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Ca^{2+}-induced Ca^{2+} Release from Internal Stores in INS-1 Rat Insulinoma Cells

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The secretion of insulin from pancreatic β-cells is triggered by the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. The resulting elevation of intracellular calcium ($[Ca^{2+}]_i$) triggers additional Ca^{2+} release from internal stores. Less well understood are the mechanisms involved in Ca^{2+} mobilization from internal stores after activation of Ca^{2+} influx. The mobilization process is known as calcium-induced calcium release (CICR). In this study, our goal was to investigate the existence of and the role of caffeine-sensitive ryanodine receptors (RyRs) in a rat pancreatic β-cell line, INS-1 cells. To measure cytosolic and stored Ca^{2+}, respectively, cultured INS-1 cells were loaded with fura-2/AM or furaptra/AM. $[Ca^{2+}]_i$ was repetitively increased by caffeine stimulation in normal Ca^{2+} buffer. However, peak $[Ca^{2+}]_i$ was only observed after the first caffeine stimulation in Ca^{2+} free buffer and this increase was markedly blocked by ruthenium red, a RyR blocker. KCl-induced elevations in $[Ca^{2+}]_i$ were reduced by pretreatment with ruthenium red, as well as by depletion of internal Ca^{2+} stores using cyclopiazonic acid (CPA) or caffeine. Caffeine-induced Ca^{2+} mobilization ceased after the internal stores were depleted by carbamylcholine (CCh) or CPA. In permeabilized INS-1 cells, Ca^{2+} release from internal stores was activated by caffeine, Ca^{2+}, or ryanodine. Furthermore, ruthenium red completely blocked the CICR response in permeabilized cells. RyRs were widely distributed throughout the intracellular compartment of INS-1 cells. These results suggest that caffeine-sensitive RyRs exist and modulate the CICR response from internal stores in INS-1 pancreatic β-cells.

Key Words: INS-1, Caffeine, Ryanodine, Calcium release, CICR

INTRODUCTION

Elevation of the intracellular free calcium concentration ($[Ca^{2+}]_i$) is a key event in insulin secretion by pancreatic β-cells. Increased glucose fueling, a major stimulus to β-cells, ultimately results in elevation of the ATP/ADP ratio, which causes plasma membrane depolarization through the closing of ATP-sensitive K^+ channels (K_{ATP}), and in turn stimulates Ca^{2+} entry through the opening of voltage-gated Ca^{2+} channels (Ca_V), which are located on plasma membranes [1-3]. Increases in $[Ca^{2+}]_i$ due to sequential activation of K_{ATP} and Ca_V channels triggers Ca^{2+} release from the endoplasmic reticulum (ER), an internal Ca^{2+} store, and this process is called calcium-induced calcium release (CICR) [4-6]. Mobilization of Ca^{2+} from internal stores can result from the opening of Ca^{2+} channels on the ER membranes such as inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) or ryanodine receptors (RyRs) [7-10]. While it is well established that $InsP_3R$-mediated Ca^{2+} release is operative in insulin secreting β-cells, the presence and role of RyRs in pancreatic β-cell signaling remains controversial.

RyRs are named because a plant alkaloid, ryanodine, binds to this channel. They are expressed abundantly in muscle cells and neurons. A critical property of RyRs is that increased cytosolic Ca^{2+}, cyclic ADP ribose, or nicotinic acid adenine dinucleotide phosphate (NAADP) can activate this channel physiologically, resulting in even greater amplification of Ca^{2+} signals elicited by Ca^{2+} entry or release [11-13]. Another key property of RyRs is that caffeine at millimolar concentrations can pharmacologically cause channel opening. In some studies, caffeine did not activate Ca^{2+} release from the internal stores of β-cells [14,15], whereas Ca^{2+} release from the internal stores was activated if caffeine-sensitive RyRs were present in intact β-cells [16,17]. In fact, the presence of RyRs in insulin secreting β-cells is well-accepted at the present time, although the expression of each type of RyRs is variable, depending on the species studied. The mRNAs for the three types of RyRs have been detected in human β-cells, whereas mRNA for RyR2 but not RyR1 was detected in INS-1 cells and rat islets using RT-PCR methods [18-20]. Since there is still no clear consensus on the role of RyR in stimulus-secretion coupling in β-cells, we determined, using in-

