake of the ion. Following exposure to high concentrations of Ins(1,4,5)P_3 these receptors may become down-regulated and therefore Ca^{2+} uptake is not counteracted, resulting in a more pronounced buffering of Ca^{2+}. By binding to these high-affinity Ins(1,4,5)P_3 receptors heparin will block the Ca^{2+} release pathway operating under basal conditions, the net effect also in this case being an increased uptake of Ca^{2+}. Prentki et al. proposed a role for basal Ins(1,4,5)P_3 in regulating Ca^{2+} cycling across endoplasmic reticulum [11] and it appears that various cells indeed contain low levels of Ins(1,4,5)P_3 even under basal conditions [18]. Hence, the continuous presence of low concentrations of Ins(1,4,5)P_3 will enable continuous activation of the high-affinity Ins(1,4,5)P_3 receptors. Spät et al. described a type of high-affinity Ins(1,4,5)P_3

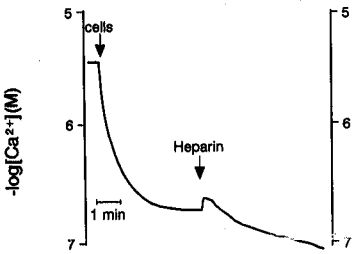

Fig. 4. The effect of heparin on the steady-state free Ca^{2+} concentration. Heparin (200 μg/ml) was added as indicated. The trace is typical of three different experiments.

receptor of unknown functional significance in neutrophils [19]. Whether such receptors also exist in RINm5F cells merits further investigations.

Another possibility whereby Ins(1,4,5)P_3 and heparin promote increased Ca^{2+} sequestration may be through the activation of receptors distinct from the traditional Ins(1,4,5)P_3 receptors. In this case heparin, by being a structural analogue of Ins(1,4,5)P_3, may actually promote Ca^{2+} uptake by acting like a partial agonist.

We have now demonstrated that the presence of Ins(1,4,5)P_3 not only leads to Ca^{2+} mobilisation but also a more effective Ca^{2+} buffering. Hence, under physiological conditions the trisphosphate may induce sequential both increase and decrease in cytoplasmic free Ca^{2+}, enabling complex regulation of intracellular processes dependent on Ca^{2+}.

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Ca^{2+}-induced Ca^{2+} release in insulin-secreting cells

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The sulphydryl reagent thimerosal (50 μM) released Ca^{2+} from a non-mitochondrial intracellular Ca^{2+} pool in a dose-dependent manner in permeabilized insulin-secreting RINm5F cells. This release was reversed after addition of the reducing agent dithiothreitol. Ca^{2+} was released from an Ins(1,4,5)P_3-insensitive pool, since release was observed even after depletion of the Ins(1,4,5)P_3-sensitive pool by a supramaximal dose of Ins(2,4,5)P_3 or thapsigargin. The Ins(1,4,5)P_3-sensitive pool remained essentially unaltered by thimerosal. Thimerosal-induced Ca^{2+} release was potentiated by caffeine. These findings suggest the existence of Ca^{2+}-induced Ca^{2+} release also in insulin-secreting cells.

Ca^{2+}-induced Ca^{2+} release; Sulphydryl reagent; Thimerosal; Intracellular Ca^{2+} transport; Insulin-secreting cell; Permeabilized cell

1. INTRODUCTION

In many cells, including insulin-secreting cells, receptor activation leads to phospholipase-C mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, resulting in generation of diacylglycerol and inositol 1,4,5-trisphosphate, (Ins(1,4,5)P_3)[1–3]. Ins(1,4,5)P_3 is a well-known second messenger that mobilizes Ca^{2+} from specific intracellular stores, which appear to be structurally related to the endoplasmic reticulum. The Ins(1,4,5)P_3-sensitive pool is well established and comprises only a part of the intracellular non-mitochondrial Ca^{2+} pool [1]. The remainder of the pool, which is Ins(1,4,5)P_3-insensitive, is less well characterized. In a number of cell types it has been shown that Ca^{2+} can be released from part of the Ins(1,4,5)P_3-insensitive Ca^{2+}-pool by a rapid increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$ [4]. This Ca^{2+}-induced Ca^{2+} release (CICR) was initially identified in striated muscle cells and later has been demonstrated in a number of non-muscle cell types [5–9]. There are at present both theoretical grounds [10,11] and experimental evidence [12,13] to indicate that CICR may be important in the generation of Ca^{2+}-oscillations. However, CICR is difficult to demonstrate directly [4]. Ca^{2+} release evoked by two pharmacological agents, caffeine and ryanodine [5,14,15], is taken as evidence for the existence of CICR mechanism in cells. Caffeine is required to be used in millimolar concentrations and an optimal concentration can often not be used, because of its solubility limitations [16,17]. Ryanodine binds very slowly to its receptor [16,18].

Moreover, in many cells Ca^{2+} release cannot be demonstrated by caffeine or ryanodine [4,19–21]. It would, therefore, be useful to have other pharmacological tools that activate CICR in caffeine-insensitive cells [21].

The structure and function of most cysteine-containing proteins critically depend on the oxidation state of the protein's sulphydryl groups (SH-groups) [22,23]. There is evidence that the receptor-channel protein that mediates CICR (ryanodine receptor) in sarcoplasmic reticulum contains 'critical' SH-groups [24]. A number of sulphydryl reagents that oxidize SH-groups, release Ca^{2+} from sarcoplasmic reticulum by opening up the CICR channel [25–27]. Thimerosal is a sulphydryl reagent that has been demonstrated to be effective, in low micromolar concentrations, in releasing Ca^{2+} from intracellular pools in several non-muscle cell types [28–31]. More recently, it has been shown that thimerosal specifically sensitizes CICR in unfertilized hamster eggs and it has been suggested that this compound can be used to demonstrate CICR in caffeine-insensitive cells [21]. In this report, we demonstrate that thimerosal releases Ca^{2+} in permeabilized RINm5F cells, an effect potentiated by caffeine, suggesting the existence of CICR in insulin-secreting cells.

2. MATERIALS AND METHODS

Clonal insulin-secreting RINm5F cells were maintained in culture in RPMI 1640 medium supplemented with 10% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), all from Flow Laboratories (Scotland). Thimerosal (sodium ethylmercurithiosalicylate), DL-dithiothreitol (DTT), Ins(1,4,5)P_3, caffeine, Ruthenium red and heparin were purchased from Sigma (St. Louis, USA). Ryanodine

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was obtained from Merck Sharp and Dohme (Rahway, NJ, USA). Calcium cocktail, containing neutral carrier ETH 1001 was from Fluka. All other chemicals were either from Sigma or Merck.

Cells were detached from culture flasks using trypsin-EDTA; washed twice with RPMI 1640 medium and twice with a cold nominally Ca^{2+}-free buffer, containing 110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin and 25 mM HEPES (pH 7.0 adjusted with KOH). Permeabilization was done by exposing cells to high-voltage electrical discharges (six pulses of 3.2 kV/cm). This treatment resulted in more than 90% permeabilized cells, as verified by Trypan blue uptake. After permeabilization, cell suspension was centrifuged and the pellet was kept on ice until use.

Eight μl of cell pellet was then added to a Plexiglass chamber containing 52 μl of incubation buffer. The incubation buffer was the same as the washing buffer, supplemented with 2 mM MgATP and an ATP-regenerating system, consisting of 10 mM phosphocreatine and 20 U of creatine kinase/ml. In addition, the incubation buffer also contained mitochondrial inhibitors consisting of 0.2 μM antimycin and 1 μg of oligomycin/ml. Additions were made from freshly prepared 100 times concentrated stock solutions. Thimerosal was dissolved in water or directly in the buffer. Changes in the ambient free Ca^{2+} concentration were recorded using a Ca^{2+}-selective mini-electrode, constructed and calibrated essentially as described by Tsien and Rink [32]. None of the substances used in the study interfered with electrode function.

3. RESULTS

Permeabilized RINm5F cells (4.2×10^7 cells/ml), in the presence of ATP and an ATP-regenerating system, sequestered Ca^{2+}, resulting in a low steady-state buffer Ca^{2+} level. Addition of thimerosal (50 μM, final concentration) resulted in a rise in Ca^{2+} within 30 s, reaching a new increased steady state Ca^{2+} level in about 4 min (Fig. 1a). Thimerosal caused Ca^{2+} release in a dose-dependent manner (Fig. 1b). The smallest dose of thimerosal eliciting a detectable rise in Ca^{2+} was 25 μM and maximum release was obtained by 100 μM. Addition of the reducing agent DTT (2 mM, final concentration) resulted in immediate onset of re-uptake of Ca^{2+}, eventually leading to complete resequestration of the ion (Fig. 1a).

Heparin binds to the Ins(1,4,5)P_3 receptor and inhibits Ins(1,4,5)P_3-induced Ca^{2+} release [33–36]. When added to the buffer in as high a dose as 1000 μg/ml, heparin did not inhibit Ca^{2+} release induced by 50 μM thimerosal (data not shown). When heparin was added at the end of completion of thimerosal-induced Ca^{2+} release, there was no re-uptake of the released Ca^{2+} (data not shown). Also, Ruthenium red (30 μM), a substance that has been shown to inhibit Ca^{2+}-induced Ca^{2+} release [37], did not block thimerosal-induced Ca^{2+} release.

As shown in Fig. 2a, 20 μM (a maximal dose) of inositol(2,4,5)-trisphosphate (Ins(2,4,5)P_3), a non-metabolizable analogue of Ins(1,4,5)P_3 was used to empty the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. Addition of thimerosal at the end of completion of Ins(2,4,5)P_3-induced Ca^{2+} release, resulted in further release of Ca^{2+}. Similarly, when thimerosal was added at the end of completion of

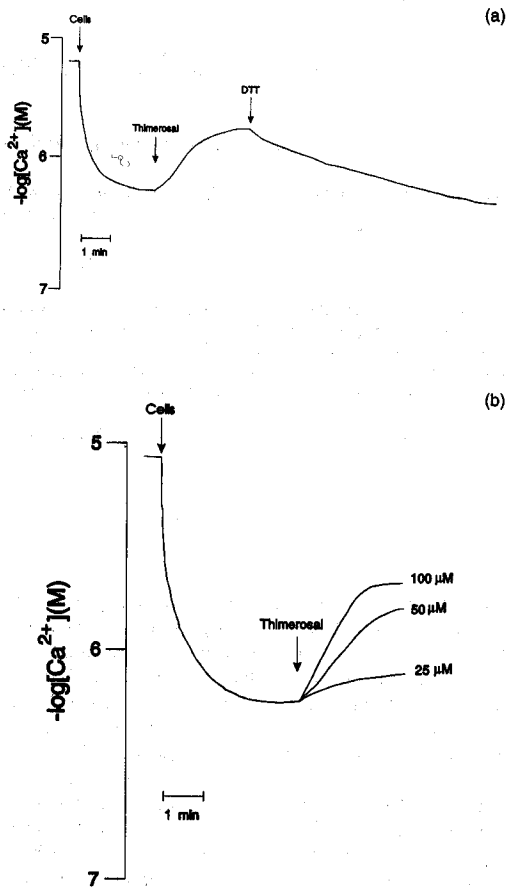

Fig. 1. Effects of thimerosal and DTT on Ca^{2+} release and re-uptake. The figure shows Ca^{2+}-electrode traces obtained under conditions described in section 2. (a) Release and re-uptake of Ca^{2+}, following additions, as indicated, of thimerosal (50 μM, final concentration) and DTT (2 mM, final concentration). The trace is representative of at least three different experiments. (b) Effects of additions of different concentrations of thimerosal on Ca^{2+} release. The arrow indicates addition of thimerosal (at the final concentrations shown). The figure shows the trace for each concentration of thimerosal superimposed and is representative of at least three different experiments.

Ca^{2+} release by thapsigargin, there was additional release of Ca^{2+} (Fig. 2b).

When Ins(1,4,5)P_3 (5 μM, final concentration) was added after thimerosal-induced Ca^{2+} release was completed, marked additional increase in the release of Ca^{2+} was observed (Fig. 3). The magnitude of this Ins(1,4,5)P_3-induced Ca^{2+} release was essentially similar to that obtained by Ins(2,4,5)P_3 in the absence of thimerosal (cf. Fig. 2a).

Caffeine (2 mM, final concentration) did not induce

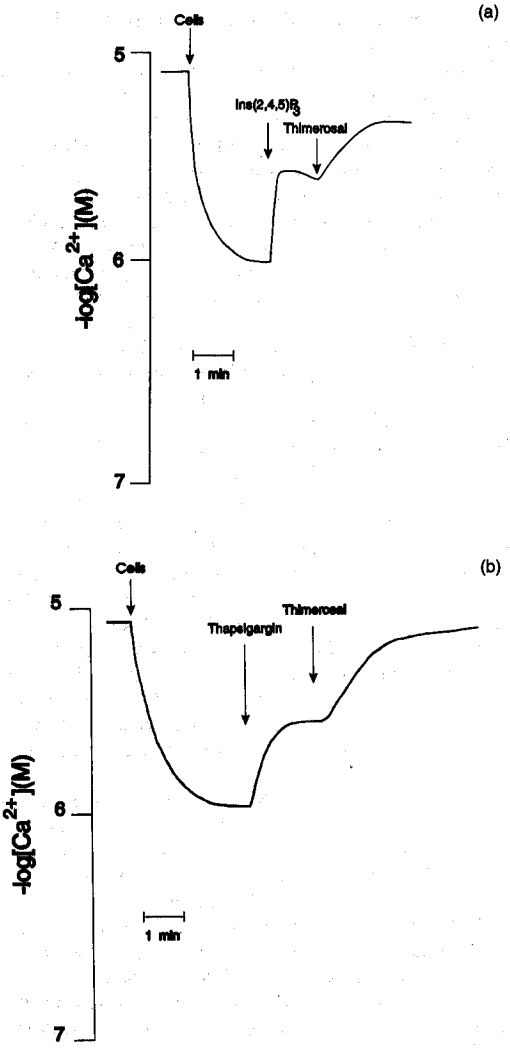

Fig. 2. Thimerosal releases Ca^{2+} from the Ins(1,4,5)P_3-insensitive pool. Conditions of experiments were the same as described in the legend to Fig. 1. As indicated, thimerosal (50 μM, final concentration) was added after emptying the Ins(1,4,5)P_3-sensitive pool by (a) addition of Ins(2,4,5)P_3 (20 μM, final concentration) and (b) thapsigargin (5 μM, final concentration). Each trace is representative of at least 3 different experiments.

Ca^{2+} release (data not shown). Under our experimental conditions the maximal final concentration of caffeine that could be achieved by addition from stock solution was 2 mM. This was due to difficulty in obtaining a concentrated enough stock solution of caffeine [16,17]. Ryanodine (100 μM, final concentration) also did not induce Ca^{2+} release (data not shown). In the presence of caffeine (50 mM, final concentration), dissolved directly in the buffer, addition of thimerosal (50 μM) caused a more pronounced release of Ca^{2+} than in the absence of caffeine (cf. Fig. 4a and b).

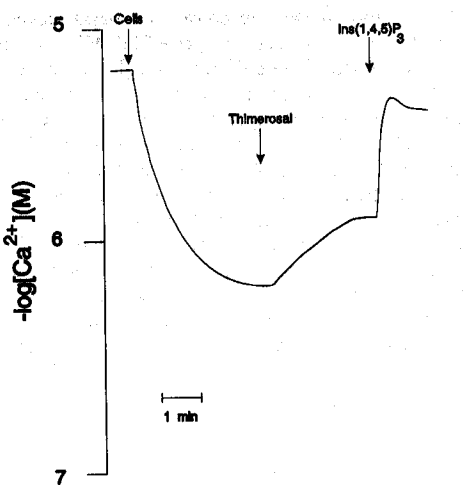

Fig. 3. Effect of addition of Ins(1,4,5)P_3 after thimerosal-induced Ca^{2+} release is completed. Conditions of experiments were the same as mentioned before. As indicated, thimerosal (50 μM, final concentration) and Ins(1,4,5)P_3 (5 μM, final concentration) were added. Each trace is representative of at least 5 different experiments.

