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Swelling-induced Ca^{2+} Release from Intracellular Calcium Stores in Rat Submandibular Gland Acinar Cells

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Abstract. The effects of osmotically-induced cell swelling on cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) were studied in acinar cells from rat submandibular gland using microspectrofluorimetry. Video-imaging techniques were also used to measure cell volume. Hypotonic stress (78% control tonicity) caused rapid cell swelling reaching a maximum relative volume of 1.78 ± 0.05 (n = 5) compared to control. This swelling was followed by regulatory volume decrease, since relative cell volume decreased significantly to 1.61 ± 0.08 (n = 5) after 10 min exposure to hypotonic medium. Osmotically induced cell swelling evoked by medium of either 78% or 66% tonicity caused a biphasic increase of $[Ca^{2+}]_i$. The rapid phase of this increase in $[Ca^{2+}]_i$ was due to release of Ca^{2+} from intracellular stores, since it was also observed in cells bathed in Ca^{2+}-free solution. The peak increase of $[Ca^{2+}]_i$ induced by cell swelling was 3.40 ± 0.49 (Fura-2 F_{340}/F_{380} fluorescence ratio, n = 11) and 3.17 ± 0.43 (n = 17) in the presence and the absence of extracellular Ca^{2+}, respectively, corresponding to an absolute $[Ca^{2+}]_i$ of around 1 μM. We found that around two-thirds of cells tested still showed some swelling-induced Ca^{2+} release (SICR) even after maximal concentrations (10^{-5} M–10^{-4} M) of carbachol had been applied to empty agonist-sensitive intracellular Ca^{2+} stores. This result was confirmed and extended using thapsigargin to deplete intracellular Ca^{2+} pools. Hypotonic shock still raised $[Ca^{2+}]_i$ in cells pretreated with thapsigargin, confirming that at least some SICR occurred from agonist-insensitive stores. Furthermore, SICR was largely inhibited by pretreatment of cells with carbonyl cyanide *m*-cholorophenyl hydrazone (CCCP) or ruthenium red, inhibitors of mitochondrial Ca^{2+} uptake. Our results suggest that the increase in $[Ca^{2+}]_i$, which underlies regulatory volume decrease in submandibular acinar cells, results from release of Ca^{2+} from both agonist-sensitive and mitochondrial Ca^{2+} stores.

Key words: Ca^{2+} — Submandibular gland — Cell swelling — Regulatory volume decrease — Carbachol — Ca^{2+} stores

Introduction

Free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is a key signal controlling the process of fluid and electrolyte secretion in exocrine cells. One of the parameters regulated by $[Ca^{2+}]_i$ in exocrine cells is cell volume. Coupling of cell volume and $[Ca^{2+}]_i$ during secretion was reported in single acinar cells from rat parotid gland (Foskett & Melvin, 1989). Fluid secretion from acinar cells is associated with an initial rapid increase in $[Ca^{2+}]_i$, (Foskett & Melvin, 1989), accompanied by a rapid, substantial cell shrinkage driven by loss of K^+ and Cl^- through ion channels (Nakahari et al., 1990; Seo et al., 1995).

The initiation of an agonist-dependent $[Ca^{2+}]_i$ response in acinar cells involves release of Ca^{2+} from intracellular stores. The major intracellular messenger mediating intracellular Ca^{2+} release in acinar cells is thought to be inositol-1,4,5-trisphosphate ($InsP_3$), although an increasing body of evidence suggests that acinar cell Ca^{2+} stores may also be under the control of other Ca^{2+}-mobilizing messengers, including cyclic ADP ribose (cADPR) and $NAADP^+$ (da Silva & Guse, 2000). There is also evidence that mitochondria may play a role in $[Ca^{2+}]_i$ regulation in acinar cells, perhaps by uptake of Ca^{2+} following high-intensity or prolonged agonist

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stimulation (Bird, Obie & Putney, Jr., 1992; Gonzalez, Schulz & Schmid, 2000).

In addition to the increase in $[Ca^{2+}]_i$ which results from stimulation with agonists, $[Ca^{2+}]_i$ also can be increased in many epithelia by mechanical stimulation or by osmotically-induced cell swelling. A variety of epithelial cell types including toad bladder cells (Wong, Debell & Chase, Jr., 1990), rabbit proximal tubule cells (McCarty and O'Neil, 1991; Breton et al., 1992), cultured intestinal 407 cells (Hazama & Okada, 1990), rabbit gastric parietal cells (Negulescu, Munck & Machen, 1992), and rat parotid gland acinar cells (Foskett et al., 1994) show a rise in intracellular free Ca^{2+} on hyposmotic cell swelling. This increase in $[Ca^{2+}]_i$ may be important in producing regulatory volume decrease (RVD), although extracellular Ca^{2+} was reported not to be an absolute requirement for RVD in collapsed rabbit proximal tubule (McCarty & O'Neil, 1991; Breton et al., 1992). In rat lacrimal acinar cells, which are physiologically similar to submandibular acinar cells, Ca^{2+} ions seem to play a critical in RVD by activating separate Ca^{2+}-dependent K^+ and Cl^- channels, although the extent and source of any increase in $[Ca^{2+}]$ remains unclear (Kotera & Brown, 1993; Park et al., 1994; Elliott, 1994; Speake, Douglas & Brown, 1998).

Previous work on single parotid acinar cells suggested that osmotically induced cell swelling raised $[Ca^{2+}]_i$ by releasing Ca^{2+} from $InsP_3$-sensitive Ca^{2+} stores (Foskett et al., 1994). However, this is in contrast to the work on lacrimal acinar cells discussed above. The purpose of the present experiments was to investigate whether $[Ca^{2+}]_i$ could be increased by osmotically induced cell swelling in rat submandibular acinar cells and, if so, what type of intracellular Ca^{2+} stores are involved. Our data suggest that agonist-insensitive, probably mitochondrial, Ca^{2+} stores coexist with agonist-sensitive Ca^{2+} stores in this cell type, and that both types of Ca^{2+} store may be involved in swelling-induced Ca^{2+} release.

Materials and Methods

Cell Preparation

Single rat submandibular acinar cells were prepared by a modification of the method of Martinez and Cassity (1985). Briefly, a male Sprague-Dawley rat (weight typically around 200g) was sacrificed by cervical dislocation and both submandibular glands were isolated and dissected free from the surrounding connective tissue. Sublingual glands were removed and discarded, and the glands were then minced in Ca^{2+}-free HEPES solution (*see below* for composition). The minced tissue was treated with 100 Units/ml collagenase (Worthington, Type IV, Lakewood, UK) in BSA-containing HEPES solution (*see below* for composition) at 37°C with continuous top-gassing with 100% O_2 for 1 hr. During incubation, the tissue was mechanically dissociated by repeated pipetting at 20-min intervals. This method produced a mixture of small cell clusters (consisting of 3–8 cells) and single cells.

Measurement of Cell Volume

For volume measurements one or two drops of the freshly isolated cell suspension were added to a coverslip precoated with Cell-Tak (Collaborative Biomedical Products, MA), which formed the base of an experimental chamber (volume 400 μl) on the stage of an Olympus IMT-2 inverted microscope. After the cells had adhered to the coverslip, the chamber was perfused at a rate of 2 ml/min with medium at 37°C. The cells were observed via a CCD camera (Watec, Japan), and video images of cells were saved on a video cassette recorder (Panasonic, Japan). In order to analyze cell volume, the area of the cell was estimated by using Scion Image software (Scion Corp., MD), which determined the number of pixels bounded by the perimeter of the cell. Cell volume was then calculated assuming that the cell was spherical. In all experiments the volume was normalized to the initial volume observed during control superfusion with isotonic solution. Cell volume was measured at 20-sec intervals and the mean value of the measured volumes at 0, 20 and 40 sec was defined as the initial volume (V_0).

