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Monitoring of Ca^{2+} Release from Intracellular Stores in Permeabilized Rat Parotid Acinar Cells Using the Fluorescent Indicators Mag-fura-2 and Calcium Green C_{18}

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The operation of intracellular Ca^{2+} stores in saponin-permeabilized rat parotid acinar cells was studied by monitoring the Ca^{2+} concentration within organelles loaded with the low affinity Ca^{2+} indicator Mag-fura-2. Inositol 1, 4, 5-trisphosphate ($InsP_3$) caused a decrease in the Mag-fura-2 ratio in a dose-dependent manner, and this effect was reversed by a removal of $InsP_3$ or by an addition of the $InsP_3$ receptor antagonist heparin. The changes in Mag-fura-2 ratio indicate the Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores and Ca^{2+} re-uptake into the stores in permeabilized acinar cells. The decrease in Mag-fura-2 ratio induced by $InsP_3$ was observed at all regions of the acinar cells, suggesting that the $InsP_3$-sensitive Ca^{2+} stores are located throughout the cells. The $InsP_3$-induced Ca^{2+} release was also monitored using the membrane-bound Ca^{2+} indicator Calcium Green C_{18} which is sensitive to the changes in Ca^{2+} concentration immediately adjacent to the membrane of intracellular Ca^{2+} stores. $InsP_3$ caused a large increase in the Calcium Green C_{18} fluorescence reflecting Ca^{2+} release from the stores. The Ca^{2+} pump inhibitor thapsigargin (ThG) itself had little or no effect on the Mag-fura-2 ratio or Calcium Green C_{18} fluorescence, but combined application of ThG with a low concentration of $InsP_3$ evoked a significant decrease in the Mag-fura-2 ratio. This result supports the hypothesis that the ThG-induced Ca^{2+} release is due to $InsP_3$-sensitive Ca^{2+} release which is mediated by the resting levels of $InsP_3$. Further, none of cyclic ADP-ribose, caffeine or ryanodine changed the Mag-fura-2 ratio and Calcium Green C_{18} fluorescence, leading to the assumption that the ryanodine-sensitive Ca^{2+} stores are minor in rat parotid acinar cells.

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The use of Ca^{2+}-sensitive fluorescent indicators such as fura-2 to measure the cytoplasmic Ca^{2+} concentration in living cells has provided much information about intracellular Ca^{2+} signaling. The Ca^{2+}-indicators can accumulate into intracellular organelles under appropriate loading conditions, and it has been attempted to show the function and distribution of intracellular Ca^{2+} stores by directly monitoring Ca^{2+} concentrations within the indicator-loaded organelles in permeabilized cells [1–7]. Rat parotid acinar cells have been extensively used as a model for the study of Ca^{2+} signaling in non-excitable exocrine cells. We loaded the low affinity Ca^{2+} indicator Mag-fura-2 (Kd for Ca^{2+} = 53 μM) [8] into the intracellular Ca^{2+} stores of rat parotid acinar cells and visualized the changes in Ca^{2+} concentration within the stores in saponin-permeabilized cells using digital imaging procedures. This dye is able to accumulate in many subcellular organelles, including mitochondria [1]. Furthermore, the lipophilic Ca^{2+} indicator Calcium Green C_{18} (Kd for Ca^{2+} = 0.23 μM) [9] was used to monitor Ca^{2+} release from intracellular Ca^{2+} stores in permeabilized parotid acinar cells. This dye consists of a Calcium Green-1 molecule conjugated to a lipophilic alkyl chain which will insert into biological membranes [9], and it can strongly label the outer-surface of intracellular compartments in permeabilized cells [10]. The changes in Ca^{2+} concentration adjacent to the membrane of intracellular organelles are detected by Calcium Green C_{18} incorporated into the membrane.

The main purpose of this study was to visualize the function of intracellular Ca^{2+} stores in permeabilized rat parotid acinar cells using a digital imaging system. The above approach clearly showed that inositol 1, 4, 5-trisphosphate ($InsP_3$) induces Ca^{2+} release from the intracellular Ca^{2+} stores at all regions of the permeabilized parotid acinar cells, but we failed to provide evidence of Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores.

