Polarized Ca^{2+} release in saponin-permeabilized parotid acinar cells evoked by flash photolysis of ‘caged’ inositol 1,4,5-trisphosphate

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In exocrine acinar cells, agonist stimulation results in a polarized Ca^{2+} signal, termed the ‘ Ca^{2+} wave’, that propagates from the apical pole towards the basolateral region. We attempted to detect the inositol 1,4,5-trisphosphate (InsP_3)-induced Ca^{2+} wave in saponin-permeabilized rat parotid acinar cells using a digital imaging system. The permeabilized acinar cells were labelled with the membrane-bound Ca^{2+} indicator Calcium Green C_{18} to detect changes in Ca^{2+} concentration adjacent to the membrane of intracellular organelles. Application of InsP_3 was made by the photolysis of InsP_3 $\text{P}^{4(5)}$ -1-(2-nitrophenyl)ethyl ester (caged InsP_3) to expose simultaneously all regions of the permeabilized acinar cells to InsP_3 . The increase in fluorescence ratio following the photolysis of 0.5 μM caged InsP_3 started at the apical region of the acinar cells within 0.1 s and spread towards the basolateral region, indicating that Ca^{2+} release from intracellular Ca^{2+} stores

was initially evoked at the apical region. Pretreatment with thapsigargin, an inhibitor of endoplasmic reticulum Ca^{2+} pumps, failed to prevent the InsP_3 -induced Ca^{2+} wave, suggesting that the generation of the Ca^{2+} wave is not attributed to the polarized distribution of the Ca^{2+} pumps. The photolysis of a high concentration (10 μM) of caged InsP_3 caused a homogeneous increase in the fluorescence ratio throughout the cells, indicating that all regions of intracellular Ca^{2+} stores similarly responded to the high concentration of InsP_3 . The present study is the first demonstration of the InsP_3 -induced Ca^{2+} wave in permeabilized exocrine acinar cells. The result provides fresh evidence that the apical region contains elements of intracellular Ca^{2+} stores particularly sensitive to InsP_3 and that the Ca^{2+} wave results from a polarized distribution of InsP_3 -sensitive Ca^{2+} stores.

INTRODUCTION

Agonist-induced cytosolic Ca^{2+} signals often result in complex temporal and spatial patterns [1]. In exocrine acinar cells, including parotid acinar cells, the increase in cytosolic Ca^{2+} concentration originates at the apical pole and then spreads towards the basolateral area [2–5]. As the polarized Ca^{2+} signal, termed the ‘ Ca^{2+} wave’, is observed both in the presence and absence of extracellular Ca^{2+} , it is thought to result from a Ca^{2+} release from intracellular Ca^{2+} stores. The characteristics of the Ca^{2+} waves have been extensively examined, but the precise mechanism underlying the Ca^{2+} waves is not clear.

Some investigators have suggested that the Ca^{2+} waves in exocrine acinar cells may be explained by subcellular and regional differences in the inositol 1,4,5-trisphosphate (InsP_3) sensitivity of intracellular Ca^{2+} stores [3,6]. This hypothesis is supported by immunocytochemical studies showing that the InsP_3 receptors are mainly localized in the apical pole of the pancreatic and submandibular acinar cells [7–9]. If the generation of the Ca^{2+} waves is associated with the heterogeneous distribution of InsP_3 -sensitive Ca^{2+} stores, direct application of InsP_3 to permeabilized acinar cells would elicit the same Ca^{2+} wave as that in intact acinar cells. In the present study, we attempted to detect the InsP_3 -induced Ca^{2+} wave in saponin-permeabilized rat parotid acinar cells using a digital imaging system. The lipophilic Ca^{2+} indicator, Calcium Green C_{18} [10], was used to monitor Ca^{2+} release from intracellular Ca^{2+} stores. This dye strongly labels the outer surface of intracellular compartments in permeabilized cells [11], and allows us to monitor the Ca^{2+} concentration immediately adjacent to the intracellular Ca^{2+} -store membrane. An application of InsP_3 was made by the flash photolysis of InsP_3

$\text{P}^{4(5)}$ -1-(2-nitrophenyl)ethyl ester (caged InsP_3), by which the permeabilized parotid acinar cells were rapidly and homogeneously exposed to InsP_3 .

MATERIALS AND METHODS

Media

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). The intracellular-like medium (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. The free Ca^{2+} and Mg^{2+} concentrations were 50 nM and 0.1 mM respectively. Calcium sponge-treated solution (CaS) was made from a solution containing 125 mM KCl, 19 mM NaCl, 10 mM Hepes (pH 7.3 with KOH) and 3 mM ATP. To remove Ca^{2+} , approx. 30 ml of this solution was passed once over a column containing 0.3 g of Calcium Sponge S (Molecular Probes, Eugene, OR, U.S.A.). Following Ca^{2+} removal, 1.4 mM MgCl_2 was added. The final Ca^{2+} concentration of CaS was 35–50 nM (determined fluorescently using fura 2).

