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Arachidonic acid is a physiological activator of the ryanodine receptor in pancreatic β-cells

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Abstract

Pancreatic β-cells have ryanodine receptors but little is known about their physiological regulation. Previous studies have shown that arachidonic acid releases Ca^{2+} from intracellular stores in β-cells but the identity of the channels involved in the Ca^{2+} release has not been elucidated. We studied the mechanism by which arachidonic acid induces Ca^{2+} concentration changes in pancreatic β-cells. Cytosolic free Ca^{2+} concentration was measured in fura-2-loaded INS-1E cells and in primary β-cells from Wistar rats. The increase of cytosolic Ca^{2+} concentration induced by arachidonic acid (150 μM) was due to both Ca^{2+} release from intracellular stores and influx of Ca^{2+} from extracellular medium. 5,8,11,14-Eicosatetraynoic acid, a non-metabolizable analogue of arachidonic acid, mimicked the effect of arachidonic acid, indicating that arachidonic acid itself mediated Ca^{2+} increase. The Ca^{2+} release induced by arachidonic acid was from the endoplasmic reticulum since it was blocked by thapsigargin. 2-Aminoethyl diphenylborinate (50 μM), which is known to inhibit 1,4,5-inositol-triphosphate-receptors, did not block Ca^{2+} release by arachidonic acid. However, ryanodine (100 μM), a blocker of ryanodine receptors, abolished the effect of arachidonic acid on Ca^{2+} release in both types of cells. These observations indicate that arachidonic acid is a physiological activator of ryanodine receptors in β-cells.

Keywords: Arachidonic acid; Calcium channels; Calcium signalling; Pancreatic β-cells; Ryanodine receptors

1. Introduction

When plasma glucose increases, cytoplasmic ATP concentration inside the pancreatic β-cells increases at the expense of ADP, inducing a closure of ATP-sensitive K^+ channels, depolarizing the membrane potential, and activating voltage-gated Ca^{2+} channels. The consequent increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) is the main trigger of exocytosis of insulin granules [1]. Glucose stimulation of β-cells, however, alters the concentration of many other active metabolites in the cytoplasm. For instance, when plasma glucose concentration increases, there is activation of phospholipase A_2 and formation of arachidonic acid (AA) [2]. AA seems to be involved in insulin secretion. Consistent with this, inhibition of phospholipase A_2 impairs glucose-induced insulin secretion [3,4].

It is known that AA increases $[Ca^{2+}]_c$ in pancreatic β-cells [2,5,6]. This increase of $[Ca^{2+}]_c$ occurs by two mechanisms: release of Ca^{2+} from intracellular stores and influx of Ca^{2+} across the plasma membrane [2,6]. However, the Ca^{2+} stores or Ca^{2+} channels involved in the Ca^{2+} release and Ca^{2+} entry induced by AA have remained unknown.

Ryanodine (RY) receptor and inositol 1,4,5-triphosphate-receptor (IP_3R), are the two major families of channels that mediate Ca^{2+} release from the endoplasmic reticulum (ER)

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[7]. The RY receptor is a large cation-selective channel that allows permeation of Ca^{2+}. One of the main properties of RY receptors is that they are activated or inhibited by cytosolic Ca^{2+} [8]. These receptors play a critical role in excitation–contraction coupling of skeletal and cardiac muscle cells [9], and are implicated in amplifying Ca^{2+} signals in neurons [10] and in pancreatic β-cells [8]. RY receptors are also expressed in many other cells, including endothelial cells, adrenal chromaffin cells, hepatocytes, and osteocytes [11].

Inositol 1,4,5-triphosphate is the physiological activator of the IP_3R [7]; however, the corresponding activator of the RY receptors remains to be elucidated. It has been proposed that cyclic-ADP-ribose could be the endogenous regulator of some types of RY receptors, but this view remains controversial [12,13]. In this study, our results suggest that AA is an endogenous activator of RY receptors in intact pancreatic β-cells.

2. Methods

2.1. Materials

Fura-2 acetoxymethyl ester was purchased from Molecular Probes, Europe. RY mixture (purity: ≥96% RY and didehydroryanodine) and thapsigargin were from Calbiochem. AA, caffeine, 2-aminoethyl diphenylborinate (2-APB), carbachol (CCh), nimodipine, nitro-L-arginine (L-NNA), and 5,8,11,14-eicosatetraynoic acid (ETYA) were from Sigma. Cell culture materials were from Invitrogen. AA was dissolved in ethanol (concentration of stock 50 mM) and stored with N_2 at −144 °C. Prior to the experiments, DMSO (final concentration 0.1%) was added to the AA solutions as a vehicle. DMSO or the diluent used for AA had no effect on $[Ca^{2+}]_c$.

2.2. Cell culture

We used highly differentiated rat insulinoma cells (S5) derived from INS-1E cell line [14]. The cells were cultured in RPMI supplemented with FBS (2.5%), penicillin (50 IU/ml), streptomycin (50 μg/ml), 2-mercaptoethanol (500 μM), HEPES (10 mM), and sodium pyruvate (1 mM). Cells were incubated at 37 °C in humidified incubator with 5% CO_2. The cells were used between 3 and 6 days after plating.