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ABBREVIATIONS: $[Ca^{2+}]_i$, intracellular calcium concentration; CICR, calcium-induced calcium release; RyRs, ryanodine receptors; CPA, cyclopiazonic acid; CCh, carbamylcholine.

sulin secreting rat pancreatic β-cells (INS-1 cells), whether caffeine mobilizes Ca^{2+} from internal stores, and if Ca^{2+} that has entered into the cell by membrane depolarization can mobilize stored Ca^{2+} through caffeine-sensitive RyR opening in ER membranes. From the results, here we report that caffeine-sensitive RyRs exist on the membrane of the internal Ca^{2+} stores and participate in the CICR response to entered Ca^{2+} in INS-1 rat pancreatic β-cell.

METHODS

Cell culture

INS-1 cells, a rat insulinoma cell line, were grown in RPMI1640 medium containing 11.1 mM glucose supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), 0.6 mM sodium bicarbonate, 2 mM L-glutamate, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM β-mercaptoethanol. The cells were subcultured every 7 days and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were performed with cells in passages 30~50. To stabilize them after Ca^{2+}-sensitive dye loading, cells were resuspended in HEPES-buffered physiological saline solution (HEPES-PSS) containing 2.5 mM glucose, 137 mM NaCl, 0.56 mM $MgCl_2$, 4.7 mM KCl, 1 mM Na_2HPO_4, 10 mM HEPES (pH 7.4), 1.28 mM $CaCl_2$, and 1% (w/v) bovine serum albumin.

Cytosolic Ca^{2+} measurement in intact cells

For measurement of $[Ca^{2+}]_i$, cultured INS-1 cells were loaded with 2 μM fura-2/AM, a Ca^{2+}-sensitive dye, for 30 min at room temperature. Fura-2 loaded cells were mounted on a glass coverslip at the bottom of perfusion chambers. Cells were continuously superfused with HEPES-PSS at a flow rate of 1 ml/min using an electronically controlled perfusion system (Warner Instrument, Hamden, CT, USA). Cytosolic Ca^{2+} imaging was performed using an inverted Olympus IX71 microscope through a 40× fluorescence objective lens. Cells were excited alternately with light at 340 nm and 380 nm, using a Polychrome V monochrometer (Till Photonics, Pleasanton, CA, USA). Fluorescence images were captured at an emitted wavelength of 510 nm using a cooled charged-coupled device, Cool-SNAP HQ2 camera (Photometrics, Tucson, AZ, USA).

Measurement of stored Ca^{2+} in permeabilized cells

INS-1 cells were loaded with 10 μM furaptra/AM for 30 min at room temperature. Cells were permeabilized by superfusion with 20 μM β-escin for 1~2 min in intracellular medium (ICM) containing 125 mM KCl, 19 mM NaCl, 10 mM HEPES, and 1 mM EGTA (pH 7.3) as described previously [21,22]. Permeabilized cells were washed in ICM without β-escin for 15 min to facilitate removal of cytosolic dye. Cells were superfused in intracellular medium containing 0.650 μM $CaCl_2$ (free $[Ca^{2+}]$=200 nM), 1.4 mM $MgCl_2$, and 3 mM Na_2ATP to activate sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and to load intracellular Ca^{2+} stores. Prior to activation of stored calcium release, cells were superfused without $MgCl_2$ to inactivate SERCA. The free Ca^{2+} concentration was changed from 0 to 10 μM according to the experimental maneuvers. We recorded fluorescence emission above 505 nm following excitation at 340 nm and 380 nm using a TILL Photonics imaging system.