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MATERIALS AND METHODS

Media. The Hanks' balanced salt solution buffered with HEPES (HBSS-H) consisted of the following : 137 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 1.03 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM glucose, and 20 mM HEPES (pH 7.4 with NaOH). Intracellular-like medium (ICM) was used to maintain permeabilized acinar cells in intracellular-like condition. ICM contained 125 mM KCl, 19 mM NaCl, 10 mM HEPES (pH 7.3 with KOH), 3 mM ATP, 1.4 mM $MgCl_2$, 0.33 mM $CaCl_2$, and 1 mM EGTA (free Ca^{2+} and Mg^{2+} concentrations were at 50 nM and 0.1 mM, respectively). In the monitoring with Calcium Green C_{18}, calcium sponge-treated medium (CaS), instead of ICM, was used, as EGTA is expected to buffer changes in Ca^{2+} concentration. CaS was prepared as described elsewhere [5]. Briefly, approximately 60 ml of a solution containing 120 mM KCl, 19 mM NaCl, 10 mM HEPES (pH. 7.3), and 3 mM ATP was passed twice over a column packed with 0.3 g of Calcium Sponge S (Molecular Probes, Eugene OR, USA) to remove residual contaminating Ca^{2+}. Following Ca^{2+} removal, 1.0 mM $MgCl_2$ was added to the solution. The final Ca^{2+} concentration of CaS was fluorescently determined using fura-2 to be ~50 nM [10].

Cell preparation. Male Wistar-strain rats were anesthetized with diethyl ether and killed by cardiac puncture. Dispersed acinar cells were prepared by the method of Merritt and Rink [11] with modifications [12]. After dispersion, the cells were washed and resuspended in a Hank's balanced salt solution buffered with HEPES (HBSS-H) containing 0.2% bovine serum albumin (BSA).

Loading of parotid acinar cells with Mag-fura-2. Dispersed parotid acinar cells were incubated with 8 μM Mag-fura-2 (Molecular Probes, Eugene OR, USA) for 30 min at 37°C and washed twice with fresh HBSS-H without BSA. The sample chambers consisting of cylinders glued to round glass coverslips were precoated with the cell adhesive Cell-Tak, and the dye-loaded cells were transferred to the chambers and attached to the bottom.

Permeabilization of acinar cells. The sample chambers were mounted on the stage of an inverted microscope (Diaphot: Nikon Inc., Tokyo, Japan) equipped with a 40 × objective, washed with BSA-free HBSS-H and then with Mg^{2+}/ATP-free ICM. The acinar cells were permeabilized by incubation with Mg^{2+}/ATP-free ICM containing 50 μg/ml saponin for 3–5 min at room temperature. Following the treatment with saponin, the cells were washed with ICM containing Mg^{2+} and ATP and incubated in the complete ICM for at least 5 min to allow complete filling of the intracellular Ca^{2+} stores.

Mag-fura-2 fluorescence measurements. Measurements of Mag-fura-2 fluorescence were made using the digital imaging system ARGUS 50 (Hamamatsu Photonics, Hamamatsu, Japan). When Mag-fura-2-loaded acinar cells were incubated with saponin as described above, the cells showed a significant decrease in fluorescence as cytosolic Mag-fura-2 was lost into the extracellular medium. Following washing with ICM, the permeabilized cells were excited alternately with light at 344 nm and 360 nm, and fluorescence emission at 510 nm was captured and digitized with the ARGUS 50 image processor (Hamamatsu Photonics, Hamamatsu, Japan) equipped with a silicon-intensified target camera. The 344 nm/360 nm ratio was recorded every 20 sec.

The volume of solution was maintained at ~40 μl by placing a vacuum line in the sample chamber. Solution changes were accomplished by the addition of 400 μl of the new solution to the sample chamber. All experiments were performed at room temperature.

Loading of permeabilized cells with Calcium Green C_{18}. Dispersed acinar cells were permeabilized with saponin as usual and then incubated with ICM containing 10 μM Calcium Green C_{18} (Molecular Probes, Eugene OR, USA) for 3 min. Following washing with dye-free ICM, the solution was switched to calcium sponge-treated medium.