2.3. Primary pancreatic β-cells

Male Wistar rats (250–280 g weight) were killed by decapitation after anesthesia with CO_2. The islets were isolated by injecting collagenase-A in Hanks' solution (9 mg/10 ml) into pancreas through the pancreatic duct. The gland was removed, incubated for 24 min at 37 °C, washed with Hanks' solution, and islets were picked up after separation on Histopaque gradient. Islets were dispersed by trypsin digestion. Cells obtained were suspended in RPMI supplemented with FBS (10%), penicillin (50 IU/ml), streptomycin (50 μg/ml), and glucose (11 mM). Cells were incubated at 37 °C in humidified environment with 5% CO_2. The $[Ca^{2+}]_c$ measurements were done within 1–3 days after obtaining the cells. The selection of the β-cells for analysis was based on the large size and the small nucleus/cytoplasm ratio in comparison with non-β-cells, as previously described [15].

2.4. Measurement of $[Ca^{2+}]_c$

Cultured cells were transferred to glass coverslips and were incubated in RPMI supplemented with 0.1% BSA and fura-2 (1 μM) for 35 min at 37 °C. Cells were then kept at room temperature for additional 10 min in a physiological solution containing 140 NaCl, 3.6 KCl, 0.5 $NaH_2PO_4{\cdot}H_2O$, 0.5 $MgSO_4{\cdot}7H_2O$, 1.5 $CaCl_2{\cdot}2H_2O$, 10 HEPES, 3 glucose (in mM), and 0.1% BSA (pH 7.4). Nominally Ca^{2+}-free medium was prepared by addition of EGTA (0.5 mM) to solution to which no $CaCl_2$ was added.

$[Ca^{2+}]_c$ was measured in single cells by microfluorometry using an inverted epifluorescence microscope (Olympus CK40) connected to a monochromator system (Photon Technology International). Coverslips were mounted as the exchangeable bottom of an open perfusion chamber on the stage of the microscope. The perfusion was controlled by a pump (Ismatec, Switzerland) at 22 μl/s. The fluorescence system was controlled by the Felix Software (Version 1.42b, PTI). The emission at the excitation wavelength of 340 nm ($F340$) and that of 380 nm ($F380$) were used to calculate the fluorescence ratio ($R340/380$). The background fluorescence was measured and subtracted from the traces. The $[Ca^{2+}]_c$ was calculated from $R340/380$ according to Grynkiewicz et al. [16]. R_{max} and R_{min} were determined by using thin films of external standards containing fura-2 and 2 M sucrose [17]. The K_d for Ca^{2+}-fura-2 was taken as 225 nM [18]. All experiments were performed at 36–37 °C.

2.5. Statistical analysis

Data were expressed as mean ± S.D. Student's t-test was used when appropriate. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of AA on $[Ca^{2+}]_c$

We used AA (1–175 μM) to determine the concentration that increases $[Ca^{2+}]_c$ reproducibly (Fig. 1A and B). A 50–150 μM AA increased $[Ca^{2+}]_c$ in a dose-dependent manner (Fig. 1B). Maximum effective concentration of AA was 150 μM and at this concentration, 95% of cells responded by an increase of $[Ca^{2+}]_c$. Lower concentrations of AA (10

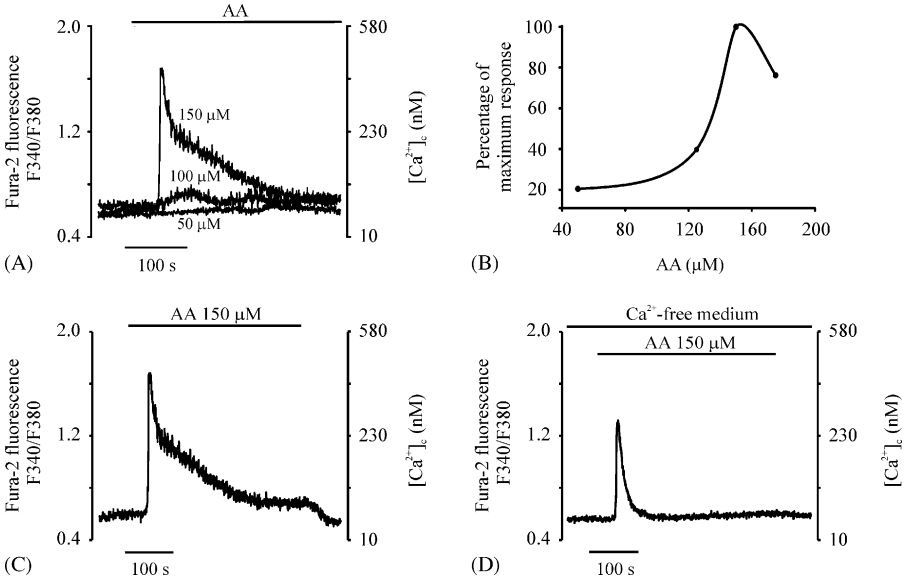

Fig. 1. Effect of AA on $[Ca^{2+}]_c$ in INS-1E cells. $[Ca^{2+}]_c$ was measured by microfluorometry using fura-2. (A) AA 150 μM increased $[Ca^{2+}]_c$ in a characteristic and reproducible manner (10 out of 10 cells). Traces for 100 and 50 μM are representative of three and five experiments, respectively. (B) Dose–response curve for AA and its effect on $[Ca^{2+}]_c$. Each dot is representative of 3–10 experiments. (C and D) Effect of AA on $[Ca^{2+}]_c$ in Ca^{2+}-containing ($n = 10$) and Ca^{2+}-free medium ($n = 7$), respectively. (D) Perfusion medium was switched to a nominally Ca^{2+}-free medium 1 min before giving AA. The increase of $[Ca^{2+}]_c$ was 376 ± 161 and 170 ± 92 nM, in (C) and (D), respectively ($P < 0.005$). These findings indicate that the increase of $[Ca^{2+}]_c$ by AA is due to Ca^{2+} release from intracellular pools and influx of Ca^{2+} from extracellular medium.