Immunocytochemistry

To determine the distribution of RyRs, immunocytochemistry was performed. Cultured INS-1 cells on a coverslip were fixed with 2% formaldehyde-PBS. After fixation, cells were permeabilized with 0.2% Triton X-100 for 5 min. Nonspecific binding was blocked by 1 hr incubation in 10% rabbit serum medium. Immunocytochemistry was done using a polyclonal RyR (N-19) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Cy-2 conjugated rabbit anti-goat secondary antibody (Jackson Immuno-Research, PA, USA). After overnight incubation in primary antibody (1 : 100 dilution) at 4°C, cells were incubated with secondary antibody (1 : 100 dilution) for 1 hr at 37°C. Immunofluorescence images for RyRs were collected using a confocal microscope (Carl Zeiss, Germany), and processed using Photoshop 7.0 software (Adobe, Mountain View, CA, USA).

Drugs

To activate calcium mobilization in intact cells, 30 mM caffeine, 10 μM carbamylcholine (CCh), or 45 mM KCl was added to HEPES-PSS. To activate calcium release from internal stores in permeabilized cells, 10 mM caffeine, 10 μM calcium or 1 μM ryanodine was added to ICM. Carbamylcholine, caffeine, ruthenium red, β-escin, and other chemicals for making buffers were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Ryanodine and cyclopiazonic acid (CPA), a SERCA inhibitor, were purchased from Calbiochem (San Diego, CA, USA). Fura 2/AM and furaptra/AM were purchased from TefLabs Inc. (Austin, TX, USA). RPMI 1640, fetal bovine serum (FBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA).

Statistical analysis

Results are presented as mean±S.E. Data were analyzed using the Student *t*-test. Rates of Ca^{2+} release were estimated from these responses by fitting the initial 10 sec period of decreasing fluorescence to a single exponential function using the Origin program as described previously [21]. Differences were considered significant when the p value was less than 0.05.

RESULTS

Caffeine mobilizes calcium from internal stores in intact INS-1 cell

First, to detect the presence of RyRs, we determined them functionally by testing the effects of 30 mM caffeine, a RyR activator, on Ca^{2+} mobilization in intact INS-1 cells. In the presence of extracellular Ca^{2+}, repetitive caffeine perfusion resulted in reiterative elevation of $[Ca^{2+}]_i$ even if those responses were slightly reduced (Fig. 1A). However, in Ca^{2+} free solution, INS-1 cells only responded to the first caffeine stimulation, and this response was transient (Fig. 1B). $[Ca^{2+}]_i$ peaks were not significantly different from each other when the same cells were stimulated with caffeine in the presence

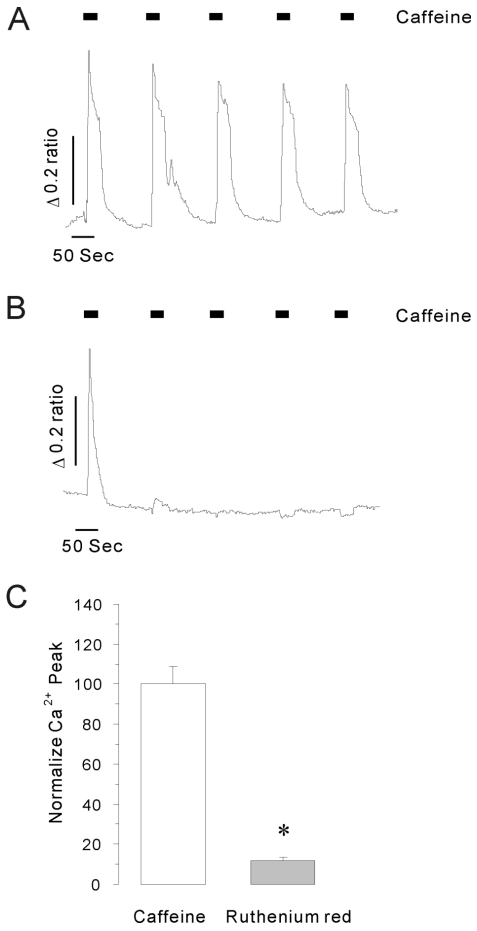

Fig. 1. Caffeine stimulated calcium mobilization from internal stores in intact INS-1 cells. The representative traces show the effects of repetitive 30 mM caffeine stimulation on $[Ca^{2+}]_i$ increases in the presence (A) and absence (B) of extracellular Ca^{2+}. The data were obtained from 5 and 7 separate experiments, respectively. INS-1 cells were responsive to repetitive caffeine stimulation in normal extracellular Ca^{2+} buffer, but only responded to the first caffeine stimulation in Ca^{2+} free solution. (C) A 50 μM of ruthenium red markedly reduced the $[Ca^{2+}]_i$ peak in the absence of extracellular Ca^{2+}. Data were normalized to control values and expressed as mean %±S.E. Asterisk indicates the value is significantly different from the corresponding value of caffeine alone ($p<0.05$).