Measurement of calcium green C_{18} fluorescence. Calcium Green C_{18} fluorescence from the permeabilized cells was detected with a confocal laser scanning microscope system (Leica TCS 4D, Leica, Heidelberg, Germany) equipped with a 40 × PL Fluotar objective. Confocal images (128 × 128 pixels) of Calcium Green C_{18} fluorescence were obtained at 488 nm excitation and >510 nm emission using a 170 μm pinhole. Confocal images of the fluorescence were obtained every 4 sec. Other parts of the protocol was similar to the experiments using Mag-fura-2.

RESULTS

Measurements of Ca^{2+} Release from Intracellular Ca^{2+} Stores Using Mag-fura-2

After permeabilization, a small cluster of three to five acinar cells were selected and stimulated with different concentrations of $InsP_3$. Fig. 1A shows typical changes in the Mag-fura-2 ratio in a small cluster stimulated by 0.1 and 0.5 μM $InsP_3$. The application of 0.1 μM $InsP_3$ had little or no effect on the Mag-fura-2 ratio (n=13), but the subsequent addition of 0.5 μM $InsP_3$ caused a rapid decrease in the Mag-fura-2 ratio reflecting a release of Ca^{2+} from intracellular Ca^{2+} stores. Similar changes induced by 0.5 μM $InsP_3$ were observed in all cell clusters examined (n=7). When acinar cells were stimulated with 0.25 μM $InsP_3$, a decrease in the Mag-fura-2 ratio was observed in six out of nine clusters of acinar cells (data not shown). To confirm that the changes in Mag-fura-2 ratio are mediated through activation of the $InsP_3$ receptor, heparin (100 μg/ml), a competitive inhibitor, was applied during stimulation with 0.5 μM $InsP_3$. As shown in Fig. 1A, the addition of heparin partly attenuated the $InsP_3$-induced decrease in the Mag-fura-2 ratio (n=3), and subsequent removal of $InsP_3$ from ICM resulted in a complete recovery.

Fig. 1B shows the changes in Mag-fura-2 ratio induced by 1 μM $InsP_3$, a concentration which produces the maximum effect. The application of $InsP_3$ caused a rapid decrease in the Mag-fura-2 ratio which was largely reversed by the removal of $InsP_3$ from ICM. If $InsP_3$ were removed together with Mg^{2+} and ATP from ICM following the decrease in the Mag-fura-2 ratio, its recovery was much smaller than the recovery in the presence of Mg^{2+} and ATP, indicating that the recovery in the ratio is due to Ca^{2+} re-uptake into the $InsP_3$-sensitive Ca^{2+} stores via the Ca^{2+} pump. Fig. 1C shows pseudocolor images taken at the times indicated. In the permeabilized acinar cells before stimulation, the Mag-fura-2 fluorescence was distributed throughout the cells, although the ratio intensity was not homogeneous (Fig. 1C-b). Stimulation with $InsP_3$ caused a decrease of the ratio in all regions of the cells (Fig. 1C-c), and subsequent removal of $InsP_3$ resulted in a spatial pattern of the Mag-fura-2 ratio almost identical to that in the pre-stimulus cells (Fig. 1C-e).

Thapsigargin (ThG), an irreversible inhibitor of the ER Ca^{2+} pump, has been shown to cause a release of Ca^{2+} from $InsP_3$-sensitive Ca^{2+} stores without activa-