and 25 μM) did not increase $[Ca^{2+}]_c$ reproducibly and only 25–50% of cells responded by a small increase of $[Ca^{2+}]_c$, the magnitude of which was 10–20% of maximal increase obtained by 150 μM AA. A typical $[Ca^{2+}]_c$ change after application of AA 150 μM consisted of two components: an initial rapid increase of $[Ca^{2+}]_c$ to a peak, followed by a sustained plateau (Fig. 1C).

$[Ca^{2+}]_c$ changes induced by AA were completely reversible and application of CCh or KCl after wash out of AA caused a normal $[Ca^{2+}]_c$ response. These observations indicate that AA 150 μM (in the background of 0.1% albumin) did not damage these cells.

Next, we investigated whether AA released Ca^{2+} from the intracellular stores or induced influx of Ca^{2+} across the plasma membrane. We compared the pattern and magnitude of $[Ca^{2+}]_c$ changes caused by AA in Ca^{2+}-containing medium (Fig. 1C) to those obtained in Ca^{2+}-free medium (Fig. 1D). The peak increase of $[Ca^{2+}]_c$ after application of AA in presence of extracellular Ca^{2+} was 376 ± 161 nM ($n = 10$) and that in Ca^{2+}-free medium was 170 ± 92 nM ($n = 7$) ($P < 0.005$). These results indicate that the increase of $[Ca^{2+}]_c$ induced by AA is due to both Ca^{2+} release from intracellular stores and influx of Ca^{2+} across the plasma membrane.

We tested nimodipine (20 μM), a blocker of L-type voltage-gated Ca^{2+} channels, to determine if these channels mediate the influx of Ca^{2+} by AA. Nimodipine reduced both the initial rapid phase (Fig. 2A; cf., Fig. 2B) and the sustained phase of $[Ca^{2+}]_c$-increase by AA (Fig. 2C), indicating that Ca^{2+} entry occurred during both of these phases. Nimodipine decreased the AA-induced initial peak Ca^{2+} response by ~65% ($P < 0.005$). These findings suggest that L-type Ca^{2+} channels are involved, at least in part, in the Ca^{2+} entry induced by AA.

3.2. AA itself rather than its metabolites increased $[Ca^{2+}]_c$

AA is converted to many metabolites in intact cells. To identify whether the effect of AA on $[Ca^{2+}]_c$ was due to AA itself or its metabolites, we tested the effect of ETYA, a non-metabolizable analogue of AA. ETYA (150 μM) also increased $[Ca^{2+}]_c$ (Fig. 3A), and the pattern and magnitude of this effect were similar to those observed after application of AA (Fig. 3B). This finding suggests that the effect of AA on $[Ca^{2+}]_c$ is mainly due to AA itself.

3.3. AA released Ca^{2+} from the ER

INS-1E cells were incubated with thapsigargin (500 nM), an irreversible blocker of the ER Ca^{2+} ATPase, for 45 min. We have previously demonstrated that such pre-treatment depletes the ER completely, without altering the basal $[Ca^{2+}]_c$ or the resting membrane potential [19]. When AA (150 μM) was added, it did not produce any increase of $[Ca^{2+}]_c$ (Fig. 4A; cf., Fig. 4B). These results indicate that AA releases Ca^{2+} from a thapsigargin-sensitive intracellular pool, most likely the ER. Acute application of thapsigargin alone (500 nM) increased $[Ca^{2+}]_c$ in a biphasic manner (Fig. 4C).