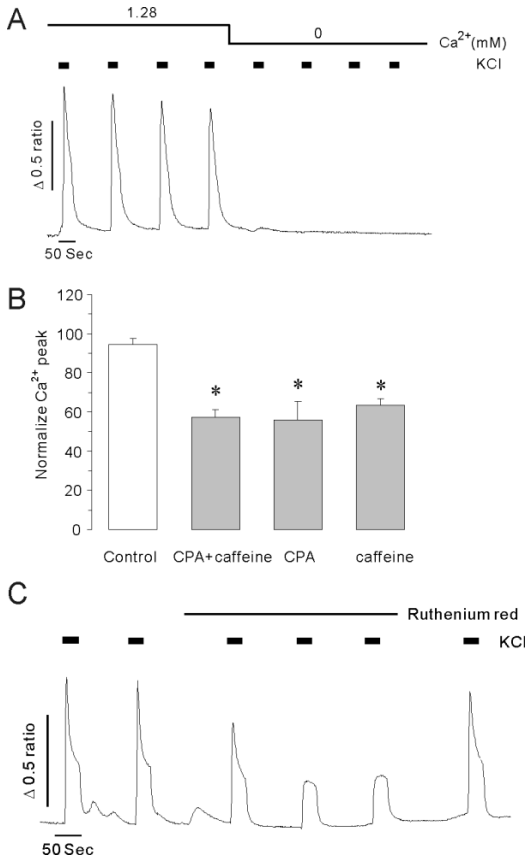

Fig. 2. KCl triggered Ca^{2+} release from internal stores in intact INS-1 cells. (A) The representative trace shows the effect of 45 mM KCl on $[Ca^{2+}]_i$ increases in the presence and absence of extracellular Ca^{2+}. The data were obtained from 6 separate experiments. $[Ca^{2+}]_i$ elevation was not observed in Ca^{2+} free medium. (B) Effects of CPA plus caffeine, CPA alone or caffeine alone on KCl-induced $[Ca^{2+}]_i$ peaks in the presence of extracellular Ca^{2+}. The data were obtained from at least 5 separate experiments. Data were normalized to the initial $[Ca^{2+}]_i$ peak and expressed as mean % ± S.E. Asterisks indicate that the values are significantly different from the corresponding value for control ($p<0.05$). Intracellular Ca^{2+} store depletion reduced depolarization-induced Ca^{2+} mobilization. (C) Representative trace shows the effect of ruthenium red on KCl-induced $[Ca^{2+}]_i$ elevations. The data were obtained from 6 separate experiments. A 50 μM of ruthenium red significantly reduced depolarization-induced Ca^{2+} mobilization; the effect was restored after washout of the ruthenium red.

and the absence of extracellular Ca^{2+} (data not shown). These results mean that caffeine can mobilize Ca^{2+} from internal stores without the presence of extracellular Ca^{2+}, and can easily deplete internal Ca^{2+} stores by repetitive stimulation. To determine the involvement of RyRs, we added ruthenium red, a RyRs blocker, to the perfusion medium. Ruthenium red markedly reduced the caffeine-induced $[Ca^{2+}]_i$ peak to 11.60±2.09% of control value in the absence of extracellular Ca^{2+} (Fig. 1C). These results suggest that caffeine can mobilize Ca^{2+} from internal stores through RyRs in intact INS-1 cells.