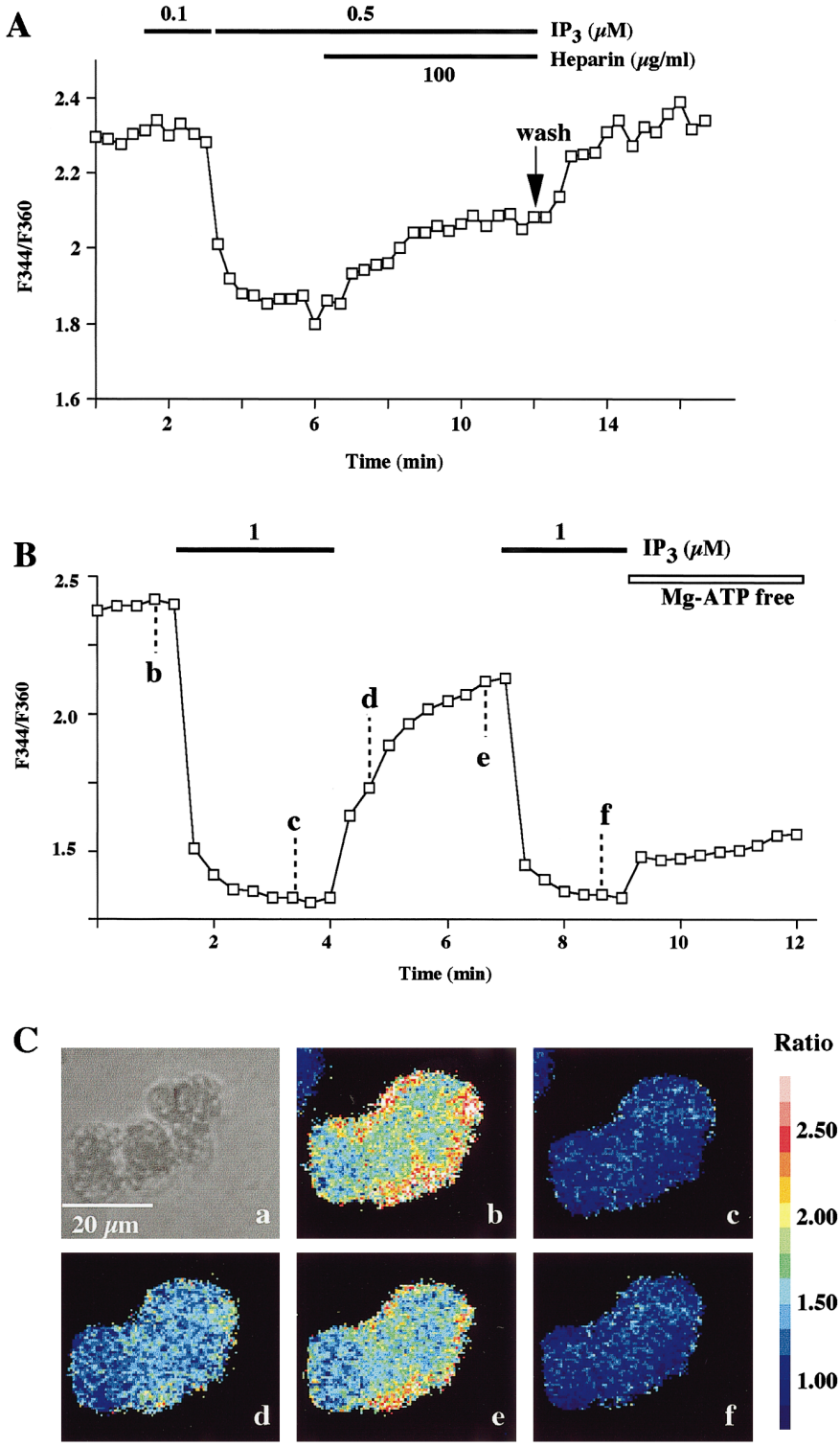

FIG. 1. $InsP_3$-induced changes in the Mag-fura-2 ratio of permeabilized rat parotid acinar cells. Mag-fura-2-loaded acinar cells were permeabilized by saponin treatment, and the fluorescence within the intracellular organelles was monitored as described in Materials and Methods. (A) A small cluster of the acinar cells were exposed to 0.1 μM $InsP_3$ for 2 min, then to 0.5 μM $InsP_3$ for 3 min and subsequently to 0.5 μM $InsP_3$ plus 100 μg/ml heparin. Further, the medium was changed to ICM without $InsP_3$ and heparin at the time indicated by the arrow. (B) A small cluster was exposed to 1 μM $InsP_3$ and then washed with complete ICM to remove $InsP_3$. After a second stimulation with 1 μM $InsP_3$, the cells were washed with Mg^{2+}/ATP-free ICM. $InsP_3$ and heparin were present for the periods shown by the solid horizontal bars at the top of (A) and (B). (C) Imaging of Mag-fura-2 ratio in the cell cluster monitored in (B). (a) brightfield image; (b–f) pseudocolour images obtained at the time points indicated by the broken lines in (B).