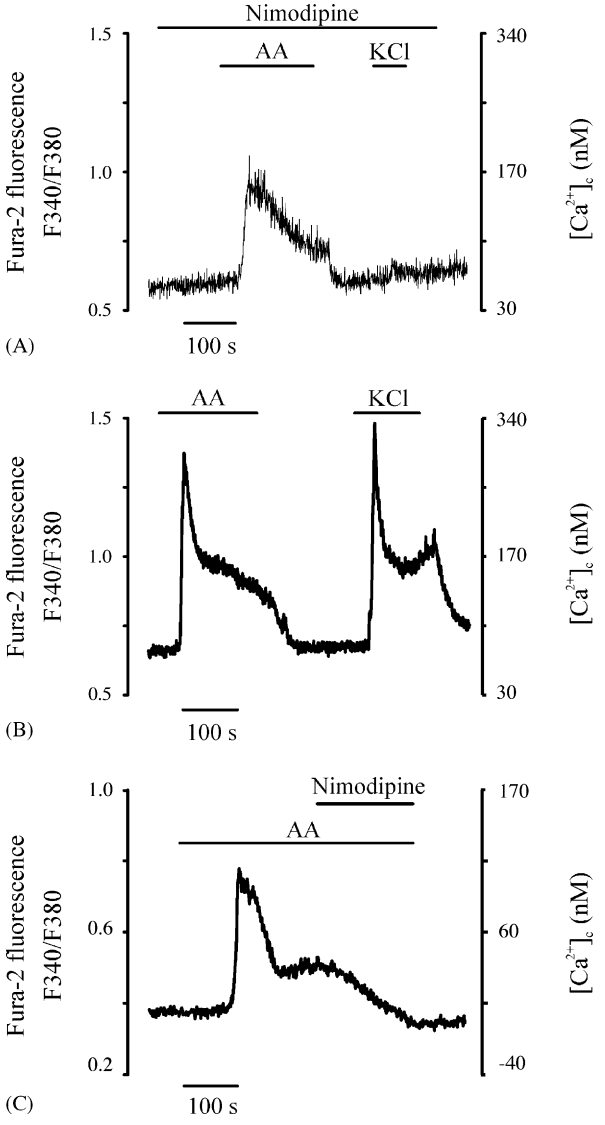

Fig. 2. Nimodipine, a blocker of L-type voltage-gated Ca^{2+} channels, decreased the effect of AA on $[Ca^{2+}]_c$ in INS-1E cells. (A) Nimodipine (20 μM) was given 2 min before application of AA (150 μM) ($n = 3$). KCl (25 mM) was applied to demonstrate the effect of nimodipine. (B) Effect of AA and KCl at the same concentrations in absence of nimodipine ($n = 5$). Nimodipine reduced the initial peak increase of $[Ca^{2+}]_c$ by ~65% ($P < 0.005$). (C) Reduction of the sustained phase of AA-induced $[Ca^{2+}]_c$ increase by nimodipine (same concentrations). The trace is representative of three experiments with similar results.

3.4. AA did not release Ca^{2+} through IP_3Rs

IP_3Rs and RY receptors are the main channels for the release of Ca^{2+} from the ER. To determine whether AA released Ca^{2+} through IP_3Rs, we used 2-APB (50 μM), a blocker of IP_3Rs. INS-1E cells were incubated for 10 min with 2-APB and the same concentration of 2-APB was present during the perfusion. As expected, 2-APB decreased the response to CCh (100 μM) by ~85%. The increase of $[Ca^{2+}]_c$ induced by CCh was 71 ± 49 nM in presence of 2-APB, compared to 485 ± 370 nM in the control group

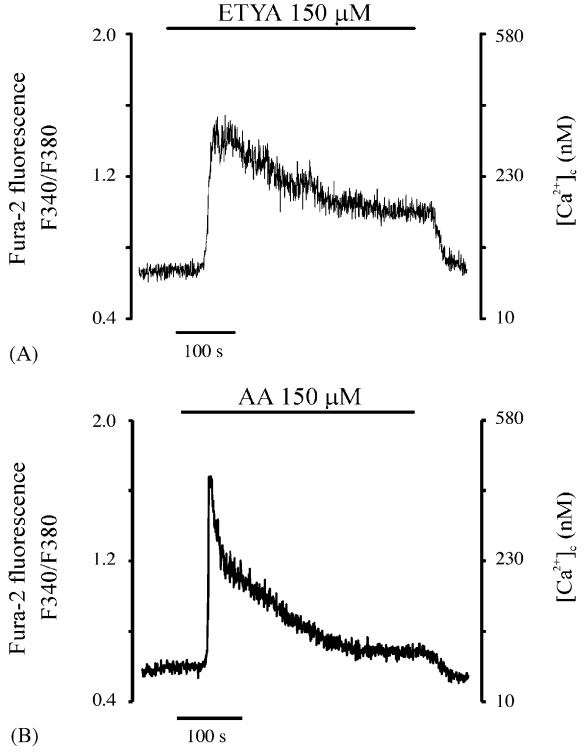

Fig. 3. AA itself increased $[Ca^{2+}]_c$. (A) ETYA (150 μM), a non-metabolizable analogue of AA, increased $[Ca^{2+}]_c$ in INS-1E cells. (B) Increase of $[Ca^{2+}]_c$ induced by AA (150 μM). Traces in (A) and (B) are representatives of 3 and 10 experiments, respectively.

($P < 0.05$) (Fig. 5A; cf., Fig. 5B). In contrast, there was no difference in the AA-induced Ca^{2+} response in presence or absence of 2-APB (199 ± 42 nM versus 281 ± 202 nM, respectively). Acute application of 2-APB (50 μM) did not increase $[Ca^{2+}]_c$ (Fig. 5C). These results indicate that IP_3Rs do not mediate the release of Ca^{2+} by AA.

3.5. AA released Ca^{2+} through RY receptors

To determine whether AA released Ca^{2+} through the RY receptors, we incubated INS-1E cells for 45 min with both ryanodine (100 μM) and caffeine (10 mM). During perfusion, only ryanodine (50 μM) was present. This treatment completely abolished the response to AA in four out of seven experiments (Fig. 6A; cf., Fig. 6B), with an overall inhibition of the increase of $[Ca^{2+}]_c$ by ~80% (59 ± 34 and 306 ± 170 nM, in ryanodine-treated cells and control experiments, respectively; $P < 0.01$).

The lack of response to AA in ryanodine-treated cells was not due to depletion of ER induced by ryanodine because CCh caused Ca^{2+} release from the ryanodine-treated cells (Fig. 6C). Moreover, the lack of response to AA in ryanodine-treated cells was not due to an interaction between AA and ryanodine in solution. This is evident from the fact that acute application of ryanodine without pre-treatment of cells, did not block $[Ca^{2+}]_c$ response to AA (Fig. 6D). It should be noted that cells pre-treated with caffeine alone (10 mM) for 45 min showed a normal response to AA (Fig. 6E).