Cell preparation

Male Wistar-strain rats were anaesthetized with diethyl ether and killed by cardiac puncture. Parotid glands were minced and digested with trypsin and collagenase as described previously [12]. Dispersed parotid acinar cells were washed and resuspended in the HBSS-H. In order to attach the cells on the coverslip,

Abbreviations used: InsP_3 , inositol 1,4,5-trisphosphate; caged InsP_3 , InsP_3 $\text{P}^{4(5)}$ -1-(2-nitrophenyl)ethyl ester; CaS, calcium sponge-treated solution; ER, endoplasmic reticulum; ICM, intracellular-like medium; ThG, thapsigargin.

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Figure 1 Digital images of Ca^{2+} release evoked by photolysis of caged InsP_3 in permeabilized parotid acinar cells

After permeabilization with saponin, the acinar cells were labelled with Calcium Green C_{18} . The permeabilized acinar cells were exposed to InsP_3 generated by the photolysis of 0.5 (A, B) or 10 (C, D) μM caged InsP_3 , and the fluorescence was captured every 0.12 (A, B) or 0.08 (C, D) s. (a) Transmission image; (b) fluorescence image excited with light at 490 nm; (c–j) pseudocolour images of the Calcium Green C_{18} ratio obtained at time points indicated in (B) and (D). The ratio is represented as relative fluorescence intensity (F/F_0). The scale bar in the fluorescence image represents 10 μm . (B, D) Time-dependent changes in the fluorescence ratio in the apical (●) and basal (○) regions of the three acinar cells. Black, red and green lines show the changes in fluorescence ratio within the black, red and green squares depicted in the fluorescence images (b). The photolysis of caged InsP_3 was made at the point indicated by the large arrow.

200 μl of cell suspension was transferred to a Cell Tak-coated sample chamber and centrifuged (50 g) for 15 s. These cells were kept in HBSS-H at room temperature until use.

Experimental protocol

The parotid acinar cells attached on the sample chamber were washed with Mg^{2+} /ATP-free ICM and permeabilized by exposure to 70–80 $\mu\text{g}/\text{ml}$ (w/v) saponin (ICN, Cleveland, OH, U.S.A.) for 1 min at room temperature. The permeabilized cells were incubated in the complete ICM containing 10–20 μM Calcium Green C_{18} (Molecular Probes) for 2 min, washed with dye-free ICM (without Calcium Green C_{18}) and then switched to CaS.

The Calcium Green C_{18} -labelled cells were stimulated with photorelease of InsP_3 from caged InsP_3 . Images of Calcium Green C_{18} fluorescence were obtained with an ARGUS HiSCA

imaging system (Hamamatsu Photonics, Shizuoka, Japan) coupled to an inverted microscope (Diaphot; Nikon, Tokyo, Japan), equipped with a Nikon CF Fluor 100 $\times$ objective, at an excitation wavelength of 490 nm and at an emission wavelength of 500–530 nm. Changes in the Ca^{2+} concentration were presented by the ratio of fluorescence values during stimulation (F) to that before the stimulation (F_0). All experiments were carried out at room temperature.

Photolysis of the caged InsP_3 compound

Caged InsP_3 (Molecular Probes) was added to the incubation medium (CaS) just before the measurement of fluorescence, and free InsP_3 was photoreleased by exposure to UV light. The photolysis was performed using a SA-200E xenon flash lamp (Eagle, Kawasaki, Japan). The UV flash light (200 J/F intensity; duration ≈ 1 ms) was guided to just above the experimental

chamber through an optic fibre. In preliminary experiments, the magnitude of the Ca²⁺ release from intracellular stores evoked by the photolysis was compared with that induced by direct application of free InsP₃, and it was estimated that the UV flash photolyses approx. 40% of the caged InsP₃ applied to the medium.

Data analysis

Image analysis was performed on a Macintosh Performa 6310 computer using the public domain NIH Image program (developed at the U.S.A. National Institutes of Health and available from the Internet by anonymous file transfer protocol from zipper.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI).