Flourescent-Dye Loading and $[Ca^{2+}]_i$ Measurement

After collagenase treatment for 1 hr, cells were washed twice with BSA-HEPES solution and then loaded with 2 μM Fura-2 acetoxymethyl ester(fura-2) in 2 ml of the same solution for 30 min at room temperature (25°C). After loading, the cells were centrifuged, washed, resuspended in the normal bath solution, and kept on ice until use. The isotonic bath solution used for fura-2 experiments contained (mM): 90 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPES, 100 mannitol, pH 7.4 (pH adjusted with NaOH) giving a measured osmolarity of 291 ± 4 mosmol/kg H_2O (n = 5). The hypotonic solution (66% of control tonicity), with a measured osmolarity of 194 ± 5 mosmol/kg H_2O (n = 4) was similar to the control solution except that mannitol was omitted. In some experiments a similar solution but containing 105 mM NaCl and 70 mM mannitol was used (78% hypotonic solution). The osmolality of all the solutions was measured by freezing-point depression using an osmometer (Precision System, Model 4002). In solutions containing 20 mM caffeine, 10 mM NaCl was replaced by 20 mM caffeine. In Ca^{2+}-free solutions, $CaCl_2$ was omitted and 0.5 mM EGTA was added.

For microspectrofluorimetric measurements of $[Ca^{2+}]_i$, the cells were allowed to settle at the bottom of a recording chamber (volume 400 μl) and viewed with a Zeiss inverted microscope (Axiovert 10, Zeiss, Germany) using a × 40 oil immersion objective lens. After the cells had adhered to the coverslip forming the base of the chamber, they were superfused with isotonic bath solution. For excitation of fura-2, a collimated beam of light from a 200W Hg arc lamp was fed to a dual spectrophotometer system (PTI, Lawrenceville, NJ), which alternated wavelengths between 340 to 380 nm by means of a spinning chopper (60 Hz). Emitted fluorescence was measured at 510 nm with a photomultiplier tube. For the recording and analysis, Felix software (Version 1.1) from PTI was used.

Calibration and Possible Effects of Ionic Strength on Fura-2 Signals

In all the figures illustrating changes in $[Ca^{2+}]_i$ the uncalibrated 340:380 nm ratio signal is shown as an index of $[Ca^{2+}]_i$. However,

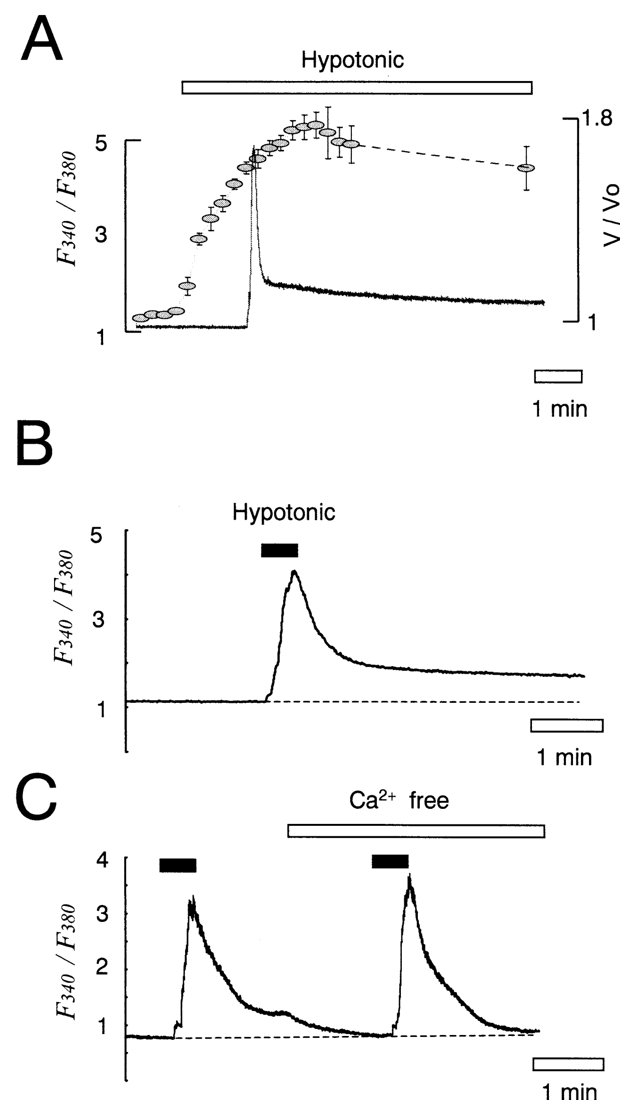

Fig. 1. Effect of osmotically-induced swelling on $[Ca^{2+}]_i$ and cell volume in rat submandibular acinar cells. The left axis in each panel shows the 340:380 nm fura-2 fluorescence ratio as an index of $[Ca^{2+}]_i$. (*A*) Superimposed records of $[Ca^{2+}]_i$ (representative trace from a single experiment typical of five cells) and cell volume during exposure to medium of 78% control hypotonicity. Data on relative cell volume are presented as mean ± SEM (n = 5, right axis). (*B*) Similar $[Ca^{2+}]_i$ response but induced by 66% hypotonicity stress. (*C*) Hypotonic stress (indicated by dark bars) also increased $[Ca^{2+}]_i$ in Ca^{2+}-free medium.

we did perform a two-point calibration of the fura-2 signal (Grynkiewicz, Poenie & Tsien, 1985) in a limited numer of cells at random throughout the experiment. We measured the value of R_{min} and S_{F380} in the Ca^{2+}-free media containing 2 mM EGTA. This was followed by the media containing 2 mM Ca^{2+} with 2 μM ionomycin for more than 15 min; then the value of R_{max} and S_{B380} were measured. $[Ca^{2+}]_i$ can be estimated from subsequent ratio values using the following equation:

$$[Ca_i^{2+}] = K_D(R - R_{min}/R_{max} - R)(S_{F380}/S_{B380})$$

where K_D = 150 nM at room temperature, R is any given 340/380 ratio value and S_{F380}/S_{B380} is the ratio of fluorescence measured at 380 nm for Ca^{2+}-free and Ca^{2+}-bound fura-2. The values were R_{min}, 0.68 ± 0.05; R_{max}, 4.20 ± 0.15; S_{F380}, 3.67 ± 0.79; S_{B380}, 1.20 ± 0.30 (n = 6). The mean resting ratio of 0.96 ± 0.05 (n = 31) obtained in isotonic Ca^{2+}-containing medium corresponds to a $[Ca^{2+}]_i$ of 39.6 nM.

The data are presented mainly as the measured 340 nm/380 nm ratio, rather than as the calculated absolute $[Ca^{2+}]_i$ value, because of the uncertainties which arise with the fura-2 calibration when the cells swell following exposure to hypotonic media, with resulting changes in ionic strength and probably cytosolic viscosity (Negulescu & Machen, 1990). In our experiments, the maximal relative increase in cell volume during exposure to hypotonic solution was 1.78 (*see* Fig. 1*A*). Assuming ionic strength is inversely proportional to the cell volume, this roughly corresponds to reduction of intracellular (cytosolic) ionic strength to a value of 82 mM. The K_D value of fura-2 can be estimated to be approximately 100 nM at this ionic strength from the data given by Williams and Fay (1990). This means that the peak fura-2 ratio during hypotonic swelling may be overestimated by up to 50% compared to the change in ratio induced by agonist stimulation. The decreased ionic strength caused by cell swelling, however, does not materially alter any of the conclusions reached.