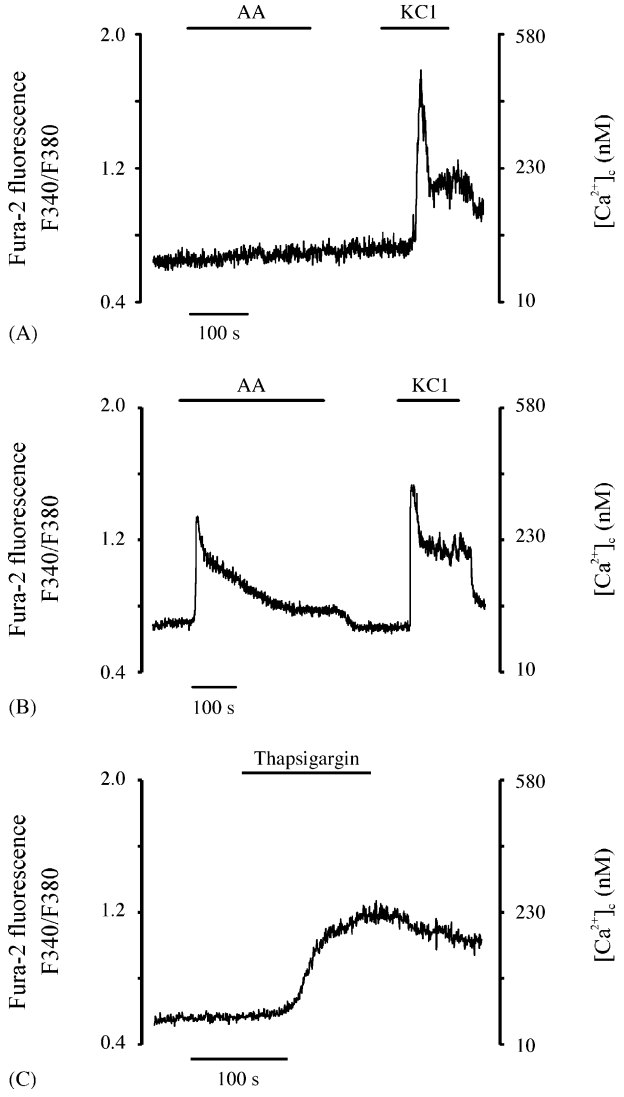

Fig. 4. AA released Ca^{2+} from the ER. (A) Effect of thapsigargin on AA. INS-1E cells were incubated with thapsigargin (500 nM) for 45 min prior to the application of AA (150 μM). Thapsigargin-treated cells responded normally to KCl (25 mM). (B) Control experiment where cells were not treated with thapsigargin. (C) Acute application of thapsigargin (500 nM) increased $[Ca^{2+}]_c$. Each trace is representative of three independent experiments.

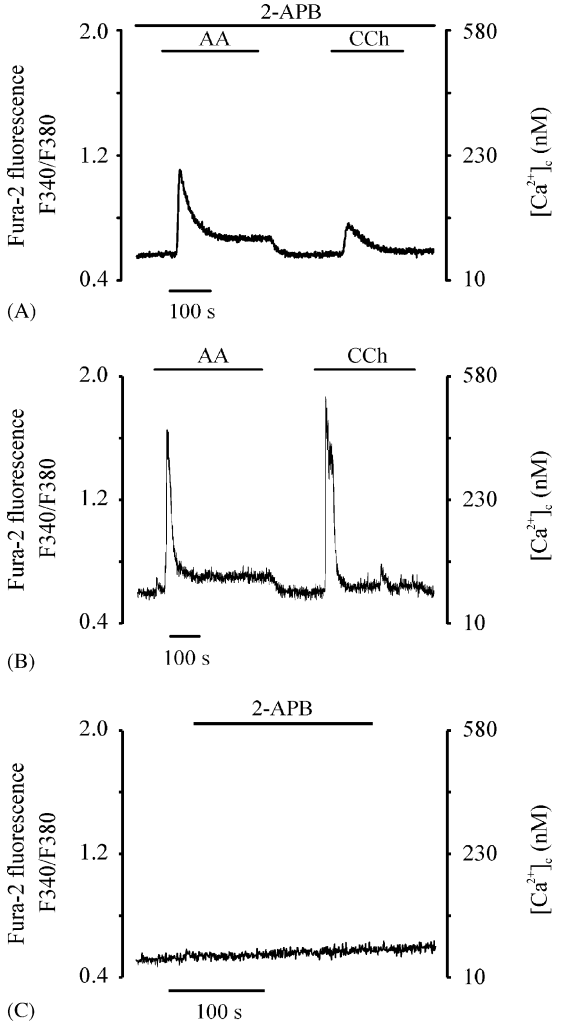

Fig. 5. AA did not release Ca^{2+} through IP_3Rs. The experiments show the effect of 2-APB, a blocker of IP_3Rs, on AA-induced Ca^{2+} response in INS-1E cells. (A) Cells were treated with 2-APB (50 μM) for 10 min during pre-incubation and during perfusion. The Ca^{2+} response to AA (150 μM) was not blocked by 2-APB but that to CCh (100 μM) was inhibited by ~85% ($P < 0.05$). (B) Control experiment shows Ca^{2+} response to CCh in the absence of 2-APB. (C) 2-APB itself (50 μM) did not increase $[Ca^{2+}]_c$. Traces are representative of five experiments in (A), four in (B), and three in (C).