RESULTS

Permeabilized parotid acinar cells were loaded with the lipophilic Ca²⁺ indicator Calcium Green C₁₈ and exposed to a single UV flash (200 J/F intensity) for about 1 ms in CaS containing 0.5 μ M caged InsP₃. The photolysis of 0.5 μ M caged InsP₃ was expected to result in a release of approx. 0.2 μ M free InsP₃. Figure 1(A) shows pseudocolour images of the Calcium Green C₁₈ fluorescence ratio (F/F_0) obtained from a small cluster of three permeabilized acinar cells. In the resting state, the fluorescence ratio was uniformly low throughout cytoplasmic regions of the acinar cells [Figure 1A(c)]. The increase in the fluorescence ratio was initiated at the edge of the apical region about 0.1 s after the photolysis of caged InsP₃ [Figure 1A(d)] and rapidly spread as a wave towards the basal area [Figure 1A(e–h)]. The response reached the maximum level throughout the cells about 0.7 s after the photolysis. The time-dependent changes in fluorescence ratio were compared for selected areas of interest in the apical and basal regions of the acinar cells. The areas are depicted as small squares in Figure 1[A(b)]. The results (Figure 1B) show that increases in the ratio are more rapid in the apical regions (●) than in the basal regions (○). A similar polarized pattern of Ca²⁺ release was observed in 23 out of 24 permeabilized acinar cells stimulated by the photolysis of 0.5 μ M caged InsP₃.

To test the effect of higher concentrations of InsP₃, the concentration of caged InsP₃ applied to the solution was increased to 10 μ M. From preliminary experiments, the photolysis of 10 μ M caged InsP₃ was expected to result in a release of $\approx$ 4 μ M free InsP₃. Figure 1(C) shows pseudocolour images of the cytoplasmic Ca²⁺ distribution in a cluster of three acinar cells. Following the photolysis, the Calcium Green C₁₈ fluorescence ratio increased simultaneously throughout the acinar cells. Changes in the ratio were compared for selected areas depicted

Figure 2 Ca²⁺ release induced by the photolysis of caged InsP₃ in the presence of ThG

The Calcium Green C₁₈-labelled acinar cells were preincubated with 4 μ M ThG for 1 min and then stimulated by the photolysis of 0.5 μ M caged InsP₃. (B) Changes in the fluorescence ratio from (A) on an expanded time scale. ●, Apical pole; ○, basal pole. The photolysis of caged InsP₃ was made at the point indicated by the large arrows.

as small squares in Figure 1[C(b)]. The result (Figure 1D) shows that there were no significant differences between the rise in ratio at the apical (●) and basal (○) areas, although the maximum level in the basal area was slightly higher than that in the apical area.

The times required to reach 50% of the maximum fluorescence ratio in the apical and basal regions were quantitatively evaluated from the time-dependent changes in the ratios. At 0.5 μ M caged InsP₃, the speed of the rise in the fluorescence ratio was significantly faster ($P < 0.05$) in the apical region than in the basal region, whereas with 10 μ M caged InsP₃ there was no statistically significant difference between the speed in the apical and basal regions (Table 1).

It has been reported that the agonist-induced Ca²⁺ wave in intact pancreatic acinar cells is prevented by a pretreatment with the endoplasmic reticulum (ER) Ca²⁺-pump inhibitor thapsigargin (ThG) [13], and the effect of ThG on the polarized Ca²⁺ release induced by the photolysis of caged InsP₃ was examined. Permeabilized parotid acinar cells were pretreated with 4 μ M ThG for 1 min and then exposed to the photolysis of 0.5 μ M caged InsP₃ (Figure 2). Following a small and sustained increase in fluorescence ratio by ThG itself, the same acinar cells strongly responded to the subsequent photolysis of caged InsP₃ (Figure 2A). Figure 2(B) shows the InsP₃-induced changes in ratio from Figure 2(A) on an expanded time scale. The increase in ratio at the apical pole preceded that at the basal pole, indicating that ThG did not significantly change the polarized

Table 1 Time to reach 50% of the maximum increase in the fluorescence ratio at the apical and basal poles

Increases in the fluorescence ratio were measured, and the time to reach 50% of the maximum increase was estimated. * $P < 0.05$, as compared with the value obtained at the apical pole (Student's t test).

	Time (ms $\pm$ S.E.)			<i>n</i>
	Apical region (A)	Basal region (B)	(B) – (A)	
Photolysis of caged InsP ₃				
0.5 μ M caged InsP ₃	237 $\pm$ 22	333 $\pm$ 26*	95 $\pm$ 15	24
10 μ M caged InsP ₃	283 $\pm$ 47	240 $\pm$ 28	–43 $\pm$ 24	9

pattern of the InsP_3 -induced Ca^{2+} signal. Similar results were observed in 15 out of 16 cells pretreated with ThG.