Solutions and Reagents

The Ca^{2+}-free HEPES solution used for cell isolation contained (mM): 130 NaCl, 4.5 KCl, 1 NaH_2PO_4, 1 $MgSO_4 \cdot 7H_2O$, 10 HEPES, 10 HEPES-Na, 10 D-glucose; pH 7.4 (adjusted with NaOH) giving an osmolarity of 290 mosmol/kg H_2O. The BSA-HEPES solution was made by adding 1% BSA, 2 mM glutamine and 1×Minimal Eagle's Medium amino acids to Ca^{2+}-free HEPES solution.

Fura-2 was obtained from Molecular Probes (Eugene, OR), trypsin, trypsin inhibitor, thapsigargin, ryanodine, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and ruthenium red from Sigma (Poole, UK), collagenase from Worthington (Lakewood, UK) and carbachol and caffeine from RBI (Poole, UK). All data are presented as means ± SEM. Statistical analysis was carried out using either paired or unpaired Student's *t*-test, as appropriate.

Results

Hypotonic Swelling-Evoked $[Ca^{2+}]_i$ Increase and RVD

Rat submandibular acinar cells consistently responded to osmotically induced swelling with a large increase in $[Ca^{2+}]_i$. Fig. 1*A* shows a typical biphasic $[Ca^{2+}]_i$ response induced by exposure to a 78% hypotonic bath solution containing 1 mM Ca^{2+}. The peak $[Ca^{2+}]_i$ increase was observed approximately 3 min (mean time to peak ratio 176 ± 23 sec, n = 5) after the onset of hypotonic stress. This peak increase was followed by a decline in $[Ca^{2+}]_i$ to a level that remained elevated above the control value. In many experiments $[Ca^{2+}]_i$ remained elevated even after return to normal tonicity, possibly implying a long-lasting activation of Ca^{2+} entry pathways.

We also measured changes in cell volume during exposure to identical hypotonic media in parallel

Table 1. Mean data on the effect of osmotically-induced changes in cell volume on $[Ca^{2+}]_i$

	Ca^{2+} in medium $[Ca^{2+}]_i(F_{340}/F_{380}$, nM)	Ca^{2+}-free bath $[Ca^{2+}]_i(F_{340}/F_{380}$, nM)
Resting state	0.96 ± 0.05 (31), 39.6 nM	0.92 ± 0.04 (42), 33.5 nM
Hypotonic (66%)	3.40 ± 0.49 (11), 1039 nM	3.17 ± 0.43 (17), 739 nM
Carbachol (10^{-5} M)	1.99 ± 0.18 (6), 185 nM	

Values given are for the mean 340:380 ratio ± SEM, with the number of individual cells studied for each condition in brackets. Absolute values of $[Ca^{2+}]_i$ derived from the mean ratio value for each condition (as described in Methods) are also shown. Note that the peak $[Ca^{2+}]_i$ induced by hypotonic cell swelling is likely to be overestimated up to 50% due to changes in intracellular ionic strength (*see* Methods). In parantheses, the number of experiments.

experiments, as shown in Fig. 1*A*. The relative cell volume (indicated by dark circles) was measured at 20-sec intervals by video imaging. The relative cell volume (V/V_0) increased substantially during the hypotonic exposure, reaching a maximum of 1.78 ± 0.05 ($n = 5$) four min after onset of hypotonic stress. Subsequently the volume slowly decreased during the remaining six minutes of exposure to the hypotonic medium, and at the end of the 10-min hypotonic exposure, the relative volume was 1.61 ± 0.08 ($n = 5$), significantly different from the maximal volume ($P < 0.05$, paired *t*-test).

To evoke a more rapid swelling-induced $[Ca^{2+}]_i$ response, we modified the protocol by reducing the osmolality of the hypotonic solution to 66%. Fig. 1*B* shows a typical increase of $[Ca^{2+}]_i$ induced by 66% hypotonic stress. The greater hypotonicity evoked a more rapid $[Ca^{2+}]_i$ response, with the peak typically occurring within 30 sec of the onset of hypotonic stress. Apart from the greater speed of the response, the overall profile of the biphasic intracellular Ca^{2+} response was very similar to that evoked by 78% hypotonic stress. Interestingly, a sustained increase of $[Ca^{2+}]_i$ above resting levels for more than 170 sec was always seen after hypotonic stress in medium containing 1 mM Ca^{2+}, even when the duration of the hypotonic exposure was limited to 30 sec (Fig. 1*B*). The average F_{340}/F_{380} ratio of all the cells examined was 0.96 ± 0.05 ($n = 31$) in resting cells, and increased to 3.40 ± 0.49 ($n = 11$) at the peak of the $[Ca^{2+}]_i$ increase induced by hypotonic stress. According to the calibration described in Methods, this corresponds to a $[Ca^{2+}]_i$ approaching 1 μM (*see* Table 1), although, as discussed, this may be a substantial overestimate.

Role of Intracellular Calcium Release

Given the rapid onset of the increase in $[Ca^{2+}]_i$ evoked by hypotonic stress, especially with 66% tonicity, we hypothesised that the peak of this $[Ca^{2+}]_i$ response probably arose by release of Ca^{2+} from intracellular stores. This was confimed by experiments in Ca^{2+}-free medium (Fig. 1*C*), since the size of the increase in ratio evoked by exposure to 66% tonicity under these conditions was 3.17 ± 0.43 ($n = 17$), not significantly different from the corresponding value in the presence of 1 mM extracellular Ca^{2+}. However, the sustained elevated $[Ca^{2+}]_i$ seen subsequent to the peak during (and after) hypotonic stress was abolished in Ca^{2+}-free medium, showing that it resulted from enhanced Ca^{2+} influx. Our results thus demonstrate that osmotic cell swelling induced mobilization of intracellular storage sites as well as sustained Ca^{2+} influx from the extracellular medium. Table 1 summarizes the effects of hypotonic stress and carbachol on $[Ca^{2+}]_i$ in rat submandibular acinar cells.

We next investigated what type or types of intracellular Ca^{2+} stores were involved in swelling-induced Ca^{2+} release (SICR). For this and all subsequent experiments we used 66% tonicity to induce SICR. Firstly, in order to assess the role of $InsP_3$-sensitive Ca^{2+} stores in SICR, hypotonic stress was applied to the cells after depletion of the Ca^{2+} store with a maximal concentration of carbachol (CCh) under Ca^{2+}-free conditions. The results of these experiments fell into two distinct groups. Fig. 2*A* shows a result typical of six of eleven cells tested with this protocol. In these cells, an initial stimulus with 10^{-5} M CCh appeared to completely deplete the store, since a second application of CCh evoked no further release. Under these conditions, subsequent hypotonic stress did not evoke SICR. This result seems to indicate that $InsP_3$-sensitive Ca^{2+} stores are the only intracellular Ca^{2+} stores involved in SICR. However, in an approximately similar number of cells (five of eleven) we obtained a quite different result, shown in Fig. 2*B*. Here, clear SICR was observed after two treatments of the cell with CCh. This SICR, though smaller than that observed in cells which had not been stimulated, was much larger than the $[Ca^{2+}]_i$ increase evoked by the second CCh stimulation.