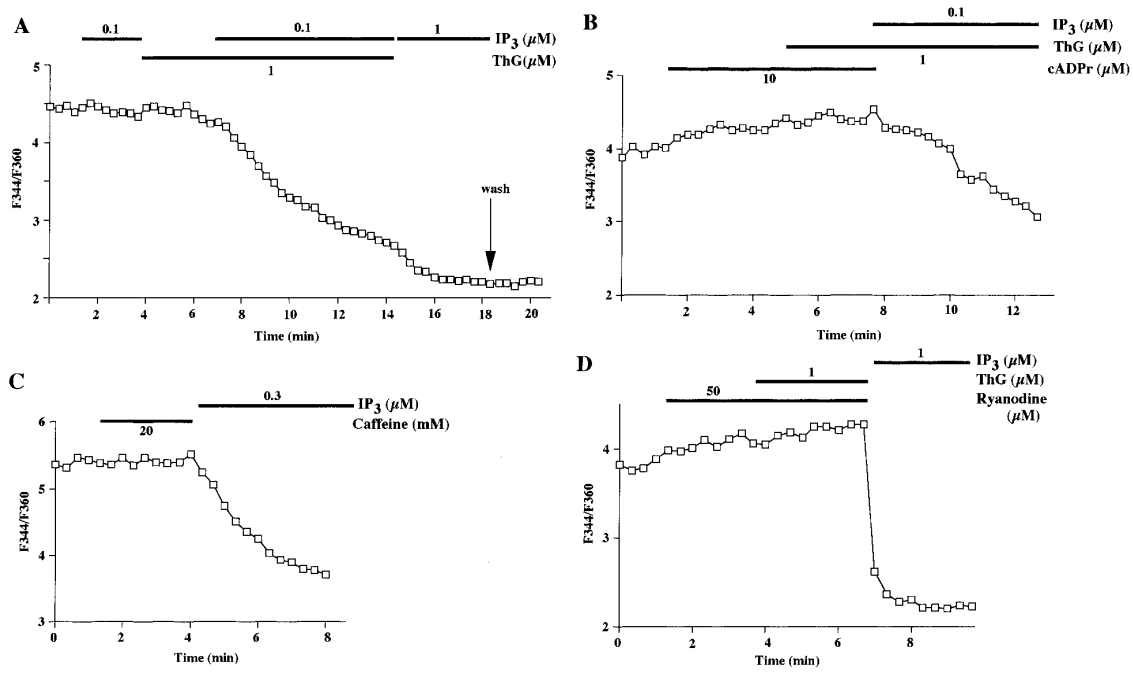

FIG. 2. Effects of various Ca^{2+}-releasing agents on the Mag-fura-2 ratio in permeabilized parotid acinar cells. Small cluster of the acinar cells were exposed to $InsP_3$ (0.1, 0.3 or 1 μM), thapsigargin (ThG; 1 μM), cyclic ADP-ribose (cADPr; 10 μM), caffeine (20 mM) or/and ryanodine (50 μM) for the periods shown by the horizontal bars at the top of each panel. In (A), the medium was switched to complete ICM without InsP3 at the time indicated by the arrow.

tion of phosphoinositide hydrolysis in rat parotid acinar cells [13, 14]. However, it is not fully understood why inhibition of the Ca^{2+} pump results in a release of Ca^{2+} from intracellular store. Some investigators suggest that the resting levels of $InsP_3$ may be involved in the ThG-induced Ca^{2+} release [15–17]. To address this possibility, we investigated whether ThG causes a decrease in the Mag-fura-2 ratio in permeabilized acinar cells. Application of 0.1 μM $InsP_3$ or 1 μM ThG did not change the Mag-fura-2 ratio in a cluster of permeabilized acinar cells (n=13) (Fig. 2A). Even if the concentration of ThG applied to the cells was increased to 2 μM, ThG did not change the ratio (n=3) (data not shown). However, the combined addition of 0.1 μM $InsP_3$ and 1 μM ThG caused a detectable decrease in the Mag-fura-2 ratio (Fig. 2A) in fourteen out of sixteen clusters. When a high concentration (1 μM) of $InsP_3$ was subsequently added, there was only a minor further decrease in the Mag-fura-2 ratio (Fig. 2A), suggesting that the combined addition of 0.1 μM $InsP_3$ and 1 μM ThG released a major portion of luminal Ca^{2+} within intracellular Ca^{2+} stores. The subsequent removal of $InsP_3$ did not reverse the decrease in the Mag-fura-2 ratio, indicating that the Ca^{2+} pump in the intracellular Ca^{2+} stores was completely blocked by ThG.