4. DISCUSSION

The present study shows that the sulphydryl reagent thimerosal, in a dose-dependent manner, releases Ca^{2+} from an intracellular Ca^{2+} pool, in permeabilized RINm5F cells. The effect was completely reversed by addition of the reducing agent DTT, implying that the effect of thimerosal was specifically due to oxidation of SH-groups and not due to non-specific and permanent damage to the membrane of Ca^{2+}-storing vesicles. The effect of thimerosal in permeabilized RINm5F cells is not likely to reflect inhibition of intracellular Ca^{2+} pumps, since the sulphydryl reagent did not alter the initial rate of Ca^{2+} uptake. There is also evidence from other studies that thimerosal, in low concentrations, does not inhibit various Ca^{2+}-pumps [21,29].

Although several studies have demonstrated mobilization of Ca^{2+} from intracellular pools by thimerosal, its mechanism of action is not well understood [28–31]. It has been suggested that in non-muscle cells, sulphydryl reagents may induce Ca^{2+} release from the Ins(1,4,5)P_3-sensitive pool, by sensitizing the Ins(1,4,5)P_3 receptor to endogenous levels of Ins(1,4,5)P_3 [38]. We and others have suggested that, under conditions of permeabilization, RINm5F cells also contain basal levels of Ins(1,4,5)P_3 [36,39]. In this study Ca^{2+} release is unlikely to be due to sensitization

of the $Ins(1,4,5)P_3$ receptor to basal levels of $Ins(1,4,5)P_3$, as has been suggested [38,40], since prior presence of heparin in buffer, in as high a concentration as 1000 μg/ml, did not prevent the release. In fact, some studies have demonstrated that sulphydryl oxidation of the $Ins(1,4,5)P_3$ receptor rather inhibits $Ins(1,4,5)P_3$ binding [41] and $Ins(1,4,5)P_3$-induced Ca^{2+} release [42]. By contrast, other studies have shown that sulphydryl oxidation increases the sensitivity of the $Ins(1,4,5)P_3$ receptor to the trisphosphate [40]. Interpretation of such conflicting data is difficult because of the use of different sulphydryl reagents and various cell types involved. However, since sulphydryl oxidation may also sensitize CICR, it cannot be ruled out that the apparent increase in sensitivity to low doses of $Ins(1,4,5)P_3$ [40] may be confounded by CICR.

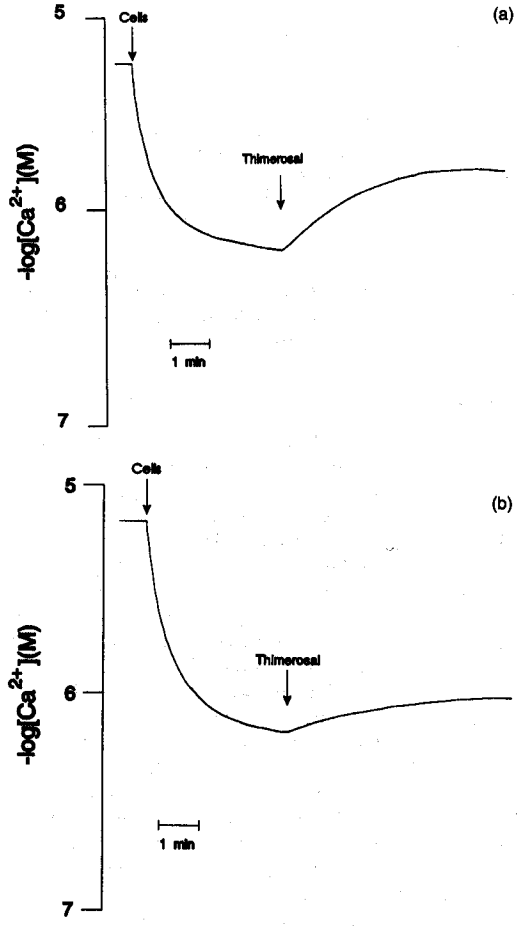

Fig. 4. Caffeine potentiates thimerosal-induced Ca^{2+} release. Conditions of experiments were the same as mentioned before except that in (a) the incubation buffer contained caffeine (50 mM, final concentration). (b) Control experiments done without caffeine in buffer. Arrows indicate additions of thimerosal (50 μM, final concentration). Each of the traces are representative of at least 3 different experiments.

Clearly, thimerosal released Ca^{2+} from an $Ins(1,4,5)P_3$-insensitive pool and not from the $Ins(1,4,5)P_3$-sensitive pool, in RINm5F cells. This pool is not equivalent to mitochondria since mitochondrial blockers were routinely used. When the $Ins(1,4,5)P_3$-sensitive pool was depleted by a supramaximal dose of $Ins(2,4,5)P_3$, addition of thimerosal still released Ca^{2+}, which in this case must have been released from an $Ins(1,4,5)P_3$-insensitive pool. In permeabilized RINm5F cells, we have demonstrated that thapsigargin [43] releases Ca^{2+} predominantly from an $Ins(1,4,5)P_3$-sensitive pool and empties the pool nearly completely (Islam and Berggren, unpublished data). Also, under these conditions, thimerosal released further Ca^{2+}. The question whether thimerosal-induced Ca^{2+} release could be due to activation of CICR was not addressed in earlier studies [28–31], although one study attributed the release to an as yet unidentified intracellular Ca^{2+} transport system [29]. Convincing and direct evidence that thimerosal sensitizes CICR has come forth only recently [21]. In view of the present findings, it is likely that the thimerosal-induced Ca^{2+} release from an $Ins(1,4,5)P_3$-insensitive pool in RINm5F cells is due to opening up of CICR channel by SH-group oxidation. Evidence that thimerosal-induced Ca^{2+} release in RINm5F cells is due to activation of CICR is reinforced by our demonstration that it can be markedly enhanced by caffeine, which is known to activate or sensitize CICR.

The existence of CICR has been inferred from caffeine-induced Ca^{2+} release in a number of non-muscle cells [16,44–46]. It is possible that CICR occurs in many cell types but is not demonstrable, because of their apparent insensitivity to caffeine, as is the case with RINm5F cells. These cells may contain a variant of the 'classical' ryanodine receptor, which mediates CICR in sarcoplasmic reticulum. In view of recent demonstrations that CICR may be involved in the generation of Ca^{2+}-oscillations and wave propagation [12,13], it is important to clarify its existence in different cells and in this context, thimerosal may be an additional tool. Although the physiological importance is far from being understood, this is the first report suggesting the existence of CICR in insulin-secreting cells.

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Sulfhydryl oxidation induces rapid and reversible closure of the ATP-regulated K^+ channel in the pancreatic β-cell

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Effects of sulfhydryl modification on the ATP regulated K^+ channel (K_{ATP} channel) in the pancreatic β-cell were studied, using the patch clamp technique. Application of the sulfhydryl oxidizing agents thimerosal and 2,2′-dithio-bis(5-nitropyridine) (DTBNP), in micromolar concentrations, caused complete inhibition of the K_{ATP} channel, in inside-out patches. The inhibition was rapid and was reversed by the disulfide reducing agents dithiothreitol and cysteine. Thimerosal, which is poorly membrane permeable, inhibited channel activity, only when applied to the intracellular face of the plasma membrane. In contrast, DTBNP, which is highly lipophilic, caused closure of the K_{ATP} channel and consequent depolarization of the membrane potential, also when applied extracellularly. Our results indicate the presence of accessible free SH groups on the cytoplasmic side of the K_{ATP} channel in the pancreatic β-cell. These SH groups are essential for channel function and it is possible that thiol-dependent redox mechanisms can modulate K_{ATP} channel activity.

ATP-regulated K^+ channel; Sulfhydryl reagent; Thimerosal; Pancreatic β-cell

1. INTRODUCTION

K^+ channels characterized by their sensitivity to intracellular ATP (K_{ATP}), play an important role in the regulation of insulin secretion from the pancreatic β-cell [1–3]. Under resting conditions, at glucose concentrations less than 5 mM, the K_{ATP} conductance dominates and therefore determines the membrane potential of the β-cell [4]. A key event in the glucose stimulation of insulin secretion is the closure of this channel. Closure of the K_{ATP} channel results in depolarization of the cell, Ca^{2+}-influx through the voltage-gated Ca^{2+} channel, increase in the cytoplasmic free Ca^{2+} concentration and insulin secretion [3,5]. The K_{ATP} channel is also the target for sulfonylureas, a class of drugs which inhibits channel activity, and are used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM) [6]. The precise signals that generate from glucose metabolism and control the activity of the K_{ATP} channel are still unknown. Currently, a change in the intracellular concentration of ATP or ATP/ADP ratio is believed to be the most important link between fuel metabolism and depolarization of the cell [2,7]. However the regulation of the channel appears to be more complex than that and may involve modulation by protein kinase C, G proteins and changes in the redox potential of the cell. [8–13]. At present little is known about the structure of the K_{ATP} channel protein, as well as about the molecular basis of its regulation.

Many biologically active proteins contain critical cysteine residues. The function of these proteins often depends on the oxidation state of sulfhydryl (thiol) groups (SH groups) [14]. Some proteins are active only when their specific SH groups remain in the reduced form, whereas for the activity of others the disulfide redox state is essential [15,16]. Selective modification of SH groups, has been extensively used to ascertain the relationship between structure and function of many biomolecules. Different types of ion channel proteins also contain SH groups, modification of which may affect channel activity [17,18]. The sulfhydryl reagent thimerosal and some 'reactive disulfides' open intracellular Ca^{2+} channels by oxidizing critical SH groups [18,19]. There is evidence to suggest, that the K_{ATP} channel of mouse skeletal muscle contains functionally important SH groups [20]. The role of SH groups in regulating the activity of the K_{ATP} channel in the pancreatic β-cell is unknown, although it is known since long that many sulfhydryl reagents stimulate insulin secretion [21–24]. In the present study we demonstrate that the sulfhydryl oxidizing agents, thimerosal and 2,2′-dithio-bis(5-nitropyridine) (DTBNP) induce rapid and reversible closure of the K_{ATP} channel in the pancreatic β-cell, indicating that this channel contains SH groups essential for the channel activity.

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2. MATERIALS AND METHODS

Thimerosal (mercury-[(*o*-carboxyphenyl)thio]-ethyl sodium), DTBNP and dithiothreitol (DTT) were from Sigma. All other chemicals were of analytical grade and were either from Sigma or Merck. DTBNP was dissolved in dimethyl sulfoxide (DMSO), the final concentration of DMSO being not more than 0.1%.

2.1. *Preparation of cells*

Pancreatic islets from adult obese mice (ob/ob) were isolated by collagenase digestion and dispersed into single cells by shaking in a Ca^{2+}- and Mg^{2+}-deficient medium, as previously described [3]. Cells were plated on petri dishes and cultured for 1–3 days in RPMI 1640 medium, containing 11 mM glucose and supplemented with fetal calf serum (10% v/v), penicillin (100 IU/ml) and streptomycin (100 μg/ml).

2.2. *Electrophysiology*

We used inside-out, outside-out and whole-cell modes of the patch-clamp technique [25]. Pipettes were pulled from borosilicate glass and coated with Sylgard resin (Dow Corning) near the tips, fire-polished and had resistances between 2 and 6 MΩ. Single-channel currents were recorded from inside-out or outside-out membrane patches and channel activity was measured at 0 mV membrane potential. The membrane potential was monitored using the whole-cell technique. Current and voltage were recorded using an Axopatch 200 patch-clamp amplifier (Axon Instruments Inc. Foster City, USA). During experiments the current and voltage signals were stored using a VR-100A digital recorder (Instrutech Corp., USA) and a high-resolution video cassette recorder (JVC, Japan). Channel records are displayed according to the convention with upward deflections denoting outward currents. K_{ATP} channel activity was identified on the basis of the sensitivity to ATP and the unitary amplitude (1.5–2 pA).

In all experiments the extracellular solution contained (in mM): 138 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, and 5 HEPES (pH 7.4 with NaOH). The 'intracellular-like' solution consisted of (in mM): 125 KCl, 1 $MgCl_2$, 10 EGTA, 30 KOH, and 5 HEPES (pH 7.15 with KOH). Patches were excised into nucleotide-free solution and ATP was first added to test for channel inhibition. ATP was then removed and patches were subsequently exposed to the test substances, as indicated in the figures. Mg-ATP (0.1 mM) was present in the intracellular solution for most of the time to reduce run-down of the K_{ATP} channel [26]. The bath had a volume of 0.4 ml and cells were perifused at a rate of 4 ml/min. All test compounds were added to the perifusion medium. Each experimental condition was tested, with identical results, in 3–6 different patches. All experiments were done at room temperature (22°C).

The current signal was filtered at 100 Hz (−3 dB value) by using an 8-pole Bessel filter (Frequency Devices, Haverhill, USA). Single-channel conductance was measured directly from a digital oscilloscope. Figures were made by plotting segments of the records on a chart recorder, scanning the segments using a HP scanner and incorporating them into Corel Draw graphics software program.

3. RESULTS

The inhibitory action of thimerosal on K_{ATP} channel activity was rapid and almost complete with 10 μM of the compound (Fig. 1). The blocking effect was dose-dependent, with a threshold concentration of thimerosal of about 1–2 μM. At higher concentrations (up to 100 μM), thimerosal invariably caused complete inhibition of channel activity (data not shown). Replacement of the thimerosal-containing solution with thimerosal-free solution did not cause spontaneous return of channel activity, even when observed for a prolonged period of time (up to 5 min). However, addition of 2 mM DTT, a disulfide reducing agent [27] readily reversed the inhibitory action of thimerosal and caused a substantial return of channel activity. Similar reversal of the inhibitory action of thimerosal was obtained by the disulfide reducing agent cysteine (100 μM) (data not shown). Although these reducing agents readily reversed the inhibitory effect of thimerosal, they themselves, did not have any effect on channel activity (Fig. 2A). DTT did not block the inhibitory effect of ATP on the K_{ATP} channel (Fig. 2B). We did not observe any effect of thimerosal (10–20 μM) on the Ca^{2+}-sensitive K^+ channel (BK channel).

Fig. 3 shows the inhibitory effect of a representative reactive disulfide, DTBNP on the K_{ATP} channel. On addition of DTBNP (20 μM), there was a rapid and pronounced reduction in channel activity. In control experiments, 0.1% DMSO (solvent for DTBNP) had no effect on channel activity. The inhibitory effect of DTBNP was also partially reversed by the reducing agents DTT (Fig. 3) and cysteine (data not shown).

Since thimerosal is hydrophilic and therefore poorly membrane permeable, it is likely that was affecting SH groups on the cytoplasmic side of the cell membrane. This was confirmed in experiments using the outside-out patch configuration, where the extracellular face of

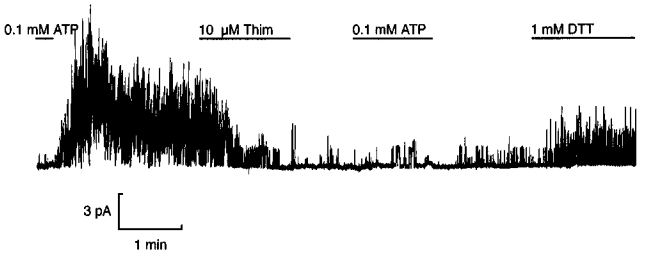

Fig. 1. Effects of thimerosal and DTT on K_{ATP} channel activity. Single-channel recordings from excised inside-out membrane patches obtained from cultured mouse β-cells. The pipette contained extracellular solution and the cell was perifused with intracellular-like solution. Thimerosal (10 μM) almost completely blocked the current through the K_{ATP} channel. The blocking effect of thimerosal was not spontaneously reversed upon withdrawal of the substance. Addition of DTT (2 mM) to the same patch, reversed the inhibitory effect of thimerosal.

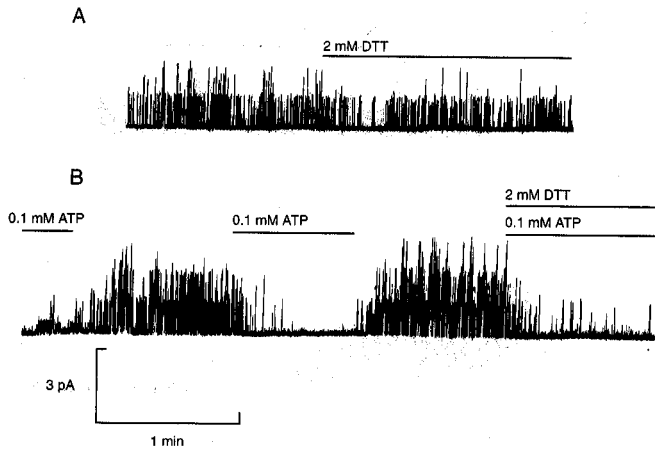

Fig. 2. Single-channel recordings from excised inside-out membrane patches. The the conditions of experiments were as described in the legend to Fig. 1. (A) DTT did not affect K_{ATP} channel activity in the absence of ATP. (B) DTT had no effect on ATP-induced (0.1 mM) inhibition of K_{ATP} channel.

the cell membrane is exposed to the perifusion medium. Addition of a high concentration of thimerosal (100 μM), no longer blocked the activity of the K_{ATP} channel (Fig. 4A). On the contrary, application of DTBNP, which is highly lipophilic, under the same conditions caused rapid inhibition of the channel (Fig. 4B).