In the protocol employed in Fig. 2*A* and 2*B*, CCh was only present briefly, which could have allowed agonist-sensitive Ca^{2+} stores to reload to varying degrees when CCh was removed. We therefore also used a different protocol, in which SICR was tested in the continuous presence of 10^{-4} M CCh. Fig. 2*C* shows a typical recording in which

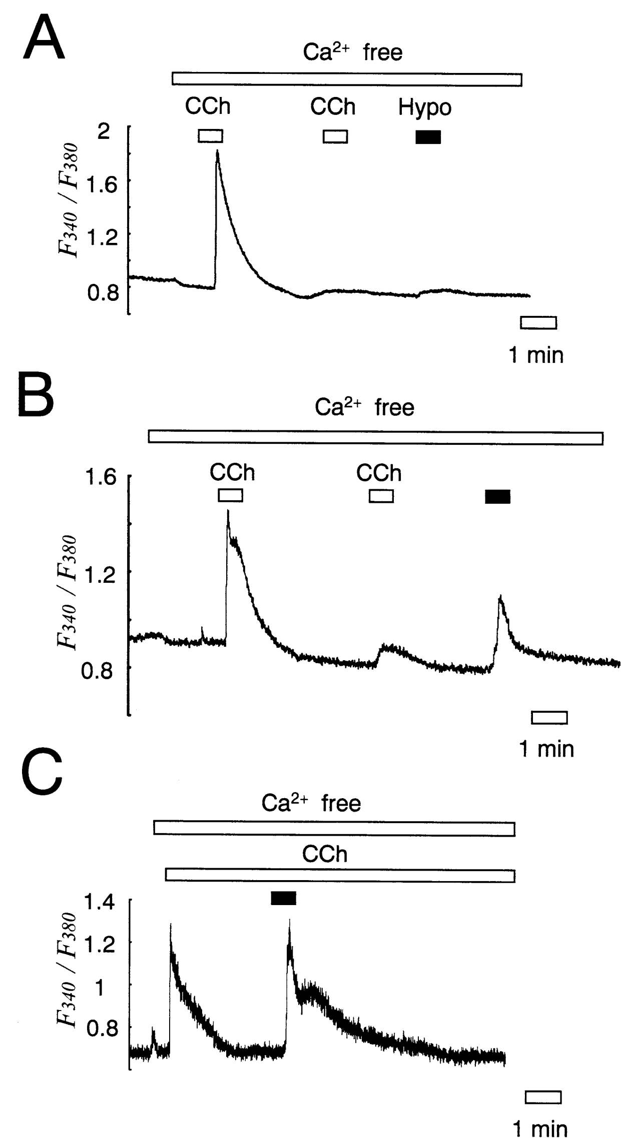

Fig. 2. Effects of depleting intracellular Ca^{2+} stores with carbachol on SICR. Dark bars in all panels indicate periods of exposure to 66% hypotonicity. (*A*) Typical response from a cell where depleting stores with 10^{-5} M CCh prevented SICR ($n = 6$). (*B*) Typical response from a cell where SICR was observed even after depletion of the $InsP_3$-sensitive Ca^{2+} store with 10^{-5} M CCh ($n = 5$). (*C*) Typical $[Ca^{2+}]_i$ response induced by hypotonic stress in the continued presence of 10^{-4} M CCh ($n = 11$).

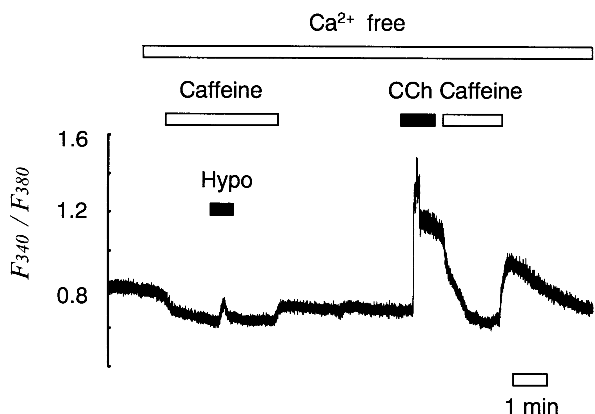

Fig. 3. Reduced SICR response in the presence of caffeine ($n = 5$). Caffeine also inhibited the increase in $[Ca^{2+}]_i$ evoked by 10^{-4} M CCh.

hypotonic shock evoked clear SICR even when CCh remained present. This pattern of response was observed in eleven of fifteen cells tested, with the other four cells showing no SICR (*data not shown*). Taken together, the results in Fig. 2*B* and 2*C* give qualified support to the idea that other types of intracellular Ca^{2+} stores, distinct from the $InsP_3$-sensitive Ca^{2+} store, exist in submandibular gland acinar cells and give rise to at least part of the SICR response. However, an alternative explanation would be that hypotonic shock was simply mobilizing $InsP_3$-sensitive stores under conditions where CCh did not evoke maximal store emptying. Although we used high (maximally effective) concentrations of CCh, it is still possible that in some cells not enough $InsP_3$ was generated to evoke maximal emptying of Ca^{2+} stores. It was noticeable that in cells where SICR could not be evoked after CCh treatment (Fig. 2*A*), the $[Ca^{2+}]_i$ transient evoked by CCh was particularly large. In the ten cells that showed responses of the Fig. 2*A* type, the mean change in fura-2 ratio with CCh was 0.58 ± 0.13 ($n = 10$). This was significantly larger ($P < 0.05$) than the peak change in ratio evoked by CCh in cells where SICR was subsequently observed (responses of the Fig. 2*B* and 2*C* type, mean change in ratio 0.35 ± 0.04, $n = 16$). We also tested the effects of ryanodine and caffeine on the SICR response. Ryanodine has been used to inhibit Ca^{2+}-induced Ca^{2+} release in salivary acinar cells, presumably via an effect on ryanodine receptors. However, ryanodine (10 μM) did not alter $[Ca^{2+}]_i$ when applied alone ($n = 4$, *data not shown*), and had no consistent effect on SICR, suggesting that ryanodine receptors were not involved in the SICR response. Caffeine (20 mM) also failed to evoke any $[Ca^{2+}]$ increase ($n = 11$, *data not shown*), in keeping with previous work in rat submandibular cells (Seo et al., 1999). However, caffeine did appear to substantially reduce SICR (Fig. 3). The mean change in ratio observed during SICR in the presence of caffeine was 0.16 ± 0.03 ($n = 5$), significantly smaller than the SICR observed in the Ca^{2+}-free control experiments ($P < 0.01$). Caffeine also clearly inhibited $InsP_3$-evoked $[Ca^{2+}]_i$ signals (Fig. 3). This is in accordance with previous work showing that caffeine acts as an inhibitor of $InsP_3$ generation in acinar cells (Toescu et al., 1992; Seo et al., 1999), although it could also reflect inhibition of the $InsP_3$ receptor.