Another Ca^{2+}-releasing messenger, cyclic ADP-ribose (cADPr), is believed to induce Ca^{2+} mobilization through action on ryanodine receptor [18]. To show whether ryanodine-sensitive Ca^{2+} stores exist in rat parotid acinar cells, the effect of cADPr on the Mag-fura-2 ratio in permeabilized acinar cell was examined. As shown in Fig. 2B, application of 10 μM cADPr had no effect on the Mag-fura-2 ratio (n=11), even when the Ca^{2+} pump of the store was blocked by 1 μM ThG. The same cluster of acinar cells displayed a slow decrease in the Mag-fura-2 ratio in response to a combination of 0.1 μM $InsP_3$ and 1 μM ThG. Further, the effects of caffeine and ryanodine, pharmacological modulators of the Ca^{2+}-induced Ca^{2+} release (or ryanodine-sensitive) channel [19], on the Mag-fura-2 ratio were examined. Application of 20 mM caffeine or 50 μM ryanodine did not evoke any changes (n=3 or 5) (Fig. 2C and D), while subsequent addition of $InsP_3$ significantly decreased the ratio. A combined addition of ryanodine and ThG also had no effect on the Mag-fura-2 ratio (Fig. 2D).

Measurements of Ca^{2+} Release from Intracellular Ca^{2+} Stores Using Calcium Green C_{18}

If Mag-fura-2 is saturated with high concentrations of Ca^{2+} in intracellular Ca^{2+} stores, a minor release of Ca^{2+} from the stores may not be reflected in Mag-fura-2 ratio. Also, the possibility is not excluded that Mag-fura-2 did not penetrate into some part of the intracellular organelles. Therefore, Calcium Green C_{18} was used to monitor Ca^{2+} concentrations immediately adjacent to the membrane of intracellular Ca^{2+} stores. The changes in Calcium Green C_{18} fluorescence were monitored using confocal microscopy. As shown in Fig.