Membrane potentials were recorded from the β-cell in the whole-cell patch clamp configuration, with the pipette containing ATP-free intracellular-like solution. The decrease in intracellular ATP caused opening of the K_{ATP} channel and membrane repolarization. Thimerosal (up to 100 μM), applied to the extracellular face of the plasma membrane did not depolarize the cell, whereas subsequent application of DTBNP (50 μM) did (Fig. 5). Application of DTBNP (50 μM) alone, without prior addition of thimerosal, had a similar effect on membrane potential (data not shown).

4. DISCUSSION

We demonstrate effects of sulfhydryl oxidation, by thimerosal and DTBNP, on K_{ATP} channel activity in the pancreatic β-cell. Thimerosal inibited channel activity in a dose-dependent manner and was effective in low micromolar concentrations, with maximal inhibitory effect obtained at 10–20 μM. Thus, thimerosal was a more potent inhibitor of this channel than for example tolbutamide, an example of the first generation of antidiabetic sulfonylureas [6]. The effect of thimerosal was reversed by addition of excess of the disulfide reducing agent DTT or cysteine. This indicates that inhibition of channel activity was caused by sulfhydryl oxidation and not due to a nonspecific effect on the channel. However, the biochemical reaction of thimerosal is not known to be specific for any particular type of SH group. Such

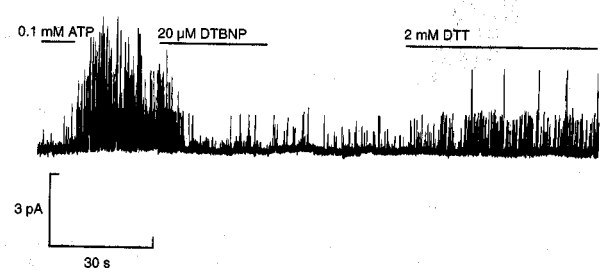

Fig. 3. Effect of DTBNP on K_{ATP} channel activity in a single-channel recording from an excised inside-out patch. The pipette contained extracellular solution and the bath was perfused with intracellular-like solution. Addition of DTBNP (20 μM) almost totally blocked channel activity. The blocking effect was not reversed following wash out of DTBNP. Upon addition of DTT (2 mM), K_{ATP} channel activity was partially restored.

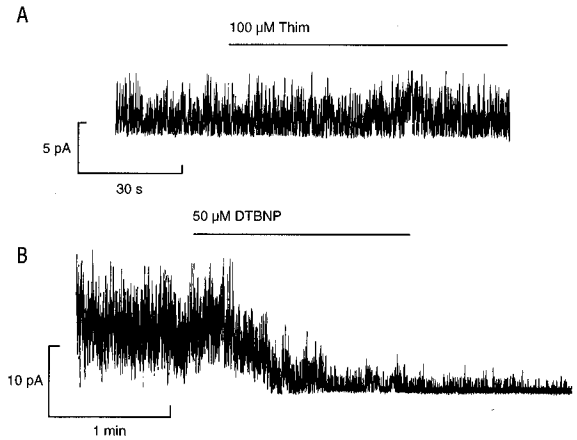

Fig. 4. Single channel recordings from excised outside-out membrane patches. The pipette was filled with intracellular-like solution and the patch was perfused with extracellular medium. (A) Thimerosal (100 μM) did not affect K_{ATP} currents when applied to the extracellular face of the membrane. (B) Application of 50 μM DTBNP completely blocked K_{ATP} channel activity in the outside-out membrane patch.

mercurial compounds may also bind to functional groups other than SH groups [28]. For this reason, we tested the effect of another sulfhydryl oxidizing agent, DTBNP. This substance belongs to a class of compounds known as 'reactive disulfides' [29]. DTBNP and related dithiopyridines are almost absolutely specific for free SH groups, which they oxidize through a thiol-disulfide exchange reaction [29,30]. Like thimerosal, DTBNP was also a potent inhibitor of the K_{ATP} channel. In contrast to the effect of thimerosal, inhibition of K_{ATP} channel activity by DTBNP was not reversed to the same extent by the reducing agents. This is not surprising, since it is known that the reaction of DTBNP with SH groups can be irreversible [31].

At the concentrations used in this study, thimerosal was effective only when applied to the cytoplasmic face of the membrane. There was no inhibition of K_{ATP} channel activity and no depolarization of the membrane, when thimerosal was applied to the extracellular side of the membrane. This suggests that the site of the critical SH groups, associated with the K_{ATP} channel, is on the cytoplasmic side of the plasma membrane. Thimerosal, being hydrophilic and poorly membrane permeable, cannot access these SH groups when applied to the extracellular side of the membrane. In contrast to thimerosal, DTBNP is lipophilic and membrane permeable. As expected, application of DTBNP to the extracellular face of the membrane also caused inhibition of the K_{ATP} channel and consequently, membrane depolarization. Since the effect of thimerosal was observed in the ex-

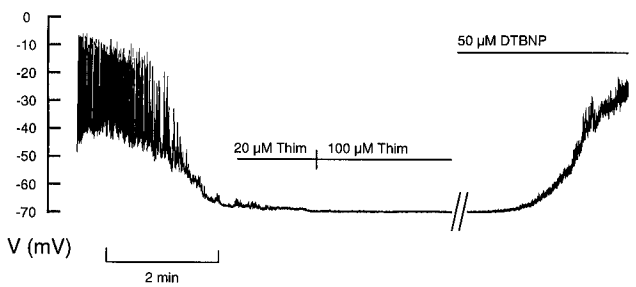

Fig. 5. Whole-cell recordings from β-cells showing effects of thimerosal and DTBNP on membrane potential. Cells were intracellularly perfused with an ATP-free intracellular-like solution and the bath was perfused with extracellular solution. Prior to the experiments, cells were cultured in media containing 11 mM glucose and because of the short time before the start of an experiment, the cells occasionally exhibited action potentials. As the pipette solution lacked ATP, the cells quickly repolarized to about −70 mV. Thimerosal, in concentrations up to 100 μM, did not affect membrane potential, whereas DTBNP (50 μM) readily induced membrane depolarization. Vertical bars in the trace indicate a brake in the record of approximately 3 min.

cised inside-out patch configuration, it is likely to represent a direct interaction with the K_{ATP} channel or a closely associated protein.

These results establish the presence of accessible free SH groups on the K_{ATP} channel in the pancreatic β-cell, as has been suggested for the K_{ATP} channel in mouse skeletal muscle [20]. Our results indicate that the SH groups are critically important for the regulation of K_{ATP} channel activity. There are many reports of alterations in the function of different ion channels caused by modification of sulfhydryl or disulfide groups on the channel protein. Such effects have been described, for instance, on the nicotinic acetylcholine receptor and on intracellular Ca^{2+} channels such as the ryanodine and the inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) receptor [17–19,30,32]. Noteworthy is, that sulfhydryl oxidation leads to opening of the intracellular Ca^{2+} channels, whereas in the case of the K_{ATP} channel the result of a similar chemical modification is the opposite [19,30].

Closure of the K_{ATP} channel may be the underlying mechanism by which some sulfhydryl reagents, that oxidize relatively superficial SH groups, also strongly stimulate insulin secretion [21–24]. The mechanism of insulin secretion by the antidiabetic sulfonylureas also involves selective inhibition of the K_{ATP} channel. However, the molecular basis of the interaction of the sulfonylureas with the K_{ATP} channel or any associated proteins is still unknown, although it has been suggested that their mechanism of action may involve an interaction with membrane-associated SH groups [33–35]. The present study also suggests that sulfhydryl modification may be a means for developing selective insulinotropic agents, as has been done for the development of some other classes of drugs [36,37]. Whether thiol-dependent redox mechanisms may play a role, also in the physiological regulation of the stimulus-secretion coupling in the pancreatic β-cell, as has been suggested before [38], is not clear. In principle, however, it is possible that the activity of the K_{ATP} channel may be modulated by metabolism-induced changes in the redox state in the close vicinity of the channel. Such switching mechanisms, by thiol-dependent redox regulation, have been postulated for other biological processes [39–41].

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Mobilization of Ca^{2+} by thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone in permeabilized insulin-secreting RINm5F cells: evidence for separate uptake and release compartments in inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool

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We characterized and directly compared the Ca^{2+}-releasing actions of two inhibitors of endoplasmic-reticulum (ER) Ca^{2+}-ATPase, thapsigargin and 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ), in electropermeabilized insulin-secreting RINm5F cells. Ambient free calcium concentration ($[Ca^{2+}]$) was monitored by Ca^{2+}-selective mini-electrodes. After ATP-dependent Ca^{2+} uptake, thapsigargin and tBuBHQ released Ca^{2+} with an EC_{50} of ~37 nM and ~2 μM respectively. Both agents mobilized Ca^{2+} predominantly from the Ins(1,4,5)P_3-sensitive Ca^{2+} pool, and in this respect thapsigargin was more specific than tBuBHQ. The total increase in $[Ca^{2+}]$ obtained with thapsigargin and Ins(1,4,5)P_3 was, on the average, only 7% greater than that with Ins(1,4,5)P_3 alone. In contrast, the total increase in $[Ca^{2+}]$ obtained with tBuBHQ and Ins(1,4,5)P_3 was 33% greater than that obtained with only InsP_3 ($P < 0.05$). Although Ca^{2+} was rapidly mobilized by thapsigargin and tBuBHQ, complete depletion of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool was difficult to achieve. After the release by thapsigargin or tBuBHQ, Ins(1,4,5)P_3 induced additional Ca^{2+} release. The additional Ins(1,4,5)P_3-induced Ca^{2+} release was not altered by supramaximal concentrations of thapsigargin and tBuBHQ, or by Bafilomycin A_1, an inhibitor of V-type ATPases, but was decreased by prolonged treatment with the ER Ca^{2+}-ATPase inhibitors. These results suggest the existence of distinct uptake and release compartments within the Ins(1,4,5)P_3-sensitive Ca^{2+} pool. When treated with the inhibitors, the two compartments became distinguishable on the basis of their Ca^{2+} permeability. Apparently, thapsigargin and tBuBHQ readily mobilized Ca^{2+} from the uptake compartment, whereas Ca^{2+} from the release compartment could be mobilized only very slowly, in the absence of Ins(1,4,5)P_3.

INTRODUCTION

The cytosolic free calcium concentration ($[Ca^{2+}]_i$) plays a key role in the regulation of diverse cellular processes, including the stimulus–secretion coupling in insulin-secreting cells [1]. Agonists can raise $[Ca^{2+}]_i$ either by stimulating influx of Ca^{2+} through voltage-gated or receptor-operated Ca^{2+} channels in the plasma membrane or by releasing Ca^{2+} from intracellular pools. Intracellular non-mitochondrial Ca^{2+} pools appear to be associated with the endoplasmic reticulum (ER), but may be distinct from ER in the form of a specialized Ca^{2+}-storing organelle [2,3]. Ins(1,4,5)P_3, formed after stimulation of surface receptors, releases Ca^{2+} from specialized intracellular stores that possess receptors for the trisphosphate. In many cells, including insulin-secreting cells, part of the intracellular Ca^{2+} pools is insensitive to Ins(1,4,5)P_3 [4,5]. The Ins(1,4,5)P_3-insensitive Ca^{2+} pools may contain a caffeine-sensitive pool, responsible for Ca^{2+}-induced Ca^{2+} release [6,7]. Both the Ins(1,4,5)P_3- and Ca^{2+}-induced Ca^{2+} release may be involved in the generation of complex patterns of Ca^{2+} signalling, such as Ca^{2+} oscillations and propagating Ca^{2+} waves [8].

Ca^{2+} is sequestered into ER by an ATP-dependent Ca^{2+} pump, embedded in the ER membrane (ER Ca^{2+}-ATPase). It has been suggested that functionally different Ca^{2+} pools may contain different isoforms of the ER Ca^{2+}-ATPase [9] or may utilize different uptake mechanisms [10]. Inhibition of ER Ca^{2+}-ATPase leads to the release of Ca^{2+}, through unknown pathways. Thapsigargin is a highly potent and selective agent for inhibition of the sarcoplasmic- and endoplasmic-reticulum Ca^{2+}-ATPase family (SERCA) [11,12]. A more recent addition to the list of ER Ca^{2+}-ATPase inhibitors is 2,5-di-(t-butyl)-1,4-benzohydroquinone (tBuBHQ) [13]. Effects of tBuBHQ have been reported in fewer studies and some aspects of actions of the two inhibitors have been compared in different cells [14–17]. Both thapsigargin and tBuBHQ are being increasingly used as tools for defining intracellular Ca^{2+} stores as well as for studying Ca^{2+} fluxes and Ca^{2+}-dependent processes [15–25]. However, it appears that the intracellular Ca^{2+} pools mobilized by these agents vary in different cell types. Thapsigargin, for instance, releases Ca^{2+} from Ins(1,4,5)P_3-sensitive pool in many cells [15,21,26], whereas in others it affects both the Ins(1,4,5)P_3-sensitive and -insensitive pools [11,27]. The clonal cell line RINm5F, established from a rat insulinoma, is a useful experimental model of insulin-secreting cells [28,29]. In the present study, we characterized and directly compared thapsigargin- and tBuBHQ-induced Ca^{2+} release in electropermeabilized RINm5F cells. Our study identifies the thapsigargin- and tBuBHQ-sensitive Ca^{2+} pools and their relationship to the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in these cells.

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; $[Ca^{2+}]$, ambient free calcium concentration; ER, endoplasmic reticulum; tBuBHQ, 2,5-di-(t-butyl)-1,4-benzohydroquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; SERCA, sarcoplasmic- and endoplasmic-reticulum Ca^{2+}-ATPase.

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MATERIALS AND METHODS

Culture and permeabilization of cells

Clonal insulin-secreting RINm5F cells were maintained in culture in RPMI 1640 medium supplemented with 10% foetal-calf serum, penicillin (100 i.u./ml) and streptomycin (100 μg/ml), in a humidified incubator at 37 °C under an atmosphere of 5% CO_2 in air. Cells were detached from culture flasks with trypsin/EDTA, washed twice with RPMI 1640 medium and twice with a cold buffer with no added Ca^{2+}, containing 110 mM KCl, 10 mM NaCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mg/ml BSA and 25 mM Hepes (pH 7.0 adjusted with KOH). The contaminating Ca^{2+} concentration in this buffer was 1–2 μM. The electropermeabilization apparatus consisted of a Plexiglass chamber with platinum electrodes placed 0.5 cm apart, a capacitor and a switch designed to discharge the capacitor in a single event [30]. For permeabilization the capacitor was charged to the required voltage and then discharged through the cell suspension in the permeabilization chamber. With six pulses of 3.2 kV/cm, more than 95% of the cells became permeable, as verified by Trypan Blue uptake. After permeabilization, the cell suspension was centrifuged and the pellet kept on ice until use. Cells remained permeabilized over the period of the experiment (about 2 h). Rat hepatocytes were isolated by collagenase digestion as described previously [31] and were electropermeabilized by the same method that was used for RINm5F cells.

Measurements of the ambient free Ca^{2+} concentration [Ca^{2+}]

Ca^{2+}-sensitive mini-electrodes were constructed and calibrated, with some modifications of the method originally described by Tsien and Rink [32]. We used borosilicate capillary tubing, 5 cm long and 0.6 mm inner diameter, with 'omega dot' for rapid filling. The tubing was cleaned, heat-polished and silicone-treated [33]. The membrane solution for Ca^{2+} electrode was prepared by dissolving premixed Calcium Cocktail I and approx. 30% (w/v) poly(vinyl chloride) in tetrahydrofuran, to obtain a thin liquid of appropriate consistency [32]. The tip of the tubing was filled by briefly dipping into the membrane solution. The reference electrode, made from similar tubing, was conventionally pulled into capillary pipettes and was filled with 1 M KCl. Electrode responses were recorded by a purpose-built high-impedance electrometer.