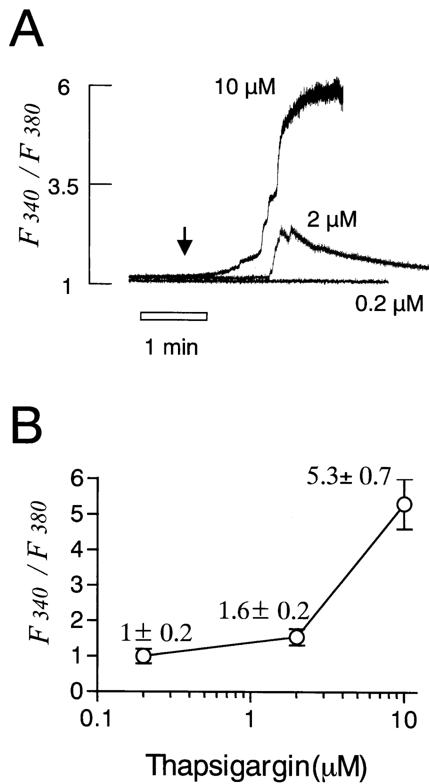

Fig. 4. Concentration dependence of the effect of thapsigargin on $[Ca^{2+}]_i$ in rat submandibular acinar cells. (*A*) Superimposed traces showing typical responses to 0.2, 2 or 10 μM thapsigargin (n = 3 to 10). The vertical arrow indicates the time when the thapsigargin was added to the superfusate. (*B*) Dose-response curve showing mean data on the $[Ca^{2+}]_i$ response to thapsigargin in the presence of 1 mM external Ca^{2+}. The half maximal concentration of thapsigargin was estimated as around 4 μM.

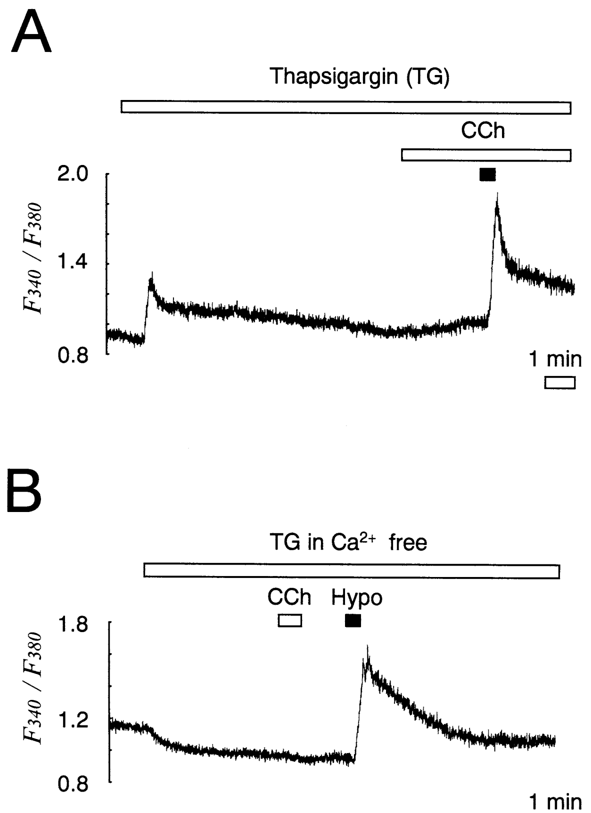

Fig. 5. Treatment of submandibular cells with thapsigargin does not prevent SICR. Dark bars indicate application of 66% hypotonic medium. Carbachol was applied at a concentration of 10^{-4} M. (*A*) Observation of SICR after emptying of Ca^{2+} stores by 2 μM thapsigargin applied for 10 min in 1 mM external Ca^{2+} (n = 10). (*B*) Observation of SICR after depleting stores with thapsigargin in Ca^{2+}-free medium (n = 4). Note that 2 μM thapsigargin had little effect on $[Ca^{2+}]_i$ under these conditions.

Are Non-$InsP_3$-Sensitive Stores Involved in SICR?

We next used the microsomal Ca^{2+}-ATPase inhibitor thapsigargin to investigate the possible role of intracellular Ca^{2+} stores distinct from the $InsP_3$-sensitive Ca^{2+} store in more detail. Since different acinar cell types vary somewhat in their "susceptibility" to thapsigargin, we first carried out a series of concentration-response experiments by examining thapsigargin's effect on $[Ca^{2+}]_i$ in the presence of Ca^{2+} in the extracellular medium. Fig. 4*A* shows typical $[Ca^{2+}]_i$ responses to three different concentrations of thapsigargin. The lowest concentration tested, 0.2 μM, hardly increased $[Ca^{2+}]_i$ (n = 3), but 2 μM thapsigargin consistently produced a substantial $[Ca^{2+}]_i$ transient, which slowly decreased back toward prestimulus levels (n = 10). The highest concentration tested, 10 μM thapsigargin, increased $[Ca^{2+}]_i$ even further, producing a sustained high plateau $[Ca^{2+}]_i$ level, and the response was irreversible (n = 3). Fig. 4*B* shows a dose-response curve for the effect of thapsigargin on $[Ca^{2+}]_i$. The half maximal concentration of thapsigargin was estimated to be approximately 4 μM.

We went on to examine whether SICR could be observed when intracellular Ca^{2+} stores loaded by SERCA-type Ca^{2+}-ATPases were depleted using thapsigargin. We applied 2 μM thapsigargin, rather than a higher concentration, in order not to produce a large increase in baseline $[Ca^{2+}]_i$. Figure 5*A* shows that SICR could still be observed following depletion of Ca^{2+} stores with 2 μM thapsigargin. However, the size of the SICR response was substantially less than in control cells (peak ratio evoked by SICR following 10 min thapsigargin was 2.03 ± 0.36, n = 10; compare with Table 1). Application of 2 μM thapsigargin for 10 min appeared sufficient to deplete SERCA-loaded Ca^{2+} stores completely, since stimulation with CCh (10^{-4} M) failed to evoke any increase of $[Ca^{2+}]_i$. We also performed similar experiments under Ca^{2+}-free conditions. Fig. 5*B* is typical of four experiments and shows that 2 μM thapsigargin had little effect on $[Ca^{2+}]_i$ in the absence of external

Ca^{2+}, presumably reflecting a relatively slow efflux of Ca^{2+} from the stores. Application of CCh (10^{-4} M) after 10-min treatment with thapsigargin did not increase $[Ca^{2+}]_i$, suggesting the agonist-sensitive Ca^{2+} stores had been depleted. However, hypotonic stress still evoked a $[Ca^{2+}]_i$ increase under these conditions (peak ratio following hypotonic stress was 1.56 ± 0.06, n = 4). This result strongly suggested that Ca^{2+} stores that are not loaded by a SERCA-type pump are also involved in SICR.

Role of Mitochondrial Calcium Stores

One candidate for a non-SERCA-loaded Ca^{2+} pool in submandibular acinar cells might be the mitochondria. We used two drugs, the uncoupling agent carbonyl cyanide *m*-cholorophenylhydrazone (CCCP) and ruthenium red, to examine the role of mitochondrial calcium stores. Like other uncoupling agents, CCCP is a protonophore that collapses the mitochondrial membrane potential. This inhibits mitochondrial Ca^{2+} uptake, which is normally driven by the negative membrane potential across the mitochondrial inner membrane (Tinel et al., 1999; Gonzalez et al., 2000). We first tested whether CCCP had any effect on Ca^{2+} release from $InsP_3$-sensitive pools. Fig. 6*A* shows a typical experiment, in which 30 sec preincubation with 100 nM CCCP had little effect on CCh-induced Ca^{2+} mobilization (compare with the subsequent CCh response recorded five min after washout of CCCP). The mean change in fura-2 ratio evoked by 10^{-4} M CCh in the presence of CCCP was 0.47 ± 0.10 (n = 6), not significantly different ($P > 0.1$) from the control CCh response in the same cells (0.44 ± 0.07, n = 6). We next tested whether CCCP altered the increase in $[Ca^{2+}]_i$ evoked by hypotonic shock (SICR) in Ca^{2+}-free medium. Fig. 6*B* shows a typical recording in which hypotonic shock hardly evoked any SICR when CCCP had previously been added together with CCh. In thirteen experiments with this protocol SICR was only observed in six of the thirteen cells, and even in these six cells the change in ratio evoked by hypotonic shock was extremely small (0.05 ± 0.02; n = 6). This was significantly smaller ($P < 0.05$) than the control SICR recorded in an identical protocol without CCCP (Fig. 2*C*, SICR change in ratio 0.35 ± 0.04, n = 16). This result clearly showed that CCCP reduced the magnitude of SICR. The effects of CCCP were similar when CCCP was added 30 sec before hypotonic stress rather than at the same time as CCh stimulation as in Fig. 6*B* (SICR was not observed in any of seven cells tested, *data not shown*).