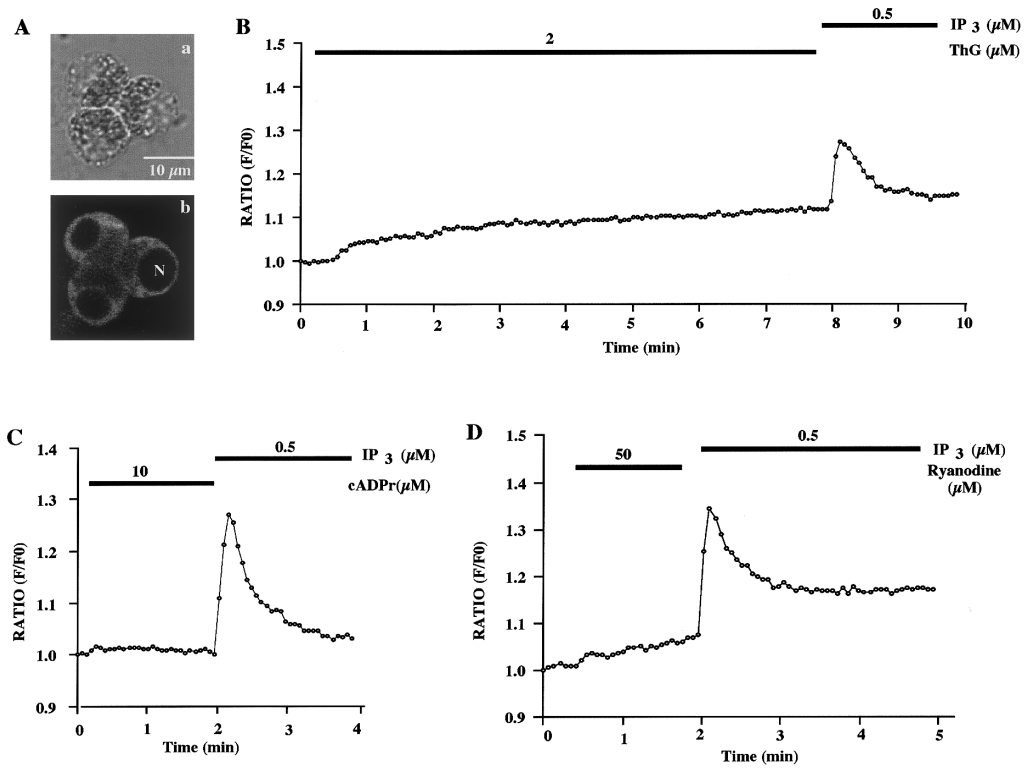

FIG. 3. Effects of various Ca^{2+}-releasing agents on Calcium Green C_{18} fluorescence in permeabilized acinar cells. The acinar cells were permeabilized with saponin and labeled with Calcium Green C_{18}. (A) Calcium Green C_{18}-labeled permeabilized acinar cells. (a) brightfield image, (b) confocal image of the fluorescence. (N) nucleus. In (B)–(D), small clusters of the acinar cells were exposed to $InsP_3$ (0.5 μM), thapsigargin (ThG; 2 μM), cyclic ADP-ribose (cADPr; 10 μM) or ryanodine (50 μM) for the periods shown by the horizontal bars at the top of each panel. The relative changes in fluorescence were determined by dividing the fluorescence intensity at each time by that at time 0.

3A(b), the confocal image of Calcium Green C_{18} fluorescence was located throughout the cytoplasmic region of permeabilized acinar cells, although the fluorescence intensity was not homogeneous. When a small cluster of permeabilized acinar cells was exposed to 2 μM ThG, a small and sustained increase in the fluorescence was observed (Fig. 3B), while addition of dimethylsulphoxide (solvent), instead of ThG, did not increase the fluorescence (data not shown). This result suggests that ThG evoked a weak but sustained leak of Ca^{2+} from cellular compartments. However, when the same cells were stimulated with 0.5 μM $InsP_3$ following incubation with ThG for 7.5 min, a clear increase in the fluorescence was observed (Fig. 3B), indicating that the exposure to ThG did not empty intracellular Ca^{2+} stores. Similar results were obtained in all acinar cells examined (n=4). The Calcium Green C_{18} fluorescence was not increased by application of 10 μM cADPr (n=6) or 50 μM ryanodine (n=3) (Fig. 3C and D), while subsequent addition of 0.5 μM $InsP_3$ evoked a large increase of the Calcium Green C_{18} fluorescence.

DISCUSSION

The present study with the Mag-fura-2 ratio clearly showed that intracellular Ca^{2+} stores of saponin-permeabilized parotid acinar cells maintain the ability to respond to $InsP_3$ and that the intracellular stores are able to sequestrate cytosolic Ca^{2+} via the Ca^{2+} pump. Since an $InsP_3$-induced Ca^{2+} release was also detected in the permeabilized cells labeled with Calcium Green C_{18}, we can conclude that the Mag-fura-2 ratio reflects changes in Ca^{2+} concentration within intracellular Ca^{2+} stores. The approach used in this study may provide useful information about subcellular Ca^{2+} signaling in parotid acinar cells.

The $InsP_3$-induced Ca^{2+} release was observed in all regions of the permeabilized cells, suggesting that $InsP_3$-sensitive Ca^{2+} stores are distributed throughout the cell cytoplasm. Based on imaging analysis of Ca^{2+} concentration within Ca^{2+} stores, van de Put and Elliot [6] reported that no intracellular differences in $InsP_3$ sensitivity were detected in permeabilized pancreatic acinar cells. However, it is not finally established whether there are subcellular regional differences in the $InsP_3$ sensitivity of the Ca^{2+} stores. Some other reports [20, 21] have suggested that the agonist-elicited Ca^{2+} wave observed in intact pancreatic acinar cells may result from the polarized distribution of $InsP_3$-sensitive Ca^{2+} stores. Further, recent immunocytochemical studies have demonstrated that $InsP_3$ recep-