The decrease of AA-induced Ca^{2+} response was greater in cells that were pre-treated with ryanodine plus caffeine, compared to cells that were treated with nimodipine or Ca^{2+}-free medium, as is shown in Fig. 6F. This could indicate that the increase of $[Ca^{2+}]_c$ by AA was mainly due to Ca^{2+} release from intracellular stores rather than due to Ca^{2+} entry across the plasma membrane.

Next, we tested the effect of AA on $[Ca^{2+}]_c$ in primary β-cells. The $[Ca^{2+}]_c$ changes obtained in primary β-cells in response to AA (150 μM) were similar in magnitude and pattern to those seen in INS-1E cells (Fig. 1B). Fifty micromolars of AA did not increase $[Ca^{2+}]_c$ in primary β-cells. Whereas 100 μM AA caused slight increase of $[Ca^{2+}]_c$ in two out of three INS-1E cells, it did not increase $[Ca^{2+}]_c$ in primary β-cells.

To determine whether AA released Ca^{2+} from RY receptors in primary β-cells, we used the same protocol to block RY receptors as in the case of INS-1E cells. Incubation of the cells with ryanodine (100 μM) and caffeine (10 mM) for 45 min, followed by ryanodine alone (50 μM) during perfusion, diminished the peak Ca^{2+} response to AA (150 μM) by 80% (Fig. 7A; cf., Fig. 7B). Taken together, the results obtained from the experiments with INS-1E cells and primary β-cells indicate that AA released Ca^{2+} from the ER by activating RY receptors.

Because it has been proposed that nitric oxide could mediate the AA-induced Ca^{2+} release in mouse parotid acini [20], we tested this possibility in pancreatic β-cells. We used L-NNA, a potent blocker of nitric oxide synthase. In the pres-

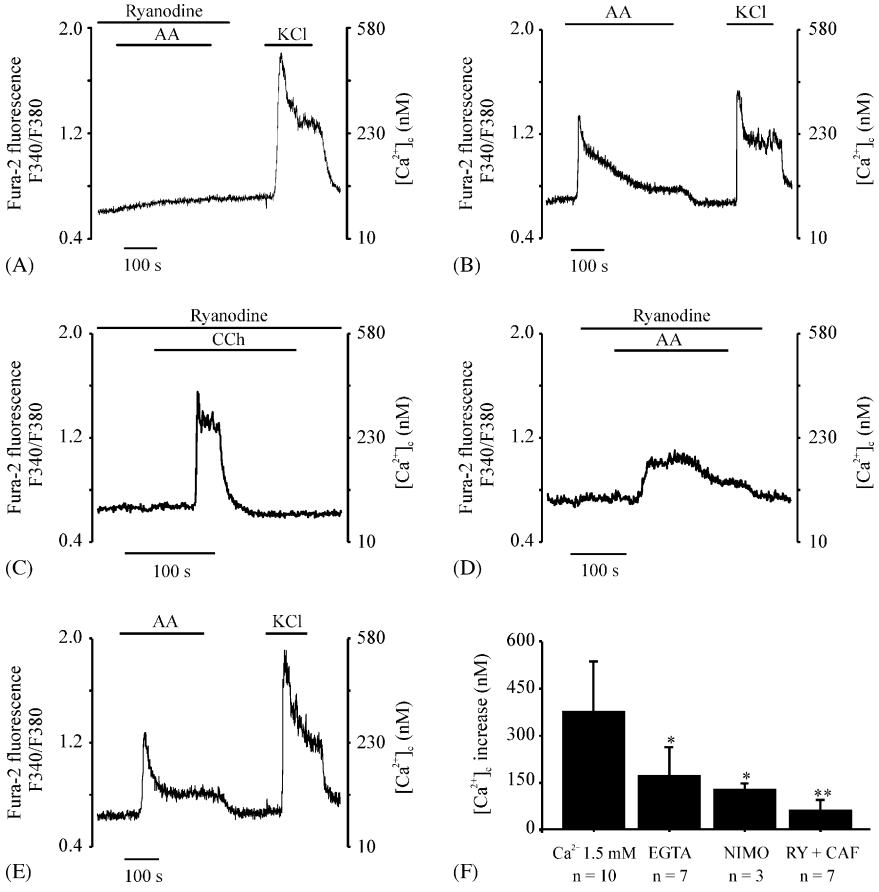

Fig. 6. AA released Ca^{2+} through RY receptors in INS-1E cells. (A) Complete inhibition of Ca^{2+} response to AA by ryanodine. Cells were incubated with ryanodine (100 μM) and caffeine (10 mM) for 45 min. Ryanodine alone (50 μM) was also present during perfusion. The trace is representative of four experiments. (B) Response to AA in cells not treated with ryanodine or caffeine ($n = 7$). (C) The effect of CCh was unaltered in cells treated with ryanodine under the same protocol as in (A). (D) Acute application of ryanodine (50 μM) did not prevent the increase of $[Ca^{2+}]_c$ by AA. (E) Pre-treatment with caffeine (10 mM) alone did not alter the response to AA. In these experiments, AA, CCh, and KCl were used at concentrations of 150, 100, and 25 mM, respectively. (F) Bars represent the mean ± S.D. of $[Ca^{2+}]_c$-increase over basal values (56 ± 22 nM) by AA (150 μM) in INS-1E cells treated by different compounds. NIMO = nimodipine; RY = ryanodine; CAF = caffeine. Marks indicate the P-values with respect to the control (left bar). $^{*}P < 0.005$; $^{**}P < 0.001$. Details about the experiments are specified in the text.

ence of L-NNA (2 mM), Ca^{2+} response to AA (150 μM) was not altered in INS-1E cells (Fig. 8A; cf., Fig. 8B). These results indicated that the effect of AA on RY receptors in β-cells was not mediated by nitric oxide.