DISCUSSION

The present study is the first to demonstrate that apical-to-basal Ca^{2+} waves can be evoked by the photolysis of caged InsP_3 in permeabilized exocrine acinar cells. The pattern of the InsP_3 -evoked Ca^{2+} wave was essentially similar to that induced by muscarinic receptor stimulation in intact parotid acinar cells [5] and other exocrine cell types [2–4,14]. The result here provides fresh evidence for the idea that the apical region of acinar cells contains elements of intracellular Ca^{2+} stores particularly sensitive to InsP_3 and that the polarized Ca^{2+} signals result from a heterogeneous cellular distribution of InsP_3 -sensitive intracellular Ca^{2+} stores. Immunocytochemical evidence demonstrates that InsP_3 receptors are localized most strikingly in the area just underneath the luminal and lateral membranes [7–9], suggesting that the high sensitivity to InsP_3 in the apical pole may be due to a high density of InsP_3 receptors. The photolysis of a high concentration (10 μM) of caged InsP_3 failed to evoke Ca^{2+} waves, indicating that all compartments of intracellular Ca^{2+} stores responded similarly to the high concentration of InsP_3 . One possible explanation for this is that the high InsP_3 concentration accelerated Ca^{2+} release from low InsP_3 -sensitive Ca^{2+} stores that are considered to be localized in the basolateral region of acinar cells [3,6]. Indeed, as shown in Table 1, when the concentration of caged InsP_3 was increased to 10 μM , the speed of rise in the fluorescence ratio was increased in the basal region rather than in the apical region.

Several studies have demonstrated that intracellular injection of InsP_3 in intact pancreatic acinar cells can produce polarized Ca^{2+} signals similar to those evoked by receptor agonists [3,6], supporting the hypothesis that the intracellular Ca^{2+} stores localized in the apical area are more sensitive to InsP_3 . In contrast, based on the finding that the kinetics of the Ca^{2+} release induced by direct application of InsP_3 were similar in the apical and basolateral regions of permeabilized pancreatic acinar cells, another group [15] suggested that there are no regional differences in InsP_3 sensitivity in permeabilized acinar cells and that additional cytosolic factors may be necessary for the agonist-induced polarized Ca^{2+} signalling in intact acinar cells. It should be noted that in the experiments on permeabilized pancreatic acinar cells [15] the Ca^{2+} release from intracellular Ca^{2+} stores was monitored by measuring the Ca^{2+} concentration within intracellular organelles every 15 s. The time intervals of 15 s for monitoring appear to be too long to detect the InsP_3 -induced Ca^{2+} waves. In addition, it has been suggested that the ER operates as one lumenally continuous Ca^{2+} store in pancreatic acinar cells [16], and it may be possible that application of InsP_3 uniformly changes the Ca^{2+} concentration within the ER in all regions of the acinar cells.

Saponin-permeabilized cells have been demonstrated to readily take up and lose macromolecules [11], and soluble cytoplasmic fractions are expected to be lost during permeabilization. Therefore, the finding that the Ca^{2+} wave was observed in permeabilized acinar cells may exclude the possibility that the cytosolic factors are essential in producing the Ca^{2+} wave. Exocrine acinar cells express types 1, 2 and 3 InsP_3 receptors [7,9]. It remains to be determined whether the expression of different InsP_3 receptors in

specific parts of the acinar cells is involved in the heterogeneity of InsP_3 -sensitive Ca^{2+} stores.

It has been proposed that the polarized distribution of the ER Ca^{2+} pumps controls initiation and propagation of the Ca^{2+} waves [13]. This is based on the finding that inhibition of the Ca^{2+} pumps by ThG resulted in elimination of the initiation site and propagated Ca^{2+} wave. In contrast, however, we previously demonstrated that treatment with ThG did not eliminate the agonist-induced Ca^{2+} wave in intact parotid acinar cells [5], and this is supported by the present study using permeabilized parotid acinar cells. As treatment with ThG delayed the initiation of the agonist-induced Ca^{2+} wave in intact acinar cells [5], we do not exclude the possibility that the Ca^{2+} wave is modulated by the ER Ca^{2+} pumps, but it seems unlikely that a polarized distribution of the ER Ca^{2+} pumps plays an essential role in producing the Ca^{2+} waves.

In addition to the InsP_3 -induced Ca^{2+} release, the Ca^{2+} -induced Ca^{2+} -release system, which is believed to be mediated by ryanodine receptors [17], has been suggested to be involved in the mechanism for the rapid spread of a Ca^{2+} wave in the granular area of pancreatic acinar cells [6]. However, this hypothesis is not still established. We recently demonstrated that cADP-ribose, caffeine and ryanodine, which are potentiators or modulators of the Ca^{2+} -induced Ca^{2+} release [17], had no effect on Ca^{2+} release from intracellular Ca^{2+} stores in permeabilized parotid acinar cells [18]. This result does not support the hypothesis that propagation of the Ca^{2+} wave in the granular area is mediated by the Ca^{2+} -induced Ca^{2+} release.

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