Depolarization-induced calcium entry triggers calcium release from internal stores

Next, the experiments were performed to clarify whether membrane depolarization can mobilize Ca^{2+} from internal stores through RyRs in INS-1 cells. A 45 mM KCl solution was perfused to mimic membrane depolarization. The repetitive depolarization by KCl perfusion resulted in a constant $[Ca^{2+}]_i$ rise in normal Ca^{2+} buffer; the response ceased when we changed to a Ca^{2+} free solution (Fig. 2A). However, INS-1 cells did not respond to KCl in the absence of extracellular Ca^{2+}. These results mean that KCl initially mobilizes Ca^{2+} from extracellular spaces *via* voltage-operated Ca^{2+} channels. To confirm whether the Ca^{2+} that entered due to depolarization can trigger Ca^{2+} release from internal stores, we examined the effects of KCl in the store-depleted condition by pretreatment cells with cyclopiazonic acid (CPA), a sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, or caffeine, a RyRs

activator. KCl-induced $[Ca^{2+}]_i$ mobilization was significantly reduced by pretreatment of cells with CPA plus caffeine (57.26±3.88% of control), CPA alone (55.96±9.42% of control) or caffeine alone (63.41±3.41% of control) in intact INS-1 cells (Fig. 2B). Similar to the effects of store depletion, ruthenium red markedly inhibited the KCl-induced $[Ca^{2+}]_i$ peak to 68.45±5.23% of control in the first stimulation (Fig. 2C). These results suggest that ryanodine-sensitive Ca^{2+} release from internal stores participates in the depolarization-induced Ca^{2+} mobilization in INS-1 insulinoma cells.

Caffeine and carbamylcholine activate the same intracellular calcium stores

We tested whether RyRs and $InsP_3Rs$ were expressed on the same membranes of intracellular Ca^{2+} stores. Pretreatment with carbamylcholine (CCh), a well known $InsP_3$ inducer, resulted in Ca^{2+} store depletion in Ca^{2+} free medium. After the store depletion by CCh, caffeine failed to elevate $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Fig. 3A). Similarly, when internal stores were depleted by pretreatment with caffeine, CCh did not elevate $[Ca^{2+}]_i$ (Fig. 3B). This phenomenon was also observed if caffeine perfused cells that had been store-depleted by pretreatment with CPA (Fig. 3C). The above results suggest that RyRs and $InsP_3Rs$ activate the same internal Ca^{2+} stores or at least are functionally cross-linked to Ca^{2+} release in INS-1 cells.

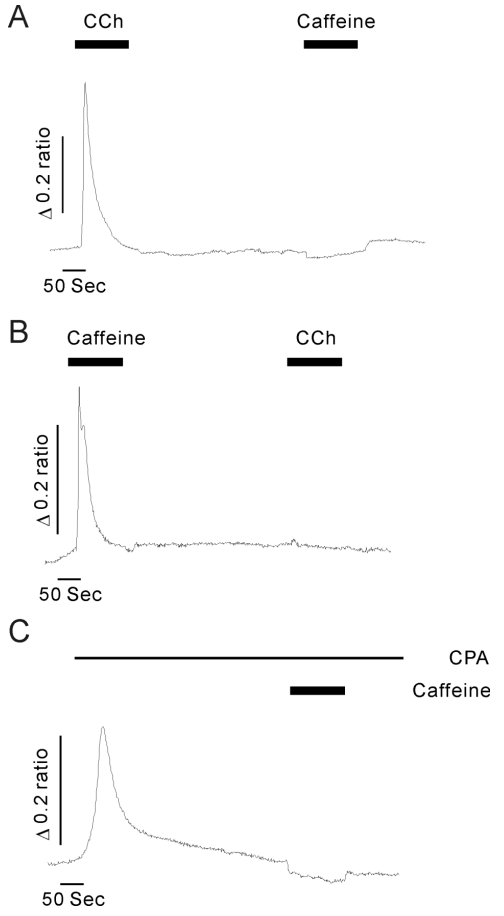

Fig. 3. Effects of internal calcium store depletion on caffeine-induced calcium release. (A) The representative trace shows the 30 mM caffeine-induced $[Ca^{2+}]_i$ rise after internal store depletion by 10 μM carbamylcholine (CCh) in Ca^{2+} free solution. (B) The representative trace shows the 10 μM CCh-induced $[Ca^{2+}]_i$ rise under store depleted conditions induced by pretreatment with 30 mM caffeine in the absence of extracellular Ca^{2+}. (C) A representative trace of the caffeine effect under store depleted condition induced by pretreatment of cells with 10 μM cyclopiazonic acid (CPA) in Ca^{2+} free solution. All data were obtained from at least 5 separate experiments. Caffeine failed to increase $[Ca^{2+}]_i$ after internal Ca^{2+} store depletion induced by pretreatment with CCh or CPA.