Cells were added to a Plexiglass chamber, containing 52 μl of incubation buffer, with continuous stirring. The incubation buffer was the same as the washing buffer, supplemented with 2 mM Mg-ATP and an ATP-regenerating system, consisting of 10 mM phosphocreatine and 20 units of creatine kinase/ml. In addition, the incubation buffer contained mitochondrial inhibitors, consisting of 0.4 μM antimycin and 2 μg of oligomycin/ml. Additions were made from 100–200-times-concentrated stock solutions, and the increase in final volume after each addition never exceeded 1%. Thapsigargin, tBuBHQ, Ca^{2+} ionophore A23187 and the Ca^{2+}-channel-blocking agent manoalide were dissolved in dimethyl sulphoxide.

All experiments were done at room temperature. None of the substances used in the study interfered with electrode function.

Materials

RPMI 1640 medium, penicillin, streptomycin, trypsin/EDTA and foetal-calf serum were from either Flow Laboratories (Irvine, Scotland) or Northumbria Biologicals (Cramlington, Northumberland, U.K.). Thapsigargin was purchased from GIBCO BRL (Gaithersburg, MD, U.S.A.) and tBuBHQ from EGA-Chemie (Steinheim, Germany). Ins(1,4,5)P_3, GTP and heparin were from Sigma (St. Louis, MO, U.S.A.). Manoalide was provided by Allergan (Irvine, CA, U.S.A.). Calcium Cocktail I, containing neutral carrier ETH 1001, was from Fluka (Buchs, Switzerland). Capillary tubing was from Federick Haer (Brunswick, ME, U.S.A.), and A23187 from Calbiochem (La Jolla, CA, U.S.A.). Bafilomycin A_1 was purchased from Dr. K. Altendorf, Osnabrück, Germany. Other chemicals were from Sigma and Merck.

Statistical analysis

Statistical significance was judged by Student's t test for unpaired data.

RESULTS

After addition of electropermeabilized RINm5F cells (final concn. 3.9×10^7 cells/ml) to the incubation buffer, containing ATP, an ATP-regenerating system and mitochondrial inhibitors, ambient [Ca^{2+}] was decreased from 6.43 ± 0.25 μM (mean ± S.E.M., $n = 45$) to a steady-state concentration of 530 ± 0.06 nM (mean ± S.E.M., $n = 45$). Addition of thapsigargin or tBuBHQ rapidly released Ca^{2+}. The rate of increase in [Ca^{2+}] and the steady-state [Ca^{2+}] reached after additions of thapsigargin or tBuBHQ, were dependent on the concentration of the two substances. The relationship between the concentration of thapsigargin or tBuBHQ and the magnitude of Ca^{2+} release is shown in Figure 1. The half-maximally effective concentration of thapsigargin was ~37 nM and the maximally effective concentration ~156 nM. For tBuBHQ, the half-maximally effective concentration and the maximally effective concentration were

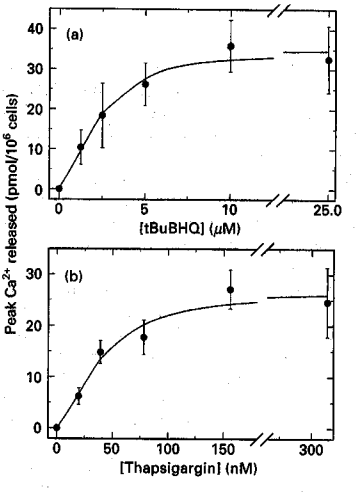

Figure 1 Concentration-response curves of (a) tBuBHQ- and (b) thapsigargin-induced Ca^{2+} release in permeabilized RINm5F cells

Electropermeabilized RINm5F cells were incubated in an intracellular-like buffer containing ATP, an ATP-regenerating system and mitochondrial blockers. Changes in medium [Ca^{2+}] were measured by Ca^{2+}-sensitive mini-electrodes. Amounts of Ca^{2+} released were estimated from the increase in medium [Ca^{2+}]. Values are means ± S.E.M. of at least three different experiments for each concentration of thapsigargin or tBuBHQ.

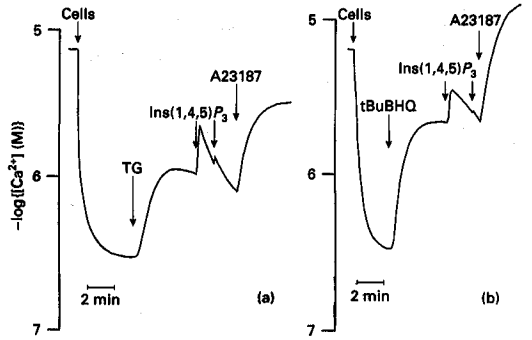

Figure 2 Effect of Ins(1,4,5)P_3 on the release of Ca^{2+} after maximal Ca^{2+} release by thapsigargin and tBuBHQ

Conditions for the experiments are as mentioned in the legend to Figure 1. After addition of thapsigargin (TG; 5 μM) (**a**) or tBuBHQ (25 μM) (**b**), $[Ca^{2+}]$ increased to a maximum. Ins(1,4,5)P_3 released additional Ca^{2+}. Other additions, as indicated by arrows, are A23187 (2 μM). The traces are representative of at least three different experiments.

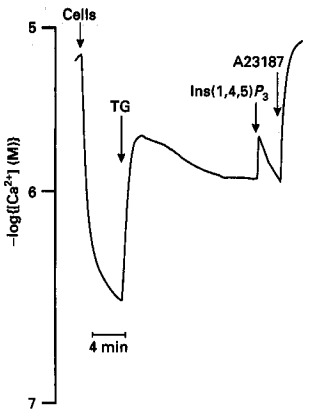

Figure 3 Ca^{2+} release by Ins(1,4,5)P_3 after prolonged treatment with thapsigargin

For details of incubation and $[Ca^{2+}]$ measurements, see the legend to Figure 1. Ins(1,4,5)P_3 (5 μM) was added 16 min after addition of thapsigargin (TG; 5 μM). Note change in the time scale in this Figure. The trace is representative of at least three different experiments.

$\sim$2 μM and $\sim$10 μM respectively. Ca^{2+} release by higher concentrations of the inhibitors (up to 10 μM thapsigargin and 100 μM tBuBHQ) was not different from the release obtained by the maximally effective concentrations of these substances. To ensure complete inhibition of the ER Ca^{2+}-ATPase, in subsequent experiments we used thapsigargin and tBuBHQ at final concentrations of 5 μM and 25 μM respectively. Addition of tBuBHQ, after maximal Ca^{2+} release by thapsigargin, or vice versa, did not cause any further increase in $[Ca^{2+}]$ (results not shown). Half of the Ca^{2+} release by maximally effective concentrations of thapsigargin and tBuBHQ was complete in about 1.5 min. At 2–4 min after addition of thapsigargin or tBuBHQ, $[Ca^{2+}]$ reached its

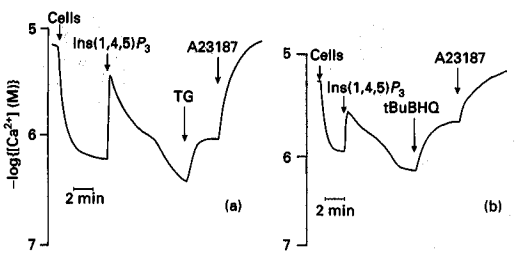

Figure 4 Effect of thapsigargin and tBuBHQ, after Ins(1,4,5)P_3-induced release and subsequent re-uptake of Ca^{2+}

For details of incubation and $[Ca^{2+}]$ measurements, see the legend to Figure 1. At points indicated by the arrows Ins(1,4,5)P_3 (5 μM) and either thapsigargin (TG; 5 μM) (**a**) or tBuBHQ (25 μM) (**b**) was added. The traces are typical of at least three different experiments.

maximum and maintained a plateau for 1–2 min. $[Ca^{2+}]$ then slowly declined to a slightly lower steady-state level. As shown in Figs. 2(a) and 2(b), $[Ca^{2+}]$ increased to its maximum after addition of thapsigargin or tBuBHQ. Addition of Ins(1,4,5)P_3 at this stage caused rapid release of further Ca^{2+}. The uptake phase of Ins(1,4,5)P_3-induced Ca^{2+} release was not affected by the presence of the inhibitors. The additional Ca^{2+} release by Ins(1,4,5)P_3, after release by thapsigargin or tBuBHQ, was modest [estimated to be about 15% of the Ca^{2+} released by Ins(1,4,5)P_3 in the absence of thapsigargin or tBuBHQ], but was seen in all experiments. The increase in $[Ca^{2+}]$ was not due to any inadvertent contamination of Ins(1,4,5)P_3 solution by Ca^{2+}, since addition of a second pulse of Ins(1,4,5)P_3 was ineffective. Furthermore, the Ca^{2+} release by Ins(1,4,5)P_3 was completely blocked by heparin (results not shown). The additional Ins(1,4,5)P_3-induced Ca^{2+} release was not decreased by 10 μM thapsigargin or 100 μM tBuBHQ and was not altered by GTP (up to 50 μM), by the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or by a specific inhibitor of V-type ATPases, Bafilomycin A_1 (10 μM) (results not shown). When added 10–18 min after thapsigargin, Ins(1,4,5)P_3 still released Ca^{2+}, although its magnitude was decreased (Figure 3). Similar results were seen with tBuBHQ (results not shown). GTP (final concn. 10–50 μM), in the presence of 3% (w/v) polyethylene glycol [34], neither released Ca^{2+} by itself, nor increased Ins(1,4,5)P_3-induced Ca^{2+} release (results not shown). In RINm5F cells, as in many other cells, Ca^{2+} released by Ins(1,4,5)P_3 is taken up, back into the Ins(1,4,5)P_3-sensitive pool ([35]; M. S. Islam and P.-O. Berggren, unpublished work). In Figures 4(a) and 4(b), thapsigargin or tBuBHQ was added after Ins(1,4,5)P_3-induced release and subsequent re-uptake of Ca^{2+}. In these experiments, maximum $[Ca^{2+}]$ obtained with thapsigargin or tBuBHQ was always less than that obtained with Ins(1,4,5)P_3.

In experiments shown in Figures 5(a) and 5(b), the Ins(1,4,5)P_3-sensitive pool was emptied by a supramaximal dose (20 μM) of Ins(2,4,5)P_3, a poorly metabolizable analogue of Ins(1,4,5)P_3 [36]. We used Ins(2,4,5)P_3 in an attempt to achieve a sustained rise in $[Ca^{2+}]$. However, in these cells, Ca^{2+} released by Ins(2,4,5)P_3 did not maintain a steady state for long. The released Ca^{2+} was slowly (in about 25 min) taken up into thapsigargin-insensitive pools (results not shown). When thapsigargin or tBuBHQ was added after maximal Ca^{2+} release by Ins(2,4,5)P_3, no increase in $[Ca^{2+}]$ was seen, although exchangeable Ca^{2+} was still present in vesicular pools, as evidenced by release in response to A23187.

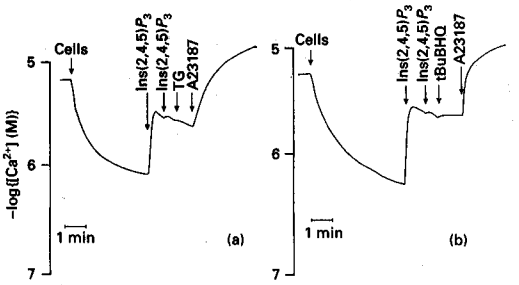

Figure 5 Effect of thapsigargin and tBuBHQ on the release of Ca^{2+}, when added after depletion of the Ins(1,4,5)P_3-sensitive pool

For details of incubation and [Ca^{2+}] measurements see the legend to Figure 1. After Ca^{2+} uptake, the Ins(1,4,5)P_3-sensitive pool was depleted by 20 μM Ins(2,4,5)P_3. No further Ca^{2+} release was seen after thapsigargin (TG; 5 μM) (**a**) or tBuBHQ (25 μM) (**b**), whereas Ca^{2+} was still releasable by A23187. The traces are representative of at least three different experiments.

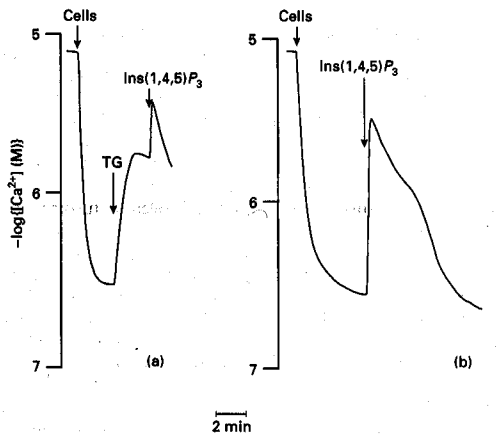

Figure 6 Comparison of Ca^{2+} release by thapsigargin and Ins(1,4,5)P_3 with that released by Ins(1,4,5)P_3 alone

For details of incubation and [Ca^{2+}] measurements, see the legend to Figure 1. (**a**) After Ca^{2+} release by thapsigargin (TG; 5 μM) reached its maximum, Ins(1,4,5)P_3 (5 μM) was added. (**b**) Control experiment done under identical experimental conditions using the same cell preparation and the same electrodes. Ins(1,4,5)P_3 was added as indicated by arrow. Total increase in [Ca^{2+}] obtained by thapsigargin and Ins(1,4,5)P_3 in (**a**) was compared with the increase in [Ca^{2+}] obtained by Ins(1,4,5)P_3 alone in (**b**). The traces are representative of experiments that have been repeated at least three times.

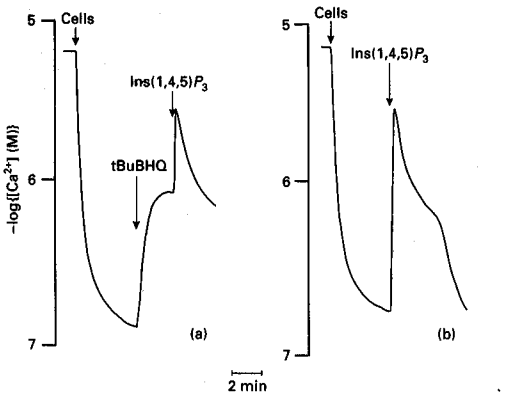

Figure 7 Comparison of Ca^{2+} release by tBuBHQ and Ins(1,4,5)P_3 with that released by Ins(1,4,5)P_3 alone

For details of incubation and [Ca^{2+}] measurements, see the legend to Figure 1. (**a**) After Ca^{2+} release by tBuBHQ (25 μM) reached its maximum, Ins(1,4,5)P_3 (5 μM) was added. (**b**) Control experiments done under identical experimental conditions, using the same cell preparation and same electrodes. Ins(1,4,5)P_3 (5 μM) was added as indicated by arrow. Increase in total [Ca^{2+}] obtained by tBuBHQ and Ins(1,4,5)P_3 in (**a**) was compared with the increase in [Ca^{2+}] obtained by Ins(1,4,5)P_3 alone in (**b**). The traces are representative of experiments that have been repeated at least three times.

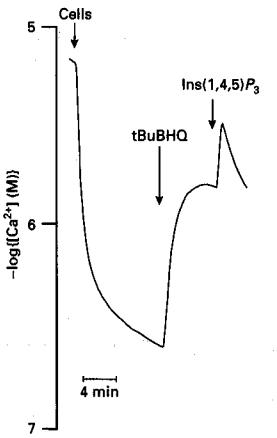

Figure 8 Manoalide did not inhibit Ca^{2+} release by tBuBHQ or Ins(1,4,5)P_3

For details of incubation and [Ca^{2+}] measurements see legend to Figure 1. Under these experimental conditions, the buffer also contained manoalide (6 μM final concn.). At 10 min after onset of Ca^{2+} uptake, tBuBHQ (25 μM) was added. Ins(1,4,5)P_3 was added at the point indicated. The trace is representative of three similar experiments.