tors are primarily detected close to luminal and lateral membranes in exocrine acinar cells [22, 23], supporting the hypothesis that a highly sensitive store to $InsP_3$ exists in the apical pole. In the present study, the intensity of Mag-fura-2 ratio and Calcium Green C_{18} fluorescence before stimulation was not necessarily homogeneous throughout the cytoplasmic region. This may imply that there are regional differences in the density of intracellular Ca^{2+} stores or the Ca^{2+} concentration in the stores. We failed to show polarized Ca^{2+} release from the $InsP_3$-sensitive stores. As the speed of agonist-elicited Ca^{2+} waves is very fast (> 10 μm/sec) [24, 25], it may be necessary to analyze the spatio-temporal pattern of the $InsP_3$-induced Ca^{2+} release in permeabilized acinar cells by monitoring with high speed.

The ThG-induced Ca^{2+} mobilization has been generally assumed to be due to an unspecific 'leak' pathway from intracellular Ca^{2+} stores. Some studies, however, have suggested that a major component of the ThG-induced increase in cytosolic Ca^{2+} concentration is due to $InsP_3$-sensitive Ca^{2+} release mediated by the resting levels of $InsP_3$ [15–17]. In the present study, ThG itself had little or no effect on the Mag-fura-2 ratio, while it induced a significant decrease in the presence of 0.1 μM $InsP_3$. This result strongly supports that the ThG-induced Ca^{2+} release involves the $InsP_3$-mediated pathway. We do not exclude the possibility that ThG causes a weak Ca^{2+} leak from the stores, because a small increase in Calcium Green C_{18} fluorescence was observed during exposure to ThG. However, subsequent addition of $InsP_3$ evoked a further, large increase in the fluorescence, indicating that the unspecific leak of Ca^{2+} is not a sufficient mechanism to empty the intracellular Ca^{2+} stores.

In pancreatic acinar cells, agonist-mediated Ca^{2+} signals are observed as a Ca^{2+} wave propagating from the luminal pole toward the basolateral region [20, 24–26]. The Ca^{2+} wave has been proposed to occur through the Ca^{2+}-induced Ca^{2+} release mechanism mediated by the ryanodine receptor [20], although this hypothesis is not established. It is therefore important to know whether ryanodine-sensitive Ca^{2+} stores are present in exocrine acinar cells. In the present study, none of cADPr, caffeine and ryanodine were effective in inducing Ca^{2+} release from intracellular stores of permeabilized acinar cells. This result is not consistent with the finding that a release of ^{45}Ca from microsomal vesicles of rat parotid acinar cells was induced by the addition of cADPr, caffeine or ryanodine [27]. In addition, based on the results obtained by immunoblot analysis and [^{3}H]ryanodine binding, ryanodine receptor type III was recently found in mouse parotid microsomal membranes [28]. Although the cause of this discrepancy is unknown, one possible explanation would be that the ryanodine receptor is expressed only at very low levels in rat parotid acinar cells. If so, it is possible that the Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores is too small to be detected with the Ca^{2+} indicators. Although the present study cannot completely exclude the possibility that ryanodine-sensitive Ca^{2+} stores exist in rat parotid acinar cells, it may be assumed that such Ca^{2+} stores are minor when compared with $InsP_3$-sensitive stores in rat parotid acinar cells.

In several studies on the suspension of exocrine acinar cells, the effects of cADPr on Ca^{2+} release have been examined using fura-2 or fluo-3, and the results appear to vary in exocrine cell types. In permeabilized lacrimal [29] and submandibular acinar cells [23], cADPr has been reported to cause a release of Ca^{2+} from intracellular Ca^{2+} stores, although the effects were much smaller than those of $InsP_3$. In contrast, the cADPr-induced Ca^{2+} release from the intracellular stores was not detected in permeabilized pancreatic acinar cells [23]. At present, there is no direct evidence showing that the ryanodine-sensitive Ca^{2+} store plays a major role in the Ca^{2+} signaling in exocrine acinar cells.

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