The results with CCCP in Fig. 2*C* are broadly consistent with CCCP preventing uptake of Ca^{2+} into a (presumably mitochondrial) intracellular Ca^{2+} pool, which becomes loaded with Ca^{2+} following CCh stimulation and can then be released by hypotonic stress. We tested this idea further by using application of CCCP to try and release mitochondrial Ca^{2+} in unstimulated cells and directly following a period of stimulation with CCh. Fig. 6*C* shows that 100 nM CCCP applied a few minutes after a 3-min stimulation with 10^{-4} M CCh caused a slow, but marked, increase in $[Ca^{2+}]_i$ (three cells of six tested showed an increase in ratio with CCCP; mean response in these three CCCP-responding cells was 0.17 ± 0.07 ratio units). In contrast, application of the same concentration of CCCP for 5 min hardly altered $[Ca^{2+}]_i$ in unstimulated cells (Fig. 6*C*). We also applied CCCP in a protocol similar to that in Fig. 6*C* but immediately after the end of CCh stim-

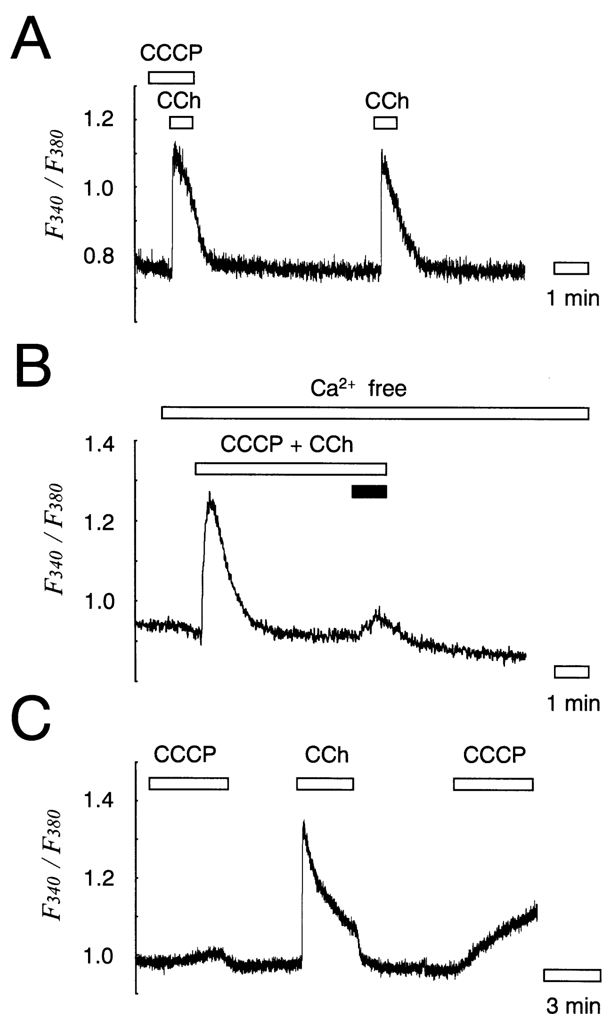

Fig. 6. Effects of CCCP on the mitochondrial Ca^{2+} store. (*A*) Lack of effect of 100 nM CCCP on Ca release evoked by 10^{-4} M CCh (typical of six experiments). (*B*) Experiment showing the inhibition of SICR by 100 nM CCCP in Ca^{2+}-free medium. The trace is typical of five other cells that showed a similar very small change in ratio, while seven other cells showed no SICR at all. (*C*) CCCP induces a gradual but substantial increase in $[Ca^{2+}]_i$ when applied after agonist stimulation, but causes little change in $[Ca^{2+}]_i$ in unstimulated cells.

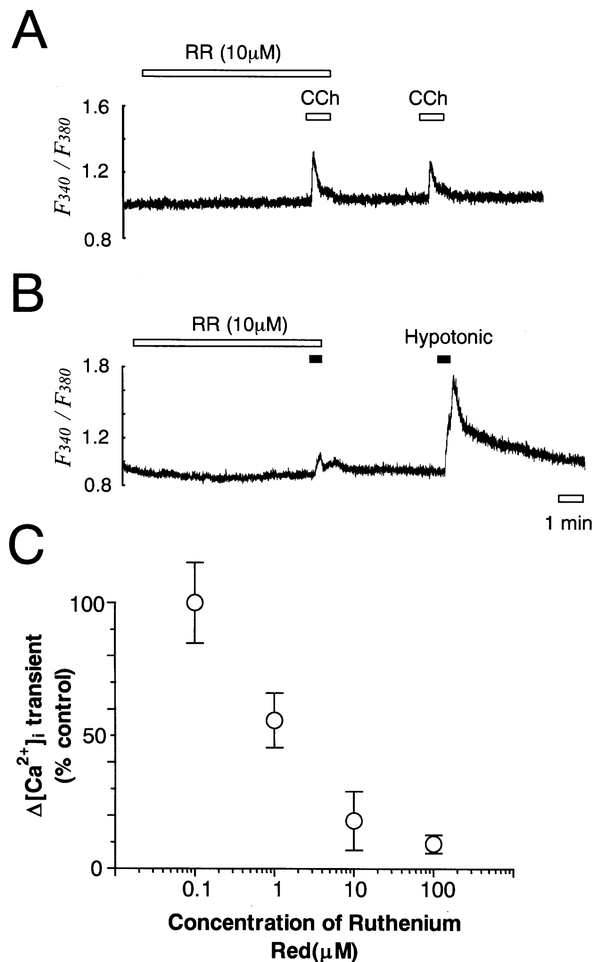

Fig. 7. Ruthenium red (RR) dose-dependently inhibits SICR. (*A*) Lack of effect of 10 μM RR on Ca release evoked by 10^{-4} M CCh ($n = 4$). (*B*) Typical experiment showing the inhibition of SICR by 10 μM RR ($n = 4$). (*C*) Dose-response curve showing the effect of preincubation with RR (for 10 min) on SICR ($n = 4$ for each concentration). RR inhibited SICR in a dose-dependent manner.

ulation. Under these circumstances the CCCP-evoked increase in $[Ca^{2+}]_i$ was slightly larger (0.25 ± 0.08 ratio units, $n = 5$; *data not shown*).