Finally, an estimate of Ca^{2+} release by thapsigargin and tBuBHQ from the Ins(1,4,5)P_3-insensitive pools was made by another approach. If thapsigargin and tBuBHQ release Ca^{2+} from the Ins(1,4,5)P_3-insensitive pools, the magnitude of Ca^{2+} release by either of these agents plus Ins(1,4,5)P_3 must be greater than that by Ins(1,4,5)P_3 alone. We compared the total change in [Ca^{2+}] by thapsigargin or tBuBHQ plus Ins(1,4,5)P_3 with that obtained by Ins(1,4,5)P_3 alone, in a series of carefully controlled experiments. The control experiments were done with the same cell preparation and the same electrodes. As shown in Figure 6(a), after maximal increase in [Ca^{2+}] by thapsigargin, addition of Ins(1,4,5)P_3 caused a further increase in [Ca^{2+}]. The total increase in [Ca^{2+}] thus obtained by thapsigargin and Ins(1,4,5)P_3 was on average about 7% greater than that obtained by Ins(1,4,5)P_3 alone in control experiments (cf. Figure 6b). This difference was

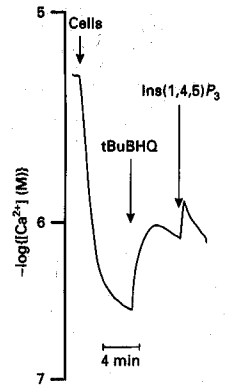

Figure 9 Ca^{2+} release by tBuBHQ and Ins(1,4,5)P_3 from electropermeabilized rat hepatocytes

For details of incubation and [Ca^{2+}] measurements, see the legend to Figure 1. Addition of tBuBHQ (25 μM) caused release of Ca^{2+}. Further Ca^{2+} was released by Ins(1,4,5)P_3 (5 μM). The trace is representative of three similar experiments.

not statistically significant. In similar experiments with tBuBHQ, the total increase in [Ca^{2+}] obtained by tBuBHQ and Ins(1,4,5)P_3 was about 33% greater than that by Ins(1,4,5)P_3 alone ($P < 0.05$) (Figures 7a and 7b). Ca^{2+} release by thapsigargin or tBuBHQ was not blocked by heparin. A marine product, manoalide, has been reported to inhibit Ca^{2+} release induced by agonists or by tBuBHQ [37,38]. Manoalide (up to 6 μM, for 10 min) [38] did not block Ca^{2+} release by the inhibitors or by Ins(1,4,5)P_3 (Figure 8). Figure 9 shows experiments done with electropermeabilized rat hepatocytes, where again Ins(1,4,5)P_3 released additional Ca^{2+} when added after Ca^{2+} release by tBuBHQ.

DISCUSSION

We defined the intracellular Ca^{2+} pools that are sensitive to thapsigargin and tBuBHQ, and studied their relationship to the Ins(1,4,5)P_3-sensitive Ca^{2+} pool in insulin-secreting cells. This is especially interesting, since mobilization of Ca^{2+} from the Ins(1,4,5)P_3-sensitive pool is part of the mechanisms that regulate Ca^{2+} oscillations in pancreatic β-cells [39]. Although the two ER Ca^{2+}-ATPase inhibitors are used to identify intracellular Ca^{2+} pools in many cells, direct comparison between these two agents in permeabilized cells has not been reported. In permeabilized RINm5F cells, the two inhibitors released Ca^{2+} from intracellular non-mitochondrial Ca^{2+} pools in a concentration-dependent manner. The half-maximally effective concentrations of tBuBHQ and thapsigargin were comparable with values reported in other cell types [11,18,22,31,37,40]. Thapsigargin and tBuBHQ released Ca^{2+} from the Ins(1,4,5)P_3-sensitive pool. In experiments where the Ins(1,4,5)P_3-sensitive pool was depleted by a supramaximal dose of the InsP_3, no further net release of Ca^{2+} was seen after addition of the inhibitors. When thapsigargin and Ins(1,4,5)P_3 were used together, the total increase in [Ca^{2+}] was not significantly greater than that released by Ins(1,4,5)P_3 alone. Thus, in RINm5F cells, as in many other cell types, the thapsigargin-sensitive and Ins(1,4,5)P_3-sensitive Ca^{2+} pools are largely coincident [21,26,41]. On the other hand, the total increase in [Ca^{2+}] by tBuBHQ plus Ins(1,4,5)P_3 was significantly greater than that caused by Ins(1,4,5)P_3 alone. Thus, although tBuBHQ was claimed to release specifically from the Ins(1,4,5)P_3-sensitive Ca^{2+} pool [31], the present study, together with others, indicate that it may also release from part of the Ins(1,4,5)P_3-insensitive pools [22,24,37]. The reason for such a difference in action between the two inhibitors is not clear. Recent reports indicate that tBuBHQ has other actions apart from inhibition of ER Ca^{2+}-ATPase [15,17,23]. It remains a possibility that tBuBHQ may also interfere with the cytosolic Ca^{2+}-binding sites.

The maximal increase in [Ca^{2+}] obtained by thapsigargin or tBuBHQ was slightly smaller than that obtained by the maximally effective concentration of Ins(1,4,5)P_3. This is in contrast with studies where thapsigargin releases substantially more Ca^{2+} than that released by Ins(1,4,5)P_3, implying a complete inclusion of the Ins(1,4,5)P_3-sensitive pool within the thapsigargin-sensitive pool [11,19,20,42,43]. One explanation for such differences may be variation in size of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool, which is often cell-specific [42,44] and may also be GTP-mediated [45,46]. It is also noteworthy that, in experiments where microsomes are used instead of cells, conspicuously less Ca^{2+} is released by Ins(1,4,5)P_3 than by thapsigargin [11,20,42]. In microsomes of bovine adrenal glomerulosa cells, Ca^{2+} release by thapsigargin is larger than that by Ins(1,4,5)P_3, whereas in intact cells of the same type the reverse is true [20]. This may be due to the fact that Ins(1,4,5)P_3 releases less Ca^{2+} from microsomes than from cells, because of the decrease in size of the Ins(1,4,5)P_3-releasable pool during preparation of microsomes [47,48].

Although a major part of the Ca^{2+} in the Ins(1,4,5)P_3-sensitive pool was rapidly mobilized by thapsigargin and tBuBHQ, complete depletion of the Ins(1,4,5)P_3-sensitive pool was difficult to achieve. When added even as long as 15–18 min after thapsigargin or tBuBHQ, Ins(1,4,5)P_3 still released further Ca^{2+}. This is in accordance with studies which show that agonists or Ins(1,4,5)P_3 release additional Ca^{2+} after Ca^{2+} release from intracellular stores by thapsigargin [20,21,26,49–51]. Other studies, however, demonstrate total depletion of the Ins(1,4,5)P_3-sensitive pool by thapsigargin or tBuBHQ, within a short time [15,16,18,22,31,52]. It is possible that the buffering action of Ca^{2+} indicators [31] or EGTA [18,52], used in some of these studies, might have masked the small additional Ins(1,4,5)P_3-induced Ca^{2+} release. Most of these studies used the Fura-2 method to measure [Ca^{2+}]. In this technique, after Ca^{2+} release by the inhibitors, [Ca^{2+}] often reaches near the saturation point of the indicator. Any additional release of Ca^{2+} by Ins(1,4,5)P_3 is difficult to appreciate. This is also because of non-linearity of fluorescence in this range, and noise. In this respect, our method of measuring Ca^{2+} by electrodes has distinct advantage. Moreover, for permeabilization these studies used detergents, a method which is difficult to control and may result in partial permeabilization of Ca^{2+}-storing vesicles [43,53,54]. Electropermeabilization is unlikely to cause leakiness of the Ca^{2+}-storing vesicles, since this technique is clean and it creates holes selectively in the plasma membrane [53]. In experiments with hepatocytes which were electropermeabilized, there was also additional release of Ca^{2+} by Ins(1,4,5)P_3 when it was added after maximal Ca^{2+} release by tBuBHQ. This is in contrast with results obtained in saponin-permeabilized hepatocytes [31], suggesting that the cause of some differences in the pattern of Ca^{2+} release may be experimental rather than biological.

We considered the possibility that the additional Ca^{2+} release by Ins(1,4,5)P_3, after thapsigargin- or tBuBHQ-induced Ca^{2+} release, was from a different Ins(1,4,5)P_3-sensitive pool. Such an Ins(1,4,5)P_3-sensitive pool might have Ca^{2+}-uptake mechanisms insensitive to these inhibitors and might even be structurally

different from ER [55]. Nicotera et al. [56] demonstrated a tBuBHQ-insensitive Ins(1,4,5)P_3-sensitive Ca^{2+} pool in rat liver cell nuclei (but see ref. [57]). Some isoforms of the SERCA family, with low sensitivity to the inhibitors, could mediate such uptake [58,59]. However, it is now established that thapsigargin inhibits completely and with equal potency all the known isoforms of the SERCA family [12]. Moreover, if the RINm5F cells possess some novel isoform of SERCA, it appears unlikely that it will not be inhibitable by two highly potent and structurally different inhibitors. Alternatively, the putative thapsigargin-insensitive Ins(1,4,5)P_3-sensitive Ca^{2+} pool may utilize H^+-dependent Ca^{2+} uptake [10]. However, in our study the thapsigargin-insensitive Ins(1,4,5)P_3 response was not decreased by the protonophore CCCP or a potent and specific inhibitor of V-type ATPases, Bafilomycin A_1 [60]. Although none of these results can rule out the existence of a different Ins(1,4,5)P_3-sensitive pool, insensitive to thapsigargin or tBuBHQ, in RINm5F cells, this does not appear very likely.

The present results fit best to a model where the Ins(1,4,5)P_3-sensitive pool is viewed to have distinct 'uptake' and 'release' compartments, as has been proposed by others [48,61,62]. When treated with thapsigargin or tBuBHQ, the two compartments can be distinguished on the basis of their permeability to Ca^{2+}. According to our model, the release compartment is impermeable to Ca^{2+}, in the absence of Ins(1,4,5)P_3. When treated with thapsigargin or tBuBHQ, the uptake compartment shows a high rate of leakage. Since thapsigargin interacts only with the ER Ca^{2+}-ATPase, the latter molecule is also most likely to be the structure that mediates the thapsigargin-induced Ca^{2+} leak. From the structural model of SERCA and from studies in sarcoplasmic reticulum, it appears that the ER Ca^{2+}-ATPase molecule may contain a channel that mediates passive Ca^{2+} efflux [63,64]. Thus the basis of high permeability may be the presence of the Ca^{2+}-ATPase molecule, which by definition is predominantly located on the uptake compartment. Thapsigargin and tBuBHQ release predominantly from this compartment. The release compartment is impermeable and can be mobilized only very slowly in the absence of Ins(1,4,5)P_3.

In the present study the evidence for the presence of uptake and release compartments within the Ins(1,4,5)P_3-sensitive pool is different from that obtained in earlier studies [48,61,62] and goes further in defining permeability of these compartments and action of the inhibitors. It is unlikely that our results are solely due to some artifact of permeabilization, as has been suggested by Menniti et al. [61]. Indeed, Brüne and Ullrich [62] demonstrated compartmentalization within the Ins(1,4,5)P_3-sensitive pool also in intact cells. The physiological significance of these findings is unclear. However, it is interesting, since the two compartments and their communicating pathway may offer additional sites for regulatory control. For instance, in permeabilized RINm5F cells, $[Ca^{2+}]_i$ oscillations linked to glycolytic oscillations may be a phenomenon of the uptake compartment [65].

To summarize, our results show that thapsigargin and tBuBHQ released Ca^{2+} mostly from the Ins(1,4,5)P_3-sensitive pool in permeabilized RINm5F cells. Thapsigargin was more specific in mobilizing Ca^{2+} from this pool than was tBuBHQ. Although the two inhibitors mobilized a large part of the Ca^{2+} from the Ins(1,4,5)P_3-sensitive pool, they did not readily deplete this pool. Additional Ca^{2+} was released by Ins(1,4,5)P_3, when added long after continued release by thapsigargin or tBuBHQ. This pattern of Ca^{2+} release from the Ins(1,4,5)P_3-sensitive pool may indicate the presence of distinct uptake and release compartments, which differ in terms of Ca^{2+} permeability when treated with the inhibitors.

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Cyclic ADP–Ribose in β Cells

Shin Takasawa *et al.* (*1*) challenge the role of inositol 1,4,5-triphosphate (IP_3) as an intracellular second messenger that mobilizes Ca^{2+} in pancreatic β cells. They found that cyclic adenosine diphosphate–ribose (cADP-ribose), but not IP_3, releases Ca^{2+} from islet microsomes. It is difficult to reconcile their results with many studies that establish IP_3 as an intracellular Ca^{2+}-mobilizing second messenger in pancreatic β cells (*2*).

Confronted with such provocative results, we performed a series of experiments to compare the Ca^{2+}-mobilizing actions of the two second messengers in β cells. We used clonal insulin-secreting RINm5F cells and cells obtained from *ob/ob* mice, where more than 95% of the islet cells correspond to normal β cells. The cells were permeabilized by high-voltage electric discharges, a technique that creates clean holes in the plasma membrane, but leaves intracellular Ca^{2+}-storing organelles in situ and undamaged (*3*). We found pronounced Ca^{2+} release when IP_3 was added to insulin-secreting RINm5F cells (Fig. 1A) or to pancreatic β cells from *ob/ob* mice (Fig. 1B). In marked contrast to the results in the report by Takasawa *et al.*, there was no Ca^{2+} release after the addition of cADP-ribose.

In experiments with β cells, we first added a low dose of caffeine to sensitize the release mechanism that presumably might respond to cADP-ribose. After maximal Ca^{2+} release by IP_3, further Ca^{2+} was released from β cells by the sulfhydryl reagent thimerosal which, as we have shown before, indicates the possible existence of a Ca^{2+}-induced Ca^{2+} release mechanism in β cells (*4*). With the use of intact β cells, we looked for the caffeine-sensitive intracellular Ca^{2+} pool on which cADP-ribose presumably acts. In small clusters of β cells that had been loaded with Fura-2, in the absence of extracellular Ca^{2+}, there was marked Ca^{2+} release from intracellular stores by IP_3-forming agonists, whereas caffeine-induced Ca^{2+} release was absent (*5*). Detailed studies using caffeine indicate that, in the β cell, caffeine increases intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) by a mechanism unrelated to its intracellular Ca^{2+}-mobilizing action (*5*). Furthermore, we used the patch-clamp technique to monitor the Ca^{2+}-sensitive K^+ conductance in β cells for detection of any small release of Ca^{2+} following the addition of cADP-ribose. This method is more sensitive than fluorimetric methods, and it has been used to record increases in $[Ca^{2+}]_i$ after intracellular application of IP_3 and guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) in the pancreatic β cell (*6*). Even so, we were unable to obtain evidence of Ca^{2+} release from intracellular stores after the addition of cADP-ribose in 14 out of 14 cells, whereas formation of IP_3 potently induced release of Ca^{2+} (Fig. 2).

These results raise several questions. First, what might be the reason for the absence of IP_3-induced Ca^{2+} release as reported by Takasawa *et al.*? Their procedure of purification of microsomes might have adversely affected the IP_3-sensitive Ca^{2+} stores, which can be easily damaged during fractionation (*7*). Second, why was cADP-ribose–induced Ca^{2+} release seen in their preparation but not in ours? We do not have a definitive answer to this question. It is possible that cADP-ribose–induced Ca^{2+} release in the β cell is small in magnitude and requires rigorous experimental conditions to be detected. Alternatively, the source of Ca^{2+} released in the experiments of Takasawa *et al.* might be cells other than β cells. It should be recalled that the islets used by Takasawa *et al.* contain a large proportion of cells that are not β cells. We avoided this potential problem by using a tumor cell line and an almost pure preparation of normal β cells as well as by performing experiments on single mouse β cells.

A possible explanation for our negative results with cADP-ribose could be that our preparation of the compound was inactive. However, precautions were taken to ensure that this was not the case. By using cADP-ribose from different sources, who verified the activity of the substance in other cell systems, we guarded against the possibility that the lack of effect in our experimental system was not simply a result of an inactive batch of the compound. Moreover, cADP-ribose seems to be a stable compound (*8*). With the aim of taking a more physiological experimental approach (that is, using cells instead of isolated organelles), we deliberately did not exactly duplicate the experiments conducted by Takasawa *et al.* Hence, there remains a possibility that some experimental factors might have adversely affected the cADP-ribose–sensitive release mechanism in our system.