Stored calcium is released by caffeine, calcium, and ryanodine in permeabilized INS-1 cells

We employed luminal Ca^{2+} measurements of ER to examine direct effects of RyRs activation on Ca^{2+} release in permeabilized INS-1 cells. Perfusion of 10 mM caffeine, 10 μM free Ca^{2+}, or 1 μM ryanodine dramatically initiated Ca^{2+}

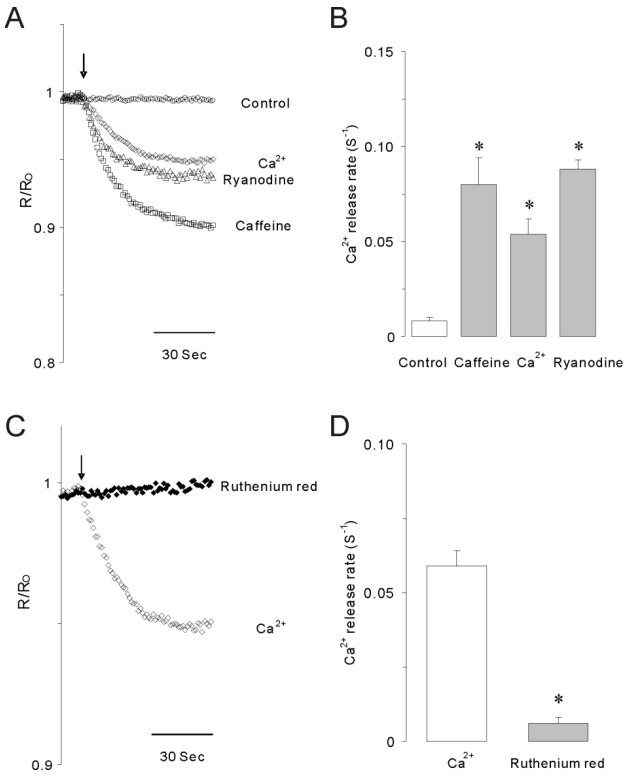

Fig. 4. Caffeine, Ca^{2+} or ryanodine-induced calcium release in permeabilized INS-1 cells. (A) 10 mM caffeine (□), 10 μM Ca^{2+} (◇) or 1 μM ryanodine (△) significantly stimulated Ca^{2+} release from internal stores in permeabilized INS-1 cells. Arrows indicate the starting point of each drug perfusion. (B) Summarized Ca^{2+} release rates (S^{-1}) induced by caffeine, Ca^{2+} or ryanodine. Data were summarized from at least 5 experiments. Asterisks indicate that the values are significantly different from the corresponding value for control ($p<0.05$). (C) The blocking effect of 50 μM ruthenium red on 10 μM Ca^{2+}-induced Ca^{2+} release in permeabilized INS-1 cells. (D) Summarized data showing the effects of ruthenium red on Ca^{2+}-induced Ca^{2+} release rates in permeabilized cells. Asterisk indicates that the value is significantly different from the corresponding value of Ca^{2+} ($p<0.05$). Ca^{2+} release induced by elevated Ca^{2+} was completely blocked by ruthenium red, a RyR blocker, in permeabilized INS-1 cells.