4. Discussion

The main finding of this study is that AA released Ca^{2+} from the ER through RY receptors in intact pancreatic β-cells. Although previous studies demonstrated that AA increases $[Ca^{2+}]_c$ by releasing Ca^{2+} from intracellular stores in β-cells, the identity of the Ca^{2+} channels and that of the Ca^{2+} stores remained unknown. In this study, we demonstrate that AA released Ca^{2+} from the ER since the release was blocked by thapsigargin. Furthermore, AA released Ca^{2+} through RY receptors since the highly specific inhibitor of RY receptors, namely ryanodine, abolished the Ca^{2+} response to AA.

The concentrations of AA used in this study are within the range observed under the in vivo conditions because it has been estimated that the intracellular concentration of AA (unesterified) in β-cells can reach more than 200 μM when β-cells are exposed to 28 mM glucose [2]. One could argue that high concentration of glucose would empty the ER by formation of AA and would impair effect of subsequent application of exogenous AA. However, it has been demonstrated in previous studies that ER is not emptied [21], even when β-cells are repeatedly exposed to high concentrations of glucose [22]. It should be noted that glucose increases Ca^{2+} by closure of the K_{ATP} channel and thereby depolarizing the cell [1] and AA increases Ca^{2+} mainly by the release of Ca^{2+} from the ER (cf., Fig. 6F).

It has been described in a previous study that AA and its metabolites 5(s)-HPETE and 12(s)-HPETE inhibit the binding of ryanodine to RY receptors in sarcoplasmic reticulum vesicles [23]. However, the study did not demonstrate inhibition of the current through RY receptors by those com-

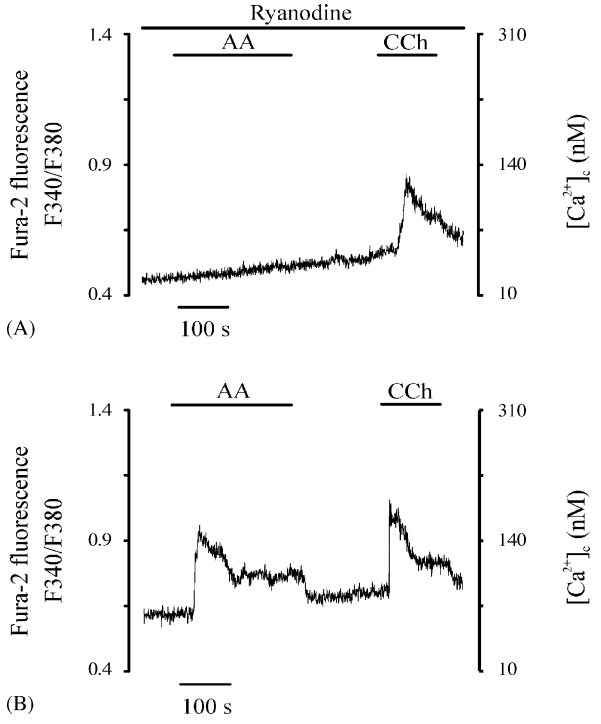

Fig. 7. AA released Ca^{2+} through RY receptors in primary pancreatic β-cells. (A) Cells ($n = 4$) were incubated with ryanodine (100 μM) and caffeine (10 mM) for 45 min. Ryanodine alone (50 μM) was present during perfusion. (B) Response to AA in cells not treated with ryanodine or caffeine ($n = 3$). Concentrations for AA and CCh were 150 and 100 μM, respectively. On the average, ryanodine diminished the maximum Ca^{2+} response to AA (150 μM) by 80%.

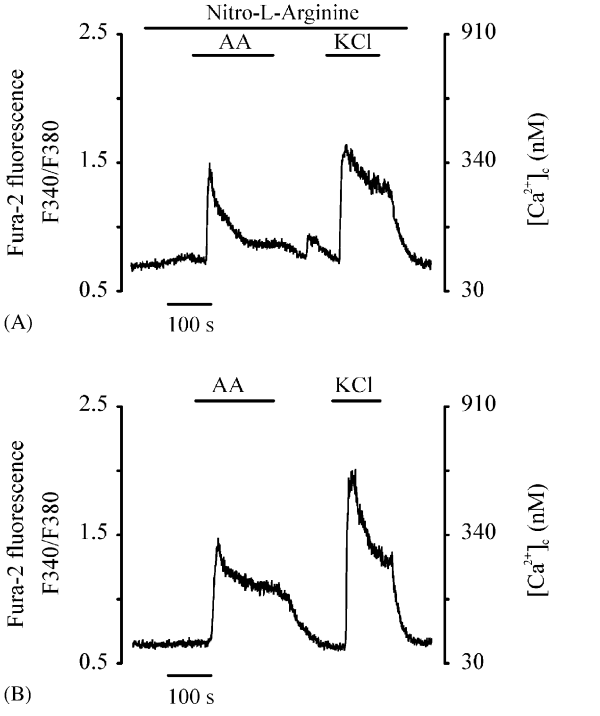

Fig. 8. AA-induced increase in $[Ca^{2+}]_c$ was not mediated by nitric oxide. (A) Blockade of nitric oxide production by L-NNA (2 mM) did not alter AA response in INS-1E cells. (B) Response to AA in absence of L-NNA. Concentrations for AA and KCl were 150 μM and 25 mM, respectively. Traces are representative of three experiments in each group.