Takasawa *et al.* also demonstrate that extracts of islets incubated in a high con-

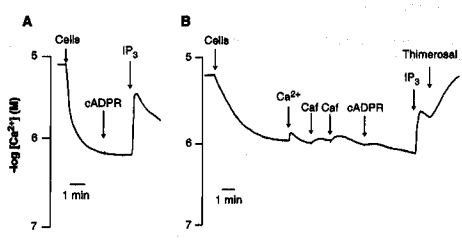

Fig. 1. Effect of cADP-ribose and IP_3 on electropermeabilized RINmF5 cells (**A**) and pancreatic β cells from *ob/ob* mice (**B**) as indicated by the release of Ca^{2+}. Pancreatic islets from fasting adult obese (*ob/ob*) mice were isolated by collagenase digestion and dispersed into small cell clusters by shaking in a Ca^{2+}- and Mg^{2+}-deficient medium (*11*). Electropermeabilized insulin-secreting cells were incubated in an intracellular-like buffer supplemented with adenosine triphosphate (ATP), an ATP-regenerating system, and the mitochondrial inhibitors antimycin and oligomycin. Changes in $[Ca^{2+}]$ were measured with a Ca^{2+}-sensitive minielectrode (*12*). Arrows indicate additions of cADPR (1 μM), $CaCl_2$ (1 nmol), IP_3 (5 μM), caffeine (4 mM), and thimerosal (50 μM). The effects of cADP-ribose were tested in three experiments with RINm5F cells and in three with pancreatic β cells from *ob/ob* mice.

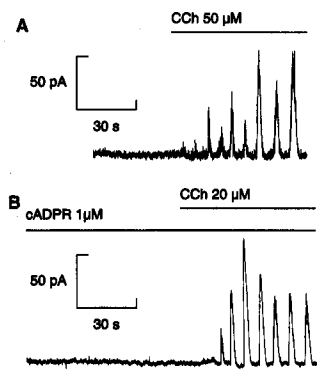

Fig. 2. Effect of cADP-ribose and carbachol (CCh) on pancreatic β cells from *ob/ob* mice as indicated by Ca^{2+}-activated K^+-currents (K_{Ca} currents). Membrane currents were recorded from single pancreatic β cells (*11*), with the use of the whole-cell configuration of the patch-clamp technique. (**A**) Oscillations in membrane K_{Ca} currents in response to CCh (50 μM) are pronounced. (**B**) Inclusion of cADP-ribose (1 μM) in the pipette solution resulted in no detectable effect. Addition of CCh (20 μM) to the same cells induced fluctuations in K_{Ca} currents. When the concentration of cADP-ribose was increased to 100 μM, there was still no effect on membrane currents (not shown).

centration of glucose release Ca^{2+} from islet microsomes and abolish Ca^{2+} release by the subsequent addition of cADP-ribose. This they attribute to the glucose-induced formation of cADP-ribose, which could not be measured. Glucose-induced arachidonic acid formation may mediate such a Ca^{2+} release (9). When multiple Ca^{2+}-mobilizing second messengers release Ca^{2+} from a common pool, the results can mistakenly be interpreted as "cross-desensitization." In a Perspective in the same issue, Antony Galione states that "glucose induces a rise in [cADP-ribose] concentrations in pancreatic β cell" (*10*). We know of no data to substantiate such a statement.

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Cyclic ADP–Ribose and Pancreatic β Cells

In their response (*1*) to our technical comment (*2*), Takasawa *et al.* state that one reason we do not observe any effect of cyclic ADP–ribose on β cells is because we are working with β cells with "negligible sensitivity to glucose," and in this context they refer to several papers, including one of ours (*3*). In this paper, we showed that β cells from *ob/ob* mice are highly sensitive to glucose as measured not only by changes in electrical activity and cytoplasmic free Ca^{2+}, but also by stimulation of insulin release.

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Caffeine induces Changes in Cytoplasmic Free Calcium Concentration in Pancreatic β-cells by Mechanisms Independent of its Action on Intracellular Ca^{2+}-pools

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In the pancreatic β-cell, an increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by caffeine is believed to indicate mobilization of Ca^{2+} from intracellular stores, through activation of a ryanodine receptor-like channel. It is not known whether other mechanisms as well, underlie caffeine-induced changes in $[Ca^{2+}]_i$. We studied the effects of caffeine on $[Ca^{2+}]_i$ by using dual wave-length excitation microfluorimetry in fura-2 loaded β-cells. In the presence of a non-stimulatory concentration of glucose, caffeine (10-50 mM) consistently increased $[Ca^{2+}]_i$. The effect was completely blocked by omission of extracellular Ca^{2+} and by blockers of the L-type voltage-gated Ca^{2+} channel, like D-600 or nifedipine. Depletion of agonist-sensitive intracellular Ca^{2+} pools by thapsigargin, did not inhibit the stimulatory effect of caffeine on $[Ca^{2+}]_i$. Moreover, this effect of caffeine was not due to a rise in cAMP, since forskolin and 3-isobutyl-1-methylxanthine (IBMX) failed to raise $[Ca^{2+}]_i$ in unstimulated β-cells. In β-cells, glucose and sulfonylureas increase $[Ca^{2+}]_i$ by causing closure of ATP-sensitive K^+ (K_{ATP}) channels. Interestingly, caffeine also caused complete inhibition of K_{ATP} channel activity, as measured in excised inside-out patches. Hence, membrane depolarization and opening of voltage-gated L-type Ca^{2+} channels were the underlying mechanisms whereby the xanthine drug increased $[Ca^{2+}]_i$.

Paradoxically, in glucose-stimulated β-cells, caffeine (>10 mM) lowered $[Ca^{2+}]_i$. This effect was due to the fact that caffeine reduced depolarization-induced whole-cell Ca^{2+} current through L-type voltage-gated Ca^{2+} channels in a dose-dependent manner. A lower concentration of caffeine (2.5-5 mM), when added after glucose-stimulated increase in $[Ca^{2+}]_i$, induced fast oscillations in $[Ca^{2+}]_i$. The latter effect was likely to be attributable to the cAMP-elevating action of caffeine, leading to phosphorylation of voltage-gated Ca^{2+} channels.

Hence, in β-cells, caffeine-induced changes in $[Ca^{2+}]_i$ are not related to any action of caffeine on the intracellular Ca^{2+} pools. In these cells, a direct interference with K_{ATP} channel- and L-type voltage-gated Ca^{2+} channel-activity is the underlying mechanism by which caffeine increases or decreases $[Ca^{2+}]_i$.

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)[1], plays a key role in the stimulation of insulin secretion from the pancreatic β-cell (1). In this cell, glucose-metabolism is coupled to an increase in $[Ca^{2+}]_i$ through the participation of at least two types of ion channels: the ATP-sensitive potassium channel (K_{ATP}) and the L-type voltage-gated Ca^{2+} channel (2-4). According to the current model, metabolism of glucose increases cytosolic ATP/ADP ratio leading to closure of the K_{ATP} channel with consequent depolarization of the cell, opening of L-type voltage-gated Ca^{2+} channels resulting in subsequent influx of Ca^{2+}, increase in $[Ca^{2+}]_i$ and finally exocytosis (3,4). While Ca^{2+} influx through the voltage-gated Ca^{2+} channels is believed to be the dominant mechanism, mobilization of Ca^{2+} from intracellular Ca^{2+} pools also contributes to the increase in $[Ca^{2+}]_i$ in the β-cell (1,5).

Stimulation of receptors linked to the phospholipase C system induces formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which upon binding to its receptor (IP_3R) triggers Ca^{2+} release from intracellular stores and Ca^{2+} influx through the plasma membrane (6,7). Another major intracellular Ca^{2+} release channel, the ryanodine receptor, is also present in many cells. The ryanodine receptor was originally described in sarcoplasmic reticulum, where it mediates Ca^{2+}-induced Ca^{2+} release (CICR) (8). The endogenous ligand for the receptor is unknown, although cyclic adenosine diphosphate ribose (cADPR) is a candidate (9). Experimentally, the receptor can be activated by nanomolar concentrations of the plant alkaloid ryanodine or millimolar concentrations of caffeine (10,11). The caffeine-ryanodine-sensitive intracellular Ca^{2+} pool is typically present in excitable cells (12-15) and has also been described in nonexcitable cells (16,17). In some cells the ryanodine receptor coexists with the IP_3R, but the distribution of the former is much restricted compared to the ubiquitous IP_3R (14,16). Three ryanodine receptors have been cloned and at least one of them is widely distributed (18,19).

[1] The abbreviations used are: $[Ca^{2+}]_i$, cytoplasmic free calcium concentration; K_{ATP}, ATP-sensitive potassium channel; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; IBMX, 3-isobutyl-1-methylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [Ethylenebis(oxyethylenenitrilo)] tetraacetic acid; NMDG, N-methyl-D-glucamine; *R*pcAMPS, *R*p-cyclic adenosine-3′,5′-monophosphorothioate.

The most commonly used pharmacological tool for the study of the ryanodine receptor is caffeine (10,11). This substance, notably, has other actions unrelated to its effect on the ryanodine receptor. These include elevation of cAMP by inhibition of cyclic nucleotide phosphodiesterases, and inhibition of plasma membrane Ca^{2+} channels (20,21). To what extent such effects of caffeine account for its effects on $[Ca^{2+}]_i$ under different experimental conditions, remains unclear. The effects of caffeine on the ryanodine receptor have been reported to vary. In addition to the stimulatory effect, an inhibitory effect of caffeine on the ryanodine receptor has been described in non-muscle cells (22). Furthermore, one type of ryanodine receptor is insensitive to the activation by caffeine (18). There is no consensus about the presence of a caffeine-sensitive intracellular Ca^{2+} pool in insulin-secreting cells. Several studies showed that in intact β-cells and islets, caffeine markedly increased $[Ca^{2+}]_i$ (23,24). On the other hand, in permeabilized insulin-secreting cells, Ca^{2+} release by caffeine is at best marginal (25,26). Because of such discrepant results, we questioned whether caffeine, in the β-cell, might cause changes in $[Ca^{2+}]_i$ by mechanisms other than those affecting Ca^{2+} mobilization from intracellular stores. In the present study we reexamined the effect of caffeine on $[Ca^{2+}]_i$ and identified the molecular mechanisms by which caffeine produced distinct changes in $[Ca^{2+}]_i$ in the pancreatic β-cell.

EXPERIMENTAL PROCEDURES

Isolation of Islets and preparation of β-cells – Pancreatic islets from obese (*ob/ob*) mice from a local colony were isolated by collagenase digestion and dispersed into small cell-clusters by shaking in a Ca^{2+}- and Mg^{2+}-deficient medium, as previously described (3). Cells were cultured on glass coverslips or in plastic petri dishes for 1-3 days, in RPMI 1640 medium, containing 5.5 mM glucose, supplemented with fetal calf serum (10% v/v), penicillin (100 IU/ml) and streptomycin (100 µg/ml).

Measurements of $[Ca^{2+}]_i$ by microfluorimetry – Cells attached to coverslips were loaded with fura-2 by incubating in basal medium (in mM): NaCl 125, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.28, HEPES 25, glucose 3, bovine serum albumin (BSA) 0.1% (pH 7.4 with NaOH) and fura-2-acetoxymethylester 2 µM, for 20 min at 37°C. Coverslips were washed twice with the buffer and mounted as the bottom of an open chamber placed on the stage of an inverted epifluorescence microscope (Zeiss, Axiovert 35M). The perifusate volume in the chamber was 0.2 ml and the perifusion rate 0.3 ml/min. After switching the perifusion solutions, there was a lag period of about 30 s before the new solution reached the chamber. The stage of the microscope was thermostatically controlled, to maintain a temperature of 37°C in the perifusate inside the chamber. The microscope was connected to a SPEX fluorolog-2 CM1T11I system, for dual wavelength excitation fluorimetry. The excitation wavelengths generated by two monochromators were directed to the cell by a dichroic mirror. The emitted light, selected by a 510 nm filter, was monitored by a photomultiplier attached to the microscope. The excitation wavelengths were alternated at a frequency of 1Hz and the length of time for data collection at each wavelength was 0.33 s. The emissions at the excitation wavelength of 340 nm (F_{340}) and that of 380 nm (F_{380}) were used to calculate the fluorescence ratio ($R_{340/380}$). Small clusters of cells (usually 3-4), isolated optically by means of the diaphragm of the microscope, were studied by using a 100x, 1.3 NA oil-immersion objective (Zeiss, Plan Neofluar). Background fluorescence was measured after quenching of the fura-2 fluorescence with manganese and was subtracted from the traces before calculation of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was calculated from $R_{340/380}$ according to Grynkiewicz *et al* (27). Maximum and minimum fluorescence ratios were determined in separate experiments using 1 µl drops of an intracellular-like buffer, containing 10 µM fura-2 free acid and either 2 mM Ca^{2+} or no Ca^{2+} in the presence of 2 mM EGTA. The K_d for the Ca^{2+}-fura-2 complex was taken as 225 nM. In order to compensate for variations in output light intensity from the two monochromators, all experiments were corrected for by the inclusion of a fluorescence ratio where both monochromators were set at 360 nm. No correction was made for interference of fura-2 fluorescence by caffeine and where $[Ca^{2+}]_i$ was measured in the presence of caffeine, the estimated $[Ca^{2+}]_i$ was probably lower than the actual $[Ca^{2+}]_i$.

Electrophysiological recordings – We used the inside-out and whole-cell configurations of the patch-clamp technique (28). Pipettes were prepared from borosilicate glass capillary tubes, coated with Sylgard resin (Dow Corning) near the tips, fire-polished and had resistances of 2-6 MΩ. For the study of the K_{ATP} channel, single-channel currents were recorded from inside-out membrane patches at 0 mV membrane potential. Currents were recorded using an Axopatch 200 patch-clamp amplifier (Axon Instruments Inc. Foster City, U.S.A.). During experiments the current signals were stored using a VR-100A digital recorder (Instrutech Corp., USA) and a high-resolution video cassette recorder (JVC, Japan). Channel records are displayed according to the convention, with upward deflections denoting outward currents. K_{ATP} channel activity was identified on the basis of sensitivity to ATP and unitary amplitude (1.5-2 pA). The extracellular solution contained (in mM): NaCl 138, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 5 (pH 7.4 with NaOH). The intracellular-like solution consisted of (in mM): KCl 125, $MgCl_2$ 1, EGTA 10, KOH 30, HEPES 5 (pH 7.15 with KOH). Patches were excised into nucleotide-free solution and ATP was first added to test for channel inhibition. ATP was then removed and patches were exposed to solutions containing caffeine. Mg-ATP (0.1 mM) was present in the intracellular solution for most of the time to reduce run-down of

K_{ATP} channel activity (29). Each experimental condition was tested, with identical results, in 3-6 different patches. The current signal was filtered at 100 Hz (-3 dB value) by using an 8-pole Bessel filter (Frequency Devices, Haverhill, U.S.A.). Single-channel conductance was measured directly from a digital oscilloscope.

For the study of the voltage-gated Ca^{2+} channels, cells were washed with a solution composed of (in mM): NaCl 138, KCl 5.6, $MgCl_2$ 1.2, $CaCl_2$ 10, tetraethylammonium-chloride 10, HEPES 5 (pH 7.4 with NaOH). The pipette solution contained (in mM): N-methyl-D-glucamine (NMDG) 150, HCl 110, $MgCl_2$ 1, $CaCl_2$ 2, EGTA 10, Mg-ATP 3, HEPES 5 (pH 7.15). NMDG was substituted for K^+ in the pipette solution to block outwardly directed K^+ currents. Voltage-steps were generated, digitized and stored using the program pClamp (Axon Instruments) and Labmaster ADC (Scientific Solutions, USA). The current responses were filtered at 2 kHz. The pulse protocol is given in the figure legends. Figures were made by plotting segments of the records on a chart recorder, scanning the segments using a HP scanner and incorporating them into Corel Draw graphics software program.

Materials – Forskolin was from Hoechst and D-600 from Knoll AG (Ludwigshaffen, Germany). *R*pcAMPS was a gift from Biolog (Bremen, Germany). All other chemicals were from Sigma.

RESULTS

Effect of caffeine on fura-2 fluorescence – In an intracellular-like solution caffeine was only weakly fluorescent (<15% increase in signal), but it affected fura-2 fluorescence considerably. In a cell-free system, caffeine (40 mM) increased fura-2 fluorescence to a variable extent, depending on the wave-lengths and ambient $[Ca^{2+}]$ (Fig.1A and B). At 100 nM Ca^{2+} caffeine caused a large increase in F_{380} (190% of control) followed by increase in F_{360} (175%) and F_{340} (160%), while net increase in fluorescence in arbitrary units was 284, 173, and 26 respectively. At a $[Ca^{2+}]$ of 1 μM, the increase in F_{380} was 132% (net increase in arbitrary units 61) and F_{360} 122% (40) with no change in F_{340}. Caffeine did not have significant effect on cellular autofluorescence.