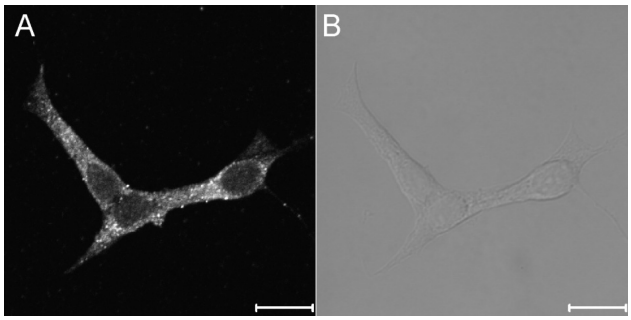

Fig. 5. The expression of ryanodine receptors in INS-1 rat insulinoma cells. The fluorescence image (A) and the bright image (B) show the expression and distribution of RyRs in the intracellular compartments. The images were obtained from 4 separate experiments. Immunocytochemistry was done using primary RyR antibody as described under experimental procedures. The scale bar is 10 μm.

release from internal stores (Fig. 4A). As shown in Fig. 4B, calcium release rates (S^{-1}) were 0.080±0.014, 0.059± 0.005, and 0.088±0.005 by perfusion of caffeine, Ca^{2+}, or ryanodine, respectively, which were markedly different from the value for control cells (0.008±0.002). Ca^{2+}-induced Ca^{2+} release (CICR) from ER was completely blocked by ruthenium red in permeabilized INS-1 cells (Fig. 4C). Ca^{2+} release rate was reduced to 0.006±0.002 by pretreatment with ruthenium red (Fig. 4D). These results strongly indicate that caffeine-sensitive RyRs modulate Ca^{2+} signals through a CICR response from the ER of INS-1 cells.

Cellular distribution of RyRs in INS-1 cells

We utilized immunocytochemical imaging to analyze cellular expression and distribution of RyRs. As shown in Fig. 5, RyRs were strongly expressed and widely distributed in intracellular compartment, and no immunoreactive signals were detected in the nuclear compartment. We clearly confirmed the existence of RyRs in the intracellular organelle of INS-1 cell.

DISCUSSION

The obvious findings of the present study, which used immunocytochemistry and calcium imaging, are that caffeine-sensitive RyRs are expressed in the subcellular compartments and that depolarization-induced Ca^{2+} entry triggers RyR-mediated CICR responses in INS-1 insulinoma cells. There are two main families of intracellular Ca^{2+} channels, $InsP_3Rs$ and RyRs in insulin secreting β-cells [7-10]. Among these channels, the critical properties of RyR are that cytosolic Ca^{2+} physiologically and caffeine pharmacologically can activate this channel [11]. In the current study, we determined the role of RyRs on Ca^{2+} mobilization in the INS-1 insulinoma cell line. To stimulate RyRs, we used caffeine, which is known to increase both the open time and the open probability of RyRs in a cooperative manner with Ca^{2+} [23]. Caffeine at 30 mM markedly stimulated Ca^{2+} mobilization in both normal and Ca^{2+} free medium of intact INS-1 cells, while failed to increase $[Ca^{2+}]_i$ after repetitive stimuli without the extracellular presence of Ca^{2+} because of internal Ca^{2+} store depletion. In the absence of external Ca^{2+}, ruthenium red, a RyR blocker, completely inhibited the caffeine-induced initial rise in $[Ca^{2+}]_i$. In some studies, caffeine increased cytosolic Ca^{2+} through activation of voltage-gated Ca^{2+} channels (Ca_V) [14,16]. However, in our study, caffeine still elicit Ca^{2+} mobilization in the absence of extracellular Ca^{2+}, and these increases were blocked by ruthenium red in intact INS-1 cells. In addition, 10 mM caffeine and 1 μM ryanodine directly released stored Ca^{2+} from ER in permeabilized INS-1 cells. These results provide evidence that caffeine induces a rise in $[Ca^{2+}]_i$ through Ca^{2+} release from RyR-sensitive internal stores. Cytosolic Ca^{2+} is known to have a dual effect on RyRs; nanomolar to micromolar concentrations of Ca^{2+} increase but millimolar concentrations decrease the open probability of RyRs [24-26]. Similar to caffeine and ryanodine, 10 μM of Ca^{2+} clearly released stored Ca^{2+}, and this CICR was completely blocked by ruthenium red in permeabilized cells. Some studies, however, failed to detect caffeine-sensitive RyR in permeabilized β-cells despite successful detection of Ca^{2+} release from the ER of intact cells [14-16]. These discrepancies may be due to differences in methods used for the permeabilization, which can cause the loss of regulatory molecules. In our study, we detected caffeine-sensitive Ca^{2+} release in both intact and permeabilized cells, which indicate that caffeine-sensitive RyRs are present and are fully operational in INS-1 cells.