We also used ruthenium red to examine the role of mitochondrial Ca^{2+} stores in SICR. Ruthenium red is probably the most widely used inhibitor of mitochondrial Ca^{2+} handling, and acts by blocking mitochondrial Ca^{2+} uptake (Gunter & Pfeiffer, 1990; Griffiths, 2000; Gonzalez et al., 2000), although it is relatively cell-impermeable. Ruthenium red is reported to have non-mitochondrial effects in some systems, so we first tested whether the compound had any effect on Ca^{2+} release from $InsP_3$-sensitive pools. Fig. 7*A* shows that preincubation with 10 μM ruthenium red had little effect on CCh-induced Ca^{2+} release (typical of four experiments). However, the preincubation with ruthenium red significantly attenuated SICR (Fig. 7*B*, typical of four experiments). The SICR response recovered after 5 min washout of 10 μM ruthenium red (Fig. 7*B*). Fig. 7*C* shows averaged results indicating that ruthenium red inhibited SICR in a dose-dependent manner ($n = 4$ for each concentration). The results with CCCP and Ruthenium red together suggest that a Ca^{2+} store which is neither loaded by a SERCA-type pump, nor depleted by CCh, coexists with the $InsP_3$-sensitive Ca^{2+} store in at least a subpopulation of rat submandibular acinar cells. This store, most likely located in the mitochondria, and the $InsP_3$-sensitive store appear to both play a role in SICR.

Discussion

Hypotonic Swelling-Evoked $[Ca^{2+}]$ Increase and RVD

Although the magnitude of the SICR response varied between cells, the present study shows that rat submandibular acinar cells consistently responded to osmotically induced cell swelling with a substantial increase in $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ is likely to be involved in activating the RVD response that was observed during exposure to the hypotonic bath solution. In accordance with this, the time course of RVD was well correlated with the period of sustained elevation in $[Ca^{2+}]_i$.

The peak of the $[Ca^{2+}]_i$ response during the hypotonic exposure was observed before maximal cell volume was attained (*see* Fig. 1*A*), suggesting that changes in Ca^{2+} may be important in initiating regulatory volume decrease. The faster $[Ca^{2+}]_i$ response evoked by a more dilute medium (66% tonicity) suggests that the response time of the $[Ca^{2+}]_i$ increase was proportional to the rate of cell swelling. The biphasic rise in $[Ca^{2+}]_i$ induced by cell swelling resulted from both Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular medium, since a sustained elevated $[Ca^{2+}]_i$ was not observed under Ca^{2+}-free conditions (*see* Fig. 1*C*).

Although there is general agreement that exocrine acinar cells undergo RVD via opening of Ca^{2+}-activated K^+ and Cl^- channels, the precise role of $[Ca^{2+}]_i$ in this process is controversial. In lacrimal acinar cells, several studies indicate that activation of Ca^{2+}-dependent K^+ and Cl^- channels, and RVD, are dependent on the presence of external Ca^{2+} (Kotera & Brown, 1993; Park et al., 1994; Speake et al., 1998). However, no increase in $[Ca^{2+}]_i$ could be detected in fura-2-loaded lacrimal acinar cells subjected to hypotonic stress (Elliott, 1994; Speake et al., 1998). The resolution of this discrepancy is unclear. However, there is evidence that the physiology of Ca^{2+} stores in acinar cells may be altered by how cells are isolated and kept (Smith et al., 2000). This has been suggested to result from alterations in the architecture of the cytoskeleton, which appears to play a role in controlling both Ca^{2+} release and Ca^{2+}

entry (for references *see* Elliott, 2001). The idea that the cytoskeleton may be a critical determinant of whether hypotonic shock causes an increase in $[Ca^{2+}]_i$ is intriguing, since cell swelling, and the consequent changes in cell membrane architecture, would be expected to have profound effects on the arrangement of cytoskeletal elements near to or attached to the plasmalemma.

Source of SICR

Several lines of evidence suggest that a large part of the Ca^{2+} release evoked by cell swelling was derived from the $InsP_3$-sensitive Ca^{2+} store. Firstly, depletion of the $InsP_3$-sensitive Ca^{2+} store with CCh reduced, and in some cases abolished, the increase of $[Ca^{2+}]_i$ produced by SICR (Fig. 2). Secondly, the increase in $[Ca^{2+}]_i$ evoked by hypotonic stress was reduced, although not abolished, by thapsigargin treatment. Thirdly, SICR was inhibited by caffeine (*see below*). Finally, if hypotonic shock causes release of $InsP_3$-sensitive Ca^{2+} stores, this would offer an explanation for the Ca^{2+} entry seen following hypotonic exposure, which would presumably be expected via depletion-activated (capacitative) Ca^{2+} entry (Putney, 1986).

We also tested for the involvement of ryanodine receptors in the SICR response, since there is evidence for the existence of caffeine- or ryanodine-sensitive intracellular Ca^{2+} stores in salivary acinar cells, particularly parotid acinar cells (Foskett & Wong, 1991; Giannini et al., 1995; DiJulio et al., 1997; Yamaki et al., 1998). Furthermore, expression of ryanodine receptors as well as $InsP_3$ receptors has been reported in rat submandibular gland (Lee et al., 1997). However, neither 10 μM ryanodine nor 20 mM caffeine produced any increase in $[Ca^{2+}]_i$ in our study, which suggests that rat submandibular cells express only very low levels of ryanodine receptors. A similar argument has been advanced for HeLa cells, which express type 2 ryanodine receptors, but in which neither caffeine nor ryanodine caused any detectable elevation of $[Ca^{2+}]_i$ (Bennett et al., 1996). Despite the absence of any Ca^{2+}-mobilizing effects of caffeine alone, caffeine inhibited SICR (Fig. 3). If SICR involves $InsP_3$-sensitive Ca^{2+} stores, as discussed above, then inhibition of SICR by caffeine might be explained by an action of caffeine on either $InsP_3$ metabolism or directly on the $InsP_3$ receptor. Caffeine has been shown to inhibit the increase in $[Ca^{2+}]_i$ induced by muscarinic and α-adrenergic agonists in submandibular cells by inhibiting $InsP_3$ production (Seo et al., 1999), while inhibitory effects of caffeine on the $InsP_3$ receptor have been described in numerous studies (for references *see* Maes et al., 1999).

If SICR does involve the $InsP_3$-sensitive Ca^{2+} store, the question arises of how Ca^{2+} is released following cell swelling. A transient increase in cellular $InsP_3$ induced by cell swelling has been reported in proximal tubule cells and in hepatocytes (Susuki et al., 1990; Baquet, Meijer & Hue, 1991), suggesting that $InsP_3$ may play the role of an intracellular messenger in SICR. An alternative would be that the swelling somehow sensitizes the stores to resting levels of $InsP_3$. The inhibitory effect of caffeine is broadly consistent with either of these possibilities, given that caffeine can inhibit both $InsP_3$ generation and InsPs-dependent Ca^{2+} release (*see above*).

Non-SERCA-loaded Intracellular Ca^{2+} Stores and their Role in SICR

Since we could find no clear functional evidence for caffeine- or ryanodine-sensitive Ca^{2+} stores, we further investigated the possible involvement of SERCA Ca^{2+}-ATPase-loaded Ca^{2+} stores in SICR using the SERCA inhibitor thapsigargin, which has been used to deplete SERCA-loaded Ca^{2+} pools in a wide variety of cell types, including salivary gland acinar cells (Foskett, Roifman & Wong, 1991; Foskett & Wong, 1991; Sakai & Ambudkar,1996; Zimmermann & Walz, 1997). We found that SICR could still be observed after cells had been treated with 2 μM thapsigargin for 10 min, either in the presence or the absence of extracellular Ca^{2+}. This thapsigargin concentration and application time seemed to be sufficient to deplete SERCA-loaded Ca^{2+} stores fully, since the $[Ca^{2+}]_i$ response to maximal concentration of CCh was abolished (*see* Fig. 5*A*). This result strongly suggests that non-SERCA-loaded Ca^{2+} stores do exist in submandibular gland and are also involved in SICR.