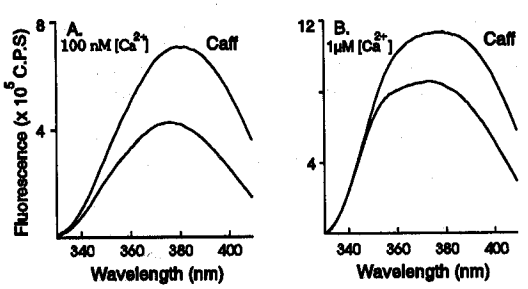

FIG. 1. **Effects of caffeine on the fluorescence excitation spectra of fura-2.** Spectra were obtained in a drop of intracellular-like solution containing 120 mM KCl, 1 mM EGTA, 25 mM HEPES (pH 7.2) and 10 μM Fura-2 free acid. Fluorescence emission was measured at 510 nm. $[Ca^{2+}]$ was adjusted to 100 nM (A) or 1 μM (B), as verified by using a Ca^{2+}-sensitive minielectrode. In caffeine containing solutions caffeine (40 mM) was added with isosmotic replacement of KCL.

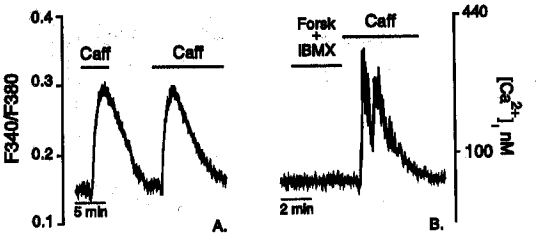

FIG. 2. **Caffeine increases $[Ca^{2+}]_i$ in pancreatic β-cells by a mechanism unrelated to cAMP formation.** $[Ca^{2+}]_i$ was measured in fura-loaded β-cells by dual wave-length microfluorimetry in the presence of 1.28 mM extracellular Ca^{2+} and 3 mM glucose. Times indicated in the labels are times of switching to the new solutions. Caffeine was dissolved in buffer with isosmotic replacement of NaCl. (A) The effect of repeated application of caffeine (50 mM) is shown. Apparent initial lowering of $[Ca^{2+}]_i$ after addition of caffeine, is an artifact due to interference of caffeine with fura-2 fluorescence. The trace is representative of 6 different experiments. (B) At points indicated, forskolin (30 μM) + IBMX (1 mM) and caffeine (50 mM) were added. The trace is representative of three different experiments.

Effects of caffeine on $[Ca^{2+}]_i$ – In the presence of extracellular Ca^{2+} and a non-stimulatory glucose concentration (3 mM), caffeine increased $[Ca^{2+}]_i$ in a concentration- dependent manner. The minimal and maximal effective concentrations of caffeine, as added to the perifusion system, was 10 mM and 50 mM respectively. At 3 mM glucose, lower concentrations of caffeine (1-5 mM) had no effect on $[Ca^{2+}]_i$. Entry of caffeine into the cytosol was signalled by an abrupt increase in F_{380} and a much smaller increase in F_{340}, giving an initial lowering of $R_{340/380}$. This was an artifact resulting from interference of caffeine with fura-2 fluorescence as mentioned above. Following this, there was a lag of 5-20 s after which $[Ca^{2+}]_i$ increased rapidly to a peak. The lag period was shorter and the rise in

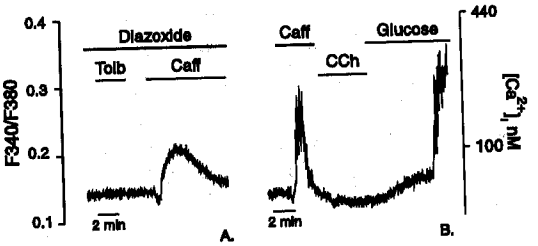

FIG. 3. **Effect of diazoxide and thapsigargin on caffeine-induced increase in the $[Ca^{2+}]_i$.** Experimental conditions were the same as mentioned in legend to figure 2. In (A) cells were exposed to diazoxide 400 μM for five minutes prior to addition of tolbutamide (100 μM) or caffeine (50 mM). In (B) cells were incubated for 20 min with thapsigargin (2.5 μM) before beginning the experiment. At points indicated, carbachol (200 μM), caffeine (50 mM) and glucose (10 mM) were added. Figures are typical of three different experiments with similar results.

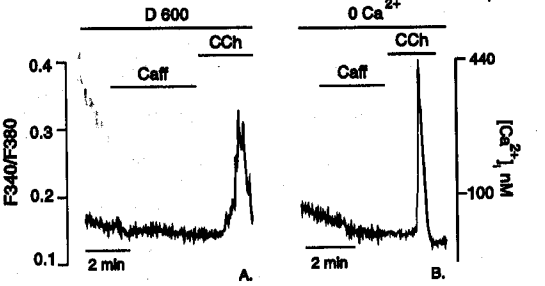

FIG. 4. **$[Ca^{2+}]_i$ increase by caffeine was blocked by L-type Ca^{2+} channel blocker or by omission of extracellular Ca^{2+}.** (A) In the presence of 1.28 mM extracellular Ca^{2+} and the L-type Ca^{2+} channel blocker D-600 (50 μM), 50 mM caffeine did not increase $[Ca^{2+}]_i$. The figure is representative of at least three different experiments. (B) Cells were superfused for 1-2 min with extracellular buffer containing EGTA (0.5-2 mM) and no added Ca^{2+}. The $[Ca^{2+}]$ of the buffer was then adjusted to 100 nM, as verified by a Ca^{2+}-selective minielectrode. Caffeine (50 mM) did not release Ca^{2+}, while marked Ca^{2+} release was obtained by carbachol (200 μM). This effect of caffeine (5- 50 mM) was seen in 18 out of 19 experiments (six preparations).

$[Ca^{2+}]_i$ faster, when basal glucose concentration was 5 mM instead of 3 mM, most likely reflecting that the β-cells are fuel-deprived at low glucose concentration (30). After the increase induced by caffeine, $[Ca^{2+}]_i$ returned to basal level in the continued presence of the substance (Fig. 2A). Repeated application of caffeine to the cells elicited similar responses, although some run-down was seen. The effect was not specific for caffeine, since the related methylxanthine aminophylline also increased $[Ca^{2+}]_i$, although the effect was less consistent (not shown). Since caffeine increases intracellular cAMP concentration by inhibiting phosphodiesterase, we tested if the increase in $[Ca^{2+}]_i$ by caffeine was mediated by cAMP. In the presence of 3 mM glucose, elevation of intracellular cAMP by forskolin (30 μM) or by forskolin plus the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (1 mM) did not affect $[Ca^{2+}]_i$ (Fig. 2B). Ryanodine (1μM) did not affect $[Ca^{2+}]_i$ (not shown).

Diazoxide, a hyperglycemic sulfonamide, inhibits glucose- and tolbutamide-induced increase in $[Ca^{2+}]_i$ in β-cell by opening the K_{ATP} channel (3,31). As shown in Fig. 3, in the presence of diazoxide (400 μM), tolbutamide-induced rise in $[Ca^{2+}]_i$ was completely blocked but caffeine-induced increase in $[Ca^{2+}]_i$ was only reduced to 64% (p = 0.12, n = 7) of that achieved in the absence of diazoxide (c.f. Fig. 2A). We also examined whether depletion of agonist-sensitive intracellular Ca^{2+} pools affected caffeine-induced increase in $[Ca^{2+}]_i$. Cells were incubated for 20 min with thapsigargin (2.5 μM), a potent inhibitor of ER Ca^{2+}-ATPase (32). Such pretreatment by thapsigargin inhibited the increase in $[Ca^{2+}]_i$ by the muscarinic agonist carbachol, but did not block the action of caffeine (Fig. 3B). The lack of effect of carbachol was not due to prior stimulation by caffeine, since in cells not treated with thapsigargin, application of carbachol after caffeine, caused release of Ca^{2+} (c.f. Fig. 4B). Dantrolene sodium (10-50 μM), which blocks Ca^{2+} release in skeletal muscle (8), also did not inhibit caffeine-induced increase in $[Ca^{2+}]_i$ (not shown).

We tested whether Ca^{2+} entry through the voltage-gated Ca^{2+} channels was involved in the caffeine-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 4A, the effect of caffeine was completely blocked by the L-type Ca^{2+} channel blocker D-600 (methoxyverapamil) (50 μM). Similar inhibition was also observed with the dihydropyridine blocker nifedipine (10 μM). To see if caffeine released Ca^{2+} from intracellular stores, its effect was tested in "low-Ca^{2+}" extracellular medium, in the presence of either 3 mM or 11 mM glucose. The "low-Ca^{2+}" solution was prepared by omitting $CaCl_2$ from the basal medium and adding 0.5-2 mM EGTA. By adding $CaCl_2$, the $[Ca^{2+}]$ of this medium was adjusted to 100 nM as measured by a Ca^{2+}-selective mini-electrode. To avoid significant depletion of intracellular Ca^{2+} stores, cells were superfused with the "low-Ca^{2+}" buffer for only 1-2 min. Under these conditions, no increase in $[Ca^{2+}]_i$ was induced by caffeine (5-50 mM) (n=18, six preparations). A small release of Ca^{2+} was seen in only one experiment. To verify that the intracellular Ca^{2+}

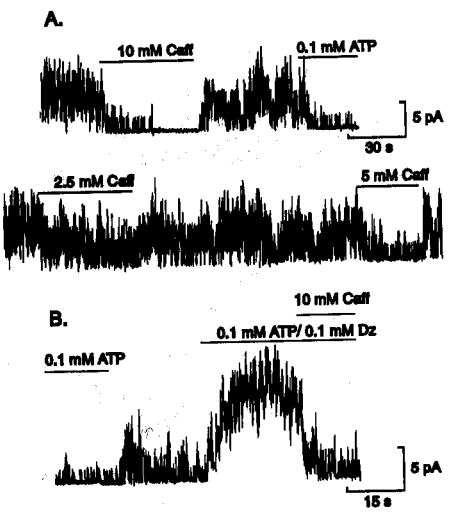

FIG. 5. **Effects of caffeine on K_{ATP} channel activity.** K_{ATP} channel activity was measured in inside-out patches shortly after excision. (A) Caffeine (10 mM) completely blocked K_{ATP} channel activity. Inhibition was fully reversible upon withdrawal of the compound. The inhibitory action of caffeine was dose-dependent, minimal effective concentration of caffeine being 2.5 mM. Addition of 5 mM caffeine to the same patch induced a further decrease in K_{ATP} channel activity. In (B), K_{ATP} channel activity stimulated by diazoxide (100 μM) was almost completely blocked by caffeine (10 mM), when added in the presence of diazoxide.

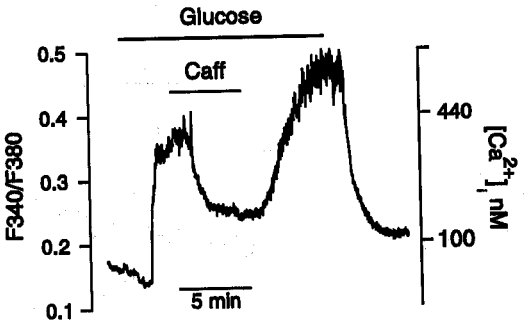

FIG. 6. **Lowering of $[Ca^{2+}]_i$ by caffeine in glucose-stimulated β-cell.** Conditions of experiment were as described in legend to figure 2. $[Ca^{2+}]_i$ was increased by stimulating the cells with glucose (8-11 mM). Addition of caffeine (10 mM), caused a reduction in $[Ca^{2+}]_i$. The trace is representative of six different experiments.

pools were not depleted by pretreatment with "low-Ca^{2+}" medium, carbachol was added. This substance caused a marked increase in $[Ca^{2+}]_i$, indicating that these pools were indeed filled with Ca^{2+} (Fig. 4B). In some experiments, $[Ca^{2+}]_i$ was first raised by depolarizing the cell with glucose, KCl or glipizide, in the presence of extracellular Ca^{2+}, in an attempt to load the intracellular Ca^{2+} pools further, and thereby maximize the possibility of detecting intracellular Ca^{2+} release. Such pretreatment also failed to elicit Ca^{2+} release by caffeine in the presence of the "low-Ca^{2+}" buffer.

The main mechanism whereby glucose and antidiabetic sulfonylureas increase $[Ca^{2+}]_i$ in β-cells, is closure of the K_{ATP} channel (3). We, therefore, examined whether caffeine also acted by a similar mechanism. In excised inside-out patches, caffeine inhibited K_{ATP} channel activity in a dose-dependent manner. The minimal concentration of caffeine for the inhibition of the K_{ATP}-channel was 2.5 mM. The inhibitory effect of 10 mM caffeine was rapid and led to a total block of K_{ATP} channel activity (Fig. 5A). Channel activity returned promptly when caffeine-containing solution was replaced by caffeine-free solution. Aminophylline (10 mM) also blocked K_{ATP} channel activity in a reversible manner. In experiments described in fig. 5B, K_{ATP} channel activity was increased by diazoxide (100 μM) and caffeine (10 mM) was added in the continued presence of diazoxide. Also, under these conditions, caffeine inhibited K_{ATP} channel activity which may suggest that caffeine interacts with a binding site separate from that for the hyperglycemic sulfonamide.

When β-cells were stimulated by glucose (8-11 mM), $[Ca^{2+}]_i$ increased rapidly to a plateau. Addition of caffeine at this stage did not increase $[Ca^{2+}]_i$ further, rather it decreased $[Ca^{2+}]_i$, as indicated by a decrease in $R_{340/380}$ and notable anti-parallel change in the F_{340} and F_{380}. The effect was reversed completely upon switching back to caffeine-free solution (Fig. 6). The inhibitory effect on $[Ca^{2+}]_i$ was always observed when caffeine was used at concentrations of 10 mM or higher. We further tested whether this reduction in $[Ca^{2+}]_i$ by caffeine could be explained by a reduction in Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channels, by using the whole-cell mode of the patch-clamp technique. Fig 7A shows the effect of caffeine on whole-cell Ca^{2+} currents in β-cells. The cells were depolarized to 0 mV from a holding potential of -70 mV. The depolarizing voltage steps were given every 20 s. Addition of 50 mM caffeine to the extracellular medium inhibited the peak Ca^{2+} current by approximately 40%. The inhibitory effect of caffeine on peak Ca^{2+} currents was fully reversible following wash-out, and was reproducible in the same cell on subsequent reexposure. In several of the cells tested, we found a small increase in the Ca^{2+} current during wash-out of caffeine from the chamber (7A, inset). As

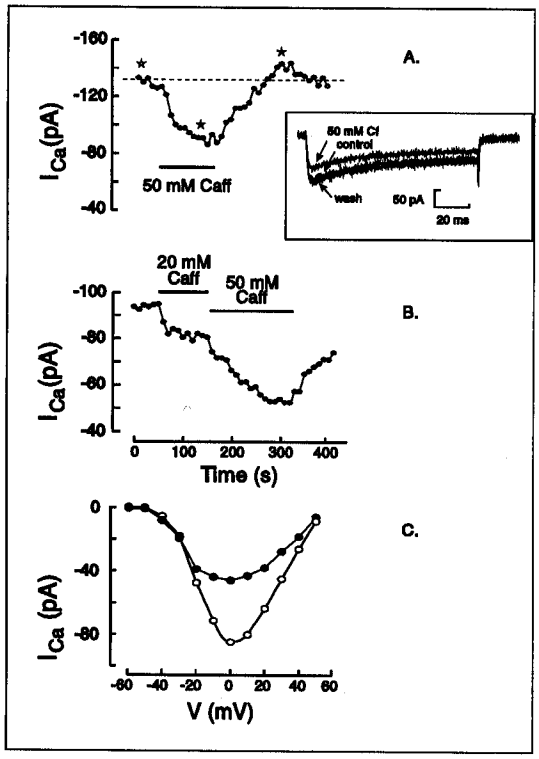

FIG. 7. **Inhibition of voltage-gated Ca^{2+} channels by caffeine.** (A) Effects of caffeine on whole-cell inward peak Ca^{2+} currents, induced by 100 ms steps to 0 mV from a holding potential of -70 mV. The depolarizing voltage step was applied every 10 s. 50 mM caffeine was present as indicated by the bar. Inset shows current traces, filtered at 2 kHz, from time points indicated by stars, prior to addition of caffeine, during exposure to caffeine and following wash-out. The peak Ca^{2+} current decreased from 133 pA to 90 pA during perifusion with 50 mM caffeine . After wash-out, the peak current amounted to 144 pA. (B) Effects of cumulative application of caffeine on inward peak Ca^{2+} current induced as described in (A). Caffeine inhibited depolarization-induced whole-cell Ca^{2+} current in a dose-dependent manner. Caffeine was present in the chamber as indicated by the bars. Prior to addition of caffeine, peak current averaged 94 pA. This was reduced to about 80 pA, after 20 mM caffeine and following 50 mM of the compound, peak current further decreased to less than 50 pA. (C) Typical example of full current-voltage relationship in the absence (open circles) and presence (filled circles) of 50 mM caffeine. Peak current in the presence of caffeine was reduced by approximately 50%.