The depolarization of plasma membranes for activation of voltage-gated Ca^{2+} channels (Ca_V) is one of the most critical events leading to a rise in $[Ca^{2+}]_i$ and insulin secretion in β-cell [1-3,27,28]. The depolarization-induced increase in $[Ca^{2+}]_i$ is not considered as being due just to Ca^{2+} entry through Ca_V. There is some evidence that Ca^{2+} signals can be modulated by CICR though Ca^{2+} release from the ER in β-cells [29,30]. One important function of CICR in β-cells is to amplify Ca^{2+} signaling for insulin secretion. To clarify the involvement of the CICR response, we evaluated KCl-induced Ca^{2+} mobilization from internal stores that have been depleted. Pretreatment of CPA, caffeine, or a combination of both resulted in a substantial reduction in depolarization-induced $[Ca^{2+}]_i$ peaks in intact INS-1 cells. In this condition, ER was completely depleted by SERCA inhibition by CPA and by pre-activation of RyRs by caffeine. These results indicate that Ca^{2+} has entered into the cells by membrane depolarization through Ca_V triggers additional Ca^{2+} mobilization from internal Ca^{2+} stores. $InsP_3Rs$ are also involved in the CICR response of insulin secreting β-cells [7,8,30]. Therefore, we used ruthenium red to distinguish RyR-mediated CICR responses from $InsP_3R$-mediated responses. A blockage of RyRs by ruthenium red significantly reduced depolarization-induced elevations in $[Ca^{2+}]_i$ in normal Ca^{2+} buffer, whereas ruthenium red had no effect on CCh-induced Ca^{2+} mobilization, which is mediated by $InsP_3Rs$ (data not shown). As mentioned above, the CICR response was completely attenuated by pretreatment with ruthenium red in permeabilized cells. This does not mean that the CICR response is fully mediated by RyRs. Actually, two types of Ca^{2+} channels are differently involved in responses to increased cytosolic Ca^{2+}. RyRs can be directly activated by cytosolic Ca^{2+}, but $InsP_3Rs$ can be potentiated by Ca^{2+} in the presence of cytosolic $InsP_3$ [24,31]. In permeabilized conditions, $InsP_3Rs$, therefore, might not be operative due to the loss of cytosolic $InsP_3$ because the plasma membrane became permeable to this molecule. Based on these results, we suggest that the Ca^{2+} that entered through Ca_V can trigger additional Ca^{2+}

release from RyR-sensitive internal stores in INS-1 cells.

One of the important issues at the present time is whether two different Ca^{2+} release channels regulate the same internal Ca^{2+} stores. Some studies have demonstrated that there is an $InsP_3$-insensitive Ca^{2+} pool in the ER that is responsive to ryanodine agonists in RINm5F cells and mouse β-cells, and it was proposed that $InsP_3Rs$ and RyRs did not regulate the same internal Ca^{2+} stores [32-34]. On the other hand, in our study, we demonstrated that caffeine had no effect after ER depletion by CPA pretreatment, and after $InsP_3R$ activation by CCh pretreatment in Ca^{2+} free medium. Moreover, pretreatment with caffeine completely eliminated the CCh-induced increase in $[Ca^{2+}]_i$. Although we did not clearly confirm the co-localization of $InsP_3R$ and RyR on ER membranes, unfortunately, our results do show that $InsP_3Rs$ and RyRs regulate the same internal Ca^{2+} stores, or at least they are functionally cross-linked. One critical role of the CICR in β-cells is that it amplifies Ca^{2+}-dependent insulin exocytosis. Further studies are now needed to clarify the roles of $InsP_3Rs$ and RyRs based on localized Ca^{2+} signals, because CICR generates large local Ca^{2+} transients [35], and their functions depend on subcellular localization of the receptors.

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