Possible Role of Mitochondria

The most obvious candidate for a non-SERCA-loaded Ca^{2+} store that might be involved in SICR, is the mitochondrion. It is increasingly clear that mitochondria can help to regulate $[Ca^{2+}]_i$ in a number of different settings. Under conditions where cytosolic Ca^{2+} is elevated for prolonged periods, loading of the mitochondrial Ca^{2+} pool occurs in many cell types, including neuron (White & Reynolds, 1997) and lacrimal gland acinar cells (Bird et al., 1992). Mitochondria can also regulate $[Ca^{2+}]_i$ in some cell types under more physiological conditions, for instance, sequestering Ca^{2+} and tuning the frequency of $[Ca^{2+}]_i$ oscillations in rat gonadotropes (Kaftan et al., 2000).

The evidence that mitochondria play a physiological role in $[Ca^{2+}]_i$ regulation in acinar cells is accumulating, although still somewhat controversial. Tinel et al. (1999) reported that mitochondria surrounding the pancreatic acinar granule region prevent the spreading of $InsP_3$-evoked local (apical

pole) $[Ca^{2+}]_i$ signals to the basolateral cytoplasm and nucleus. These authors found that, when mitochondrial function was inhibited, agonist stimulation resulted in a global increase of $[Ca^{2+}]_i$, due to the loss of this "barrier" function of the mitochondria. The findings of this study have subsequently been confirmed and extended by Straub et al. (2000) and Park et al. (2001), although they were disputed by Gonzalez et al. (2000), who reported a more homogeneous distribution of mitochondria in the pancreatic acinar cell. Gonzalez et al. (2000) demonstrated that mitochondria in pancreatic acinar cells did not contain releasable Ca^{2+} at rest but did accumulate releasable Ca^{2+} when $[Ca^{2+}]_i$ was increased by stimulating cells with a physiological agonist or a SERCA inhibitor, a finding also confirmed by Park et al. (2001). Our results with CCCP application (Fig. 6*C*) similarly suggest that mitochondria in submandibular acinar cells only become loaded with Ca^{2+} following stimulation.

Inhibiting Mitochondrial Ca^{2+} Uptake with CCCP or Ruthenium Red Inhibits SICR

The most compelling piece of evidence for mitochondrial Ca^{2+} stores playing a role in SICR was the inhibitory effect of CCCP and ruthenium red on SICR. Preincubation with CCCP or ruthenium red did not affect the CCh-induced $[Ca^{2+}]_i$ transient. This suggested that the drugs did not inhibit ER Ca^{2+} release, notwithstanding the evidence that Ruthenium Red can inhibit ryanodine receptor Ca^{2+} release channels in muscle sarcoplasmic reticulum (Garcha & Hughes, 1994; Kanmura, Raeymaekers & Casteels, 1989; Netticadan, Xu & Narayanan, 1996; Vites & Pappano, 1994; Kargacin, Ali & Kargacin, 1998). Given the lack of effect of CCCP and ruthenium red on ER Ca^{2+} release, the inhibitory effect of both drugs on SICR clearly implies a role for mitochondrial stores. By analogy with the work of Gonzalez et al. (2000) and Park et al. (2001) on pancreatic acinar cells, we suggest that these mitochondrial stores gradually become loaded with Ca^{2+} over 1–2 min when $[Ca^{2+}]_i$ is raised by an agonist, by a SERCA inhibitor, or by swelling-induced Ca^{2+} release from the $InsP_3$-sensitive store.

As with the $InsP_3$-sensitive store, the question arises of how cell swelling evokes mitochondrial Ca^{2+} release. Our study does not give any clear indication on this point. Again, it is tempting to suggest that the effect of cell swelling might be transduced via cytoskeletal attachment, in this case of mitochondria. Gonzalez et al. (2000) and Park et al. (2001) recently reported that mitochondria are often found in submembrane locations in pancreatic acinar cells, an architecture that is presumably maintained by cytoskeletal or membrane attachment.

Does Variable Mitochondrial Function Give Rise to the Variability in SICR Following Store Depletion?

The interpretation of experiments examining the source of the Ca^{2+} giving rise to SICR was complicated by the fact that we observed two distinct types of $[Ca^{2+}]_i$ response to hypotonic stress after depletion of $InsP_3$-sensitive Ca^{2+} stores with CCh. In one group of cells, SICR could be observed, while in the other, it could not. These two types of $[Ca^{2+}]_i$ response, which occurred in roughly equal proportions, were observed on the same day in cells prepared from the same rat. Furthermore, there was no difference in the number of cells in the cell cluster, or in cell size, between the two groups. Interestingly, there was a significant difference in the magnitude of the $[Ca^{2+}]_i$ transient induced by CCh in cells in the two groups, with the $[Ca^{2+}]_i$ transient induced by CCh in the cells that subsequently showed SICR being smaller than in the cells that did not show SICR. This result could be explained if mitochondria in the second group of cells did not take up Ca^{2+} when $[Ca^{2+}]_i$ was raised by CCh stimulation. This would have the dual result that the CCh-evoked $[Ca^{2+}]_i$ transient would be larger and that no Ca^{2+} would be sequestered in mitochondria for subsequent release by SICR. This hypothesis is also supported to some extent by the fact that SICR was consistently observed in cells pretreated with 2 μM thapsigargin for 10 min. These cells had a persistently elevated $[Ca^{2+}]_i$ throughout the exposure to thapsigargin, which may have favored mitochondrial Ca^{2+} loading (*see* Gonzalez et al., 2000). An alternative hypothesis would be based on the observation that, following mitochondrial Ca^{2+} loading in pancreatic acinar cells, mitochondria subsequently lose Ca^{2+} over minutes to tens of minutes (Gonzalez et al., 2000; Park et al., 2001). The cells where no SICR was observed could thus simply be those cells where loss of Ca^{2+} from mitochondria was relatively fast, so that mitochondria no longer contained releasable Ca^{2+} when the hypotonic shock was applied. Finally, Gonzalez et al. (2000) have suggested that the extent and effect of mitochondrial Ca^{2+} uptake in pancreatic acinar cells may depend on the precise morphological arrangement of the mitochondria in a given cell, and this has recently been confirmed experimentally (Park et al., 2001). The presently available evidence suggests that a large global increase in $[Ca^{2+}]_i$, such as that produced by maximal agonist stimulation, will probably lead to all mitochondria becoming loaded with Ca^{2+} to some extent (e.g., Park et al., 2001). Nonetheless, it remains possible that the different classes of SICR response we observed might be produced by a subtle difference in the subcellular distribution of mitochondria, which in turn could determine the precise extent and time course of mitochondrial Ca^{2+} loading following stimulation.

In summary, we have demonstrated that hypotonic cell swelling activates intracellular Ca^{2+} release and prolonged Ca^{2+} entry in rat submandibular acinar cells. All the results we have obtained are consistent with hypotonic stress inducing Ca^{2+} release both from $InsP_3$-sensitive Ca^{2+} stores and from mitochondria. However, SICR from the mitochondria seems only possible when the mitochondria have been loaded with Ca^{2+} by elevation of $[Ca^{2+}]_i$. As in other systems, this would be consistent with one role of mitochondria being to limit the increase in $[Ca^{2+}]_i$ induced by high levels of stimulation.

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