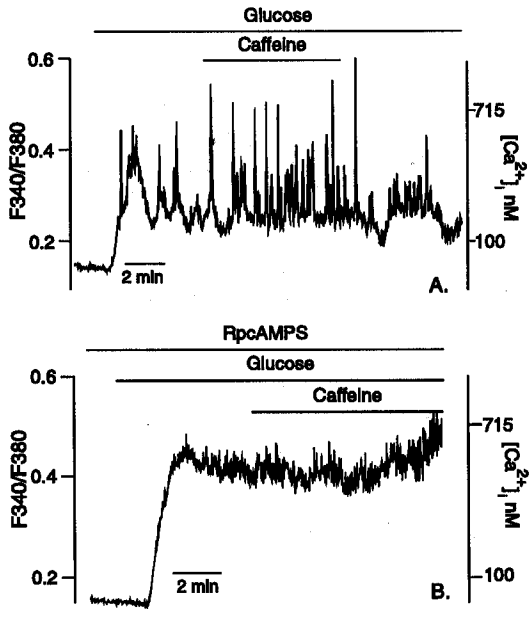

Fig. 8. **Caffeine induced fast $[Ca^{2+}]_i$ oscillations in glucose-stimulated β-cells.**
Fura-2 loaded β-cell clusters (2-4 cells) were stimulated by glucose (8-10 mM). (A). Addition of caffeine (5 mM), at point indicated, induced fast $[Ca^{2+}]_i$ oscillations which reversed upon withdrawal of the compound. Similar results were observed in eight out of nine different experiments. In (B), *R*pcAMPS (50 μM) were present during loading with fura-2 (for 20 min) and the rest of the experiment. The trace is representative of three different experiments. Control experiments using cells from the same cell preparation showed fast $[Ca^{2+}]_i$ oscillations.

exemplified in Fig 7B, the inhibitory effect of caffeine on peak Ca^{2+} currents was dose-dependent. When 20 mM caffeine was administered, a reduction of about 10-20 % of the whole-cell Ca^{2+} current was regularly observed. Increasing the concentration to 50 mM, further reduced the depolarization-evoked Ca^{2+} currents to about 40-50%. The current-voltage relationship is shown in Fig 7C. The inhibitory effect of 50 mM caffeine was most pronounced at around 0 mV.

In experiments described in fig. 8A, $[Ca^{2+}]_i$ was first raised by stimulating the cells with glucose (8-10 mM). This resulted in sustained elevation of $[Ca^{2+}]_i$ or slow oscillations in $[Ca^{2+}]_i$ (0.1-0.9/min). After the increase in $[Ca^{2+}]_i$ by glucose, caffeine was added at a low concentration (2.5-5 mM). This induced fast $[Ca^{2+}]_i$ oscillations (0.9-3.5/min). Characteristically, these oscillations were superimposed on an elevated level of $[Ca^{2+}]_i$ contained large amplitude Ca^{2+} transients (6-12 s) and disappeared after withdrawal of caffeine. Caffeine-induced oscillations were seen in 8 out of

9 experiments. In one experiment, 5 mM caffeine did not induce $[Ca^{2+}]_i$ oscillations but instead caused a reduction in $[Ca^{2+}]_i$, as described above for higher concentrations of the compound.

The effects of *R*pcAMPS, an antagonist of PKA (33), on caffeine-induced $[Ca^{2+}]_i$ oscillation were tested in fig. 8B. In these experiments, cells were pretreated with *R*pcAMPS (50 μM) for 20 min by adding the substance to the buffer during incubation with fura-2. *R*pcAMPS was continuously present in the perifusion during the rest of the experiment. With this treatment, cells responded to glucose with an increase in the $[Ca^{2+}]_i$, but $[Ca^{2+}]_i$ oscillations induced by 5 mM caffeine was largely prevented.

DISCUSSION

Because of lack of more suitable pharmacological tools, caffeine is often used for investigating problems related to Ca^{2+} signalling that involve Ca^{2+} induced Ca^{2+} release mediated by the ryanodine receptor. In such studies, it is often necessary to use caffeine at concentrations as high as 40-50 mM to obtain maximal Ca^{2+} release (34-36). The most well-known mechanism by which caffeine increases $[Ca^{2+}]_i$, is through the activation of the ryanodine receptor. Hence, an increase in $[Ca^{2+}]_i$ by caffeine is generally taken as indicative of Ca^{2+} release from intracellular Ca^{2+} stores (15,22-24). The results in the present study caution against such a simplistic view and demonstrate novel mechanisms by which caffeine can affect $[Ca^{2+}]_i$.

It was claimed that depending on the concentration, caffeine can either stimulate or inhibit intracellular Ca^{2+} release (22,24). In the present study, we therefore used caffeine in a wide range of concentrations. However, it is to be noted that in our microfluorimetric experiments, concentrations of caffeine refer to the concentrations as added to the perifusion media. The concentration in the perifusion chamber, at the onset of a response, was probably lower. Most responses to caffeine occurred within 1-2 min of switching to the caffeine-containing solution, whereas an estimated time of about 3.45 min would be required before 90% of the intended concentration of the compound could be attained in the perifusion chamber.

Interference of caffeine with fluorescence of Ca^{2+}-indicators has been appreciated in several studies (36,37). In our study, caffeine increased the fluorescence of fura-2 in a $[Ca^{2+}]$- and wavelength-dependent manner. A much greater increase of F_{380} compared to F_{340}, after addition of caffeine, resulted in a reduction in basal $R_{340/380}$ in all experiments. This reduction in $R_{340/380}$ was not due to a decrease in $[Ca^{2+}]_i$, where F_{340} should decrease. The opposing effects of caffeine and Ca^{2+} on F_{380}, may make it difficult to detect a small change in $[Ca^{2+}]_i$.

Our study showed that in unstimulated β-cells, in the presence of extracellular Ca^{2+}, caffeine consistently increased $[Ca^{2+}]_i$. This confirms earlier reports which attributed similar effects to the intracellular Ca^{2+}-mobilizing action of caffeine (23,24). In the present study, however, three lines of evidence indicated that Ca^{2+} release from intracellular stores was not involved. Firstly, in the presence of a "low-Ca^{2+}" solution extracellularly, there was no increase in $[Ca^{2+}]_i$, as tested with various concentrations of caffeine in a large number of experiments, despite the presence of releasable Ca^{2+} in the intracellular Ca^{2+} pools. Secondly, when cells were treated for a prolonged period of time with the potent SERCA inhibitor thapsigargin, caffeine still increased $[Ca^{2+}]_i$. Thirdly, the $[Ca^{2+}]_i$-increase by caffeine was completely blocked by the L-type voltage-gated Ca^{2+} channel blockers D-600 and nifedipine, indicating that Ca^{2+} entry through this channel was involved. Moreover, dantrolene, a blocker of ryanodine receptor did not block caffeine-induced increase in $[Ca^{2+}]_i$.

Since caffeine is a well-known inhibitor of phosphodiesterase, our first thought was that caffeine might increase $[Ca^{2+}]_i$ by elevating cAMP and thereby phosphorylating L-type voltage-gated Ca^{2+} channels. However, this seemed unlikely since in the presence of a non-stimulatory concentration of glucose, forskolin and IBMX did not increase $[Ca^{2+}]_i$, whereas caffeine always did. It has been reported that, under resting conditions, cAMP increases $[Ca^{2+}]_i$ by stimulating Ca^{2+} entry through voltage-gated Ca^{2+} channels in a hamster β-cell line (HIT T-15) (38), but not in normal β-cells (38,39) or insulin-secreting RINm5F cells (41). Hence, our results indicated that caffeine triggered events by mechanisms not dependent on cAMP, resulting in opening of voltage-gated Ca^{2+}-channels.

In β-cells, resting membrane potential is maintained by K_{ATP} conductance. Closure of this channel causes cell depolarization, opening of voltage-gated L-type Ca^{2+} channels and Ca^{2+}-influx (3,42). The $[Ca^{2+}]_i$-increase caused by glucose and antidiabetic sulfonylureas, both of which act by closing the K_{ATP} channel, is inhibited by diazoxide, an opener of this channel. In our experiments, the inhibitory effect of diazoxide on the caffeine-induced increase in $[Ca^{2+}]_i$ was small. But single channel recordings in excised inside-out patches confirmed that caffeine was an efficient blocker of the K_{ATP} channel. In spite of being highly membrane-permeable (37), the concentration of caffeine required for maximal $[Ca^{2+}]_i$ increase in intact cells was five times greater than that required for maximal inhibition of K_{ATP} channel activity in excised patches. This may be due to the fact that in intact cells, the inhibitory effect is opposed by the stimulatory effect of intracellular ADP on K_{ATP} channel activity (43). The inhibitory effect of caffeine on the K_{ATP} channel was unlikely to be mediated by cAMP, since previous studies showed that even a 60 fold increase in cAMP did not significantly affect the activity of the channel (40, Islam, Larsson and Berggren, unpublished observations). The lack of complete inhibition by diazoxide of the $[Ca^{2+}]_i$-increasing effect of caffeine, was consistent with the finding that caffeine inhibited K_{ATP} channel activity in excised patches, even under conditions of stimulation by

diazoxide. Henquin used $^{86}Rb^+$ efflux as a measure of K_{ATP} channel activity and reported a reduction in $^{86}Rb^+$ efflux from rat islet cells by another xanthine, theophylline (44). Our present report is the first direct demonstration of an inhibitory effect of caffeine on K_{ATP} channel activity, an effect that might underlie the observed elevation in $[Ca^{2+}]_i$ in intact β-cells by caffeine. ATP and xanthine derivatives share the purine ring which may be a possible basis for the inhibitory action of caffeine on the K_{ATP} channel, since at a high concentration (5 mM), the purine base adenine also blocks channel activity (45). An inhibitory effect of caffeine on K_{ATP} channel activity is consistent with a previous report, which showed that caffeine may induce modest insulin secretion also in the absence of glucose (46).

The effects of caffeine were complex in that it could not only increase but also decrease $[Ca^{2+}]_i$. The latter phenomenon was observed when caffeine was added subsequent to a glucose-induced increase in $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$-lowering effect was unlikely to be due to interference of caffeine with glucose metabolism, since such reduction was seen even when $[Ca^{2+}]_i$ was raised by depolarization with a high concentration of KCl (not shown). Moreover, at least one previous study has examined the effect of caffeine on glucose metabolism and excluded any significant effect (47). A reduction of $[Ca^{2+}]_i$ in glucose-stimulated islets by caffeine was described by Roe *et al* , an effect interpreted as a reduction in Ca^{2+} release from intracellular stores (24). However, since mobilization of Ca^{2+} from intracellular stores is not a major mechanism of glucose-induced increase in $[Ca^{2+}]_i$ and since caffeine did not release Ca^{2+} from intracellular stores, in the first place, we needed to find another mechanism for this phenomenon. In the β-cell, Ca^{2+} entry through the L-type voltage-gated Ca^{2+} channel is the major mechanism by which $[Ca^{2+}]_i$ is increased after glucose stimulation. We tested whether a reduction in Ca^{2+} entry through this channel might underlie the lowering effect of caffeine on $[Ca^{2+}]_i$. Patch-clamp studies on the effects of caffeine on the L-type voltage-gated Ca^{2+} channel in the β-cell, confirmed that caffeine indeed reduced depolarization-induced whole-cell Ca^{2+} current. Inhibition of I_{Ca} by caffeine has also been reported in smooth muscle cells, sympathetic neurones and purkinje fibers (13,48,49). Thus caffeine, by closing the K_{ATP} channel, caused cell depolarization and opening of the L-type voltage-gated Ca^{2+} channel in unstimulated β-cells. On the other hand, caffeine inhibited influx through the same Ca^{2+} channel, when the latter was already activated by depolarization. This apparent paradox can be explained by the fact that the former action was due to depolarization brought about by inhibition of K_{ATP} channel by caffeine, while the latter was a direct inhibitory effect of caffeine on the L-type Ca^{2+} channel.

In β-cells stimulated by glucose, a low concentration of caffeine (2.5-5 mM) induced fast $[Ca^{2+}]_i$ oscillations and Ca^{2+} transients superimposed on an elevated $[Ca^{2+}]_i$. This is likely to be attributable to cAMP-elevating action of caffeine, since previous studies have demonstrated that administration of cAMP analogues or cAMP-elevating agents like GLP-1 (7-36) amide, to glucose-stimulated β-cells, induces similar fast $[Ca^{2+}]_i$ oscillations (50, and Juntti-Berggren and Berggren, unpublished data). Here, we further show that this effect of caffeine is inhibited by blocking PKA with *R*pcAMPS. The precise mechanisms underlying this phenomenon is unclear, but a possible explanation lies in the fact that cAMP also increases electrical activity and Ca^{2+} action potentials in glucose-stimulated β-cells (51). It is well documented that a cAMP-dependent phosphorylation of the L-type Ca^{2+} channel, which may be favored by a voltage-dependent change in the conformation of the subunits, increases the number of active channels and their probability of opening (52-54). In β-cells, PKA phosphorylation increases depolarization-evoked Ca^{2+} current (55). An increase of depolarization-evoked Ca^{2+} current was also observed in our experiments, following wash-out of caffeine from the chamber (Fig. 7A inset), which may be induced by a transient period of a residual low concentration of caffeine, inherent in the wash-out process. Under these experimental conditions, the inhibitory effect of higher concentrations of the substance was of course, reversed.

Our previous studies showed that in permeabilized insulin-secreting cells, Ca^{2+} release by caffeine was, if anything, very small (25,26). In those studies, an optimal concentration of caffeine could not be used because of difficulty in obtaining a concentrated enough stock solution of the substance. In the present study caffeine was used at concentrations as high as 50 mM, by dissolving it directly in the perifusion buffer. Even in this situation, a small Ca^{2+} release was observed only rarely. We can not exclude the possibility that under these conditions, caffeine released a small amount of Ca^{2+} which remained undetected because of uptake into other pools or was masked due to the action of the plasma membrane Ca^{2+} pump and/or interference of caffeine with fura-2 fluorescence. Herchuelz *et al*, by measuring $^{45}Ca^{2+}$ outflow, demonstrated a small Ca^{2+} release from rat pancreatic islets by caffeine (23). However, a large proportion of cells in rat islets are non-β cells and might constitute the caffeine-sensitive pool in their experiments. In some cells, $[Ca^{2+}]_i$ oscillations have been demonstrated in the presence of high extracellular Ca^{2+}, even after depletion of intracellular Ca^{2+} stores by ER Ca^{2+}-ATPase inhibitors (56). A caffeine-sensitive Ca^{2+} store is implicated, because the oscillations are stimulated by low concentrations of caffeine and inhibited by high concentrations of ryanodine (57). The identity and mechanism of filling of the putative caffeine-sensitive pool in these cells are unresolved. From our study it appears that, as far as β-cells are concerned, the mechanisms responsible for caffeine sensitivity are located in the plasma membrane.

In summary, caffeine-induced changes in $[Ca^{2+}]_i$ in β-cells were not mediated by its action on

intracellular Ca^{2+} pools. Inhibition of K_{ATP} channel activity was a distinct mechanism by which caffeine induced an increase in $[Ca^{2+}]_i$. In these cells a caffeine-sensitive intracellular Ca^{2+} pool was either absent or very small, compared to Ins(1,4,5)P_3-sensitive stores. The basis for the $[Ca^{2+}]_i$-lowering effect of caffeine in glucose-stimulated β-cells, was inhibition of L-type voltage-gated Ca^{2+} channels. Furthermore, in glucose-stimulated β-cells, caffeine may induce fast Ca^{2+} oscillations possibly by a cAMP-dependent phosphorylation of voltage-gated Ca^{2+} channels.

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