pounds. Based on our results, the report that AA decreased the ryanodine binding could be explained by a competitive mechanism and not by inhibition of RY receptors. On the other hand, Striggow and Ehrlich reported that leukotriene B4, an AA metabolite, but not AA, does activate RY receptors in microsomes of cerebellum [24]. The reasons for such differences may lie in the fact that different cells have different isoforms of RY receptors [8]. Moreover, it should be noted that all of these previous studies were done using broken cell preparations. Here, we tested activation of RY receptors in their native environment in intact cells, an approach that allows study of the regulation of the channels under more physiological conditions.

Pancreatic β-cells express both IP_3Rs and RY receptors. In our study, Ca^{2+} release induced by AA was not blocked by 2-APB, a known blocker of IP_3Rs. One of the difficulties in interpreting results obtained with 2-APB is that the substance may block ER Ca^{2+} ATPases. However, it is unlikely that 2-APB inhibited the ER Ca^{2+} ATPase in our study, because at the concentration used to block IP_3Rs (50 μM), 2-APB did not increase $[Ca^{2+}]_c$ by itself, whereas thapsigargin did. In fact, the EC50 of 2-APB for blocking ER Ca^{2+} ATPases has been reported to be ~300 μM in intact cells [25], and ~90 μM in permeabilized cells [26]. Our findings indicate that Ca^{2+} release induced by AA was not due to the formation of 1,4,5-inositol-triphosphate neither was it due to activation of IP_3Rs.

On the other hand, ryanodine significantly inhibited Ca^{2+} response to AA (Fig. 6A). Inhibition of RY receptors was not due to a depletion of the Ca^{2+} store by ryanodine (Fig. 6C), neither was it due to an interaction between AA and ryanodine in solution (Fig. 6D). It should be noted that we have used ryanodine to block the RY receptors in a use-dependent manner. Thus, we used a protocol where the channels were first activated by caffeine to allow ryanodine to bind to the open channels. Such careful attention to protocols is essential to obtain inhibition of RY receptors by ryanodine [27].

It is known that the G-protein-coupled receptor GPR40 is activated by different free fatty acids [28–30]. It has been reported that palmitate increases $[Ca^{2+}]_c$ in β-cells from $GPR40^{+/+}$-mice but not from $GPR40^{-/-}$-mice [31]. GPR40 is postulated to be linked to the 1,4,5-inositol-triphosphate pathway [28]. Since our results demonstrated that AA-induced Ca^{2+} release is a phenomena independent of 1,4,5-inositol-triphosphate formation, GPR40 is unlikely to mediate AA-induced Ca^{2+} release.

In different kinds of cells, it has been found that AA metabolites increased $[Ca^{2+}]_c$ by activation of Ca^{2+} influx from extracellular medium [32–34] and release of Ca^{2+} from intracellular stores [35]. To test whether the increase of $[Ca^{2+}]_c$ in pancreatic β-cells was due to AA itself or due to its metabolites, we used a non-metabolizable analogue of

AA, ETYA. This compound increased $[Ca^{2+}]_c$ to the same extent as that obtained with AA, suggesting that Ca^{2+} increase was mediated by AA itself. Other authors have reported that AA rather than its metabolites can induce Ca^{2+} release [2,6,36,37]. It has been postulated that nitric oxide could mediate the effect of AA on RY receptors [20]. We tested L-NNA, an inhibitor of nitric oxide synthase, and we found that Ca^{2+} response to AA response was not altered by L-NNA (Fig. 8A and B), suggesting that AA activates RY receptors directly rather than via production of nitric oxide.

We found that AA not only released Ca^{2+} from the ER but also induced Ca^{2+} entry from the extracellular medium. Nimodipine diminished both the initial rapid phase (Fig. 2A) and the sustained phase of $[Ca^{2+}]_c$-increase by AA (Fig. 2C). We found that nimodipine decreased by 65% the peak increase of $[Ca^{2+}]_c$ induced by AA. This indicates that the initial response to AA is due to Ca^{2+} release (through the RY receptors) and Ca^{2+} entry (through the L-type voltage-gated Ca^{2+} channels). However, it is unlikely that AA directly activated L-type Ca^{2+} channels. Instead, our interpretation of this finding is that activation of RY receptors by AA leads to activation of plasma membrane currents which in turn leads to membrane depolarization and thereby activation of L-type Ca^{2+} channels. This explanation is based on our previous observations that activation of RY receptors leads to membrane depolarization in pancreatic β-cells [38].

In summary, we provided evidence that in intact rat insulinoma cells and in primary β-cells AA releases Ca^{2+} from the ER by activating RY receptors. Previous studies have already established that glucose stimulation increases AA concentrations in β-cells and that both AA and RY receptors are involved in insulin secretion. Taken together, these results indicate that AA is a physiological activator of RY receptors in β-cells, and that it could also be the activator of RY receptors in other types of cells. Further studies are needed to establish whether activation of RY receptors by AA is a property limited to the novel β-cell isoform of RY receptor or shared by other isoforms too.

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