The Route of Ca$^{2+}$ Entry during Reloading of the Intracellular Ca$^{2+}$ Pool in Pancreatic Acini*

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To trace the route of Ca$^{2+}$ entry and the role of the cytosolic Ca$^{2+}$ pool in reloading of the internal stores of pancreatic acinar cells, Mn$^{2+}$ influx into Fura 2-loaded cells and the effect of 1,2-bis(2-aminophenoxyethane-$N,N',N'$)-tetracetic acid (BAPTA) on Ca$^{2+}$ storage in intracellular stores and reloading were examined. Treatment of acini suspended in Ca$^{2+}$-free medium with carbachol (cell stimulation) or carbachol and atropine (reloading period) resulted in 2-fold increase in the rate of Mn$^{2+}$ influx. Increasing Ca$^{2+}$ permeability of the plasma membrane by elevation of extracellular pH from 7.4 to 8.2 further increased the rate of Mn$^{2+}$ influx observed during cell stimulation and the reloading period. Loading the acini with BAPTA by incubation with 50 μM of the acetomethoxy form of BAPTA (BAPTA/AM) was followed by a transient reduction in free cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$). To compensate for the increased Ca$^{2+}$ buffering capacity in the cytosol the acini incorporated Ca$^{2+}$ from the external medium. Although BAPTA prevented changes in free cytosolic Ca$^{2+}$ concentration during carbachol and atropine treatment, it had no apparent effect on Ca$^{2+}$ content of the internal stores or the ability of agonists to release Ca$^{2+}$ from these stores. Loading the cytosol with BAPTA considerably reduced the rate of Ca$^{2+}$ reloading. These observations are not compatible with direct communication between the medium and the inositol 1,4,5-trisphosphate releasable pool and provide direct evidence for Ca$^{2+}$ entry into the cytosol prior to its uptake into the intracellular pool, both during cell stimulation and the Ca$^{2+}$ reloading.

Stimulation of non-exitable cells by Ca$^{2+}$ mobilizing agonists results in an increase of [Ca$^{2+}$], due to activation of at least 2 passive pathways. The first involves release of Ca$^{2+}$ from intracellular stores by In-1,4,5-P$_3$ (1). This second messenger activates a Ca$^{2+}$ channel (2, 3) either in a discrete component of the endoplasmic reticulum (4, 5) or in a separate organelle, the calciosome (6). Because this is a limited source of [Ca$^{2+}$], the change in intracellular Ca$^{2+}$ is transient. The second pathway is across the plasma membrane and therefore results in Ca$^{2+}$ entry into the cells by a receptor operated Ca$^{2+}$ channel or pathway (7-14). In the case of pancreatic exocrine cells, the latter pathway has been shown to be essential for the sustained phase of secretion, the former for the initial phase of secretion (15, 16).

During stimulation, regulation of intracellular Ca$^{2+}$ also involves active transport. It has been shown that stimulation also results in changes in the turnover rate of both the plasma membrane Ca$^{2+}$ pump (17, 18) and the Ca$^{2+}$ pump in the membrane of the intracellular Ca$^{2+}$ store (19-21).

Restoration of the resting state in such cells requires reloading of the intracellular Ca$^{2+}$ store which has been shown universally to depend on Ca$^{2+}$ uptake from the medium, thus requiring Ca$^{2+}$ transit across the plasma membrane. Indeed it has been shown that stimulation enhances plasma membrane Ca$^{2+}$ entry by 5-7-fold (7, 18) and that this increased influx is maintained as long as the internal stores are depleted of Ca$^{2+}$. Even long after cell stimulation has terminated (9, 13). Reuptake of Ca$^{2+}$ into the intracellular pool also maintains activity of the pool Ca$^{2+}$ pump which is increased during stimulation (19-21). The mechanism of regulation of Ca$^{2+}$ reloading has remained controversial. Reloading of the intracellular store occurs at a constant level of [Ca$^{2+}$], identical to the resting level (9, 22-24). Since Ca$^{2+}$ entry is required for reloading, this finding has been taken to mean that there was a direct entry of medium Ca$^{2+}$ into the intracellular store, without transit through the cytoplasm, a “privileged” pathway for Ca$^{2+}$ (22, 25), or that Ca$^{2+}$ entry occurs through a restricted region where the endoplasmic reticulum and the plasma membrane are closely opposed (26). This hypothesis would require direct contact between the Ca$^{2+}$ stores membrane and the plasma membrane or a vesicle shuttling mechanism between the two membranes. However, if the pool Ca$^{2+}$ pump remains activated during reloading and is dominant with respect to Ca$^{2+}$ entry, it is quite possible to conceive of reloading at resting or even below resting [Ca$^{2+}$].

Using Mn$^{2+}$ entry as an index of plasma membrane Ca$^{2+}$ permeability and a cytosolic Ca$^{2+}$ chelator such as BAPTA, it is possible to exclude a direct pathway for Ca$^{2+}$ reloading. During reloading of pancreatic acini, we have shown an increased rate of Mn$^{2+}$ influx. Further, BAPTA decreases the rate but not the extent of reloading of the cell Ca$^{2+}$ store. The pathway for reloading must therefore transit the cytosolic compartment of the cell, and regulation of [Ca$^{2+}$] during reloading is due to the properties of the plasma membrane entry pathway and the pool Ca$^{2+}$ pump.

**EXPERIMENTAL PROCEDURES**

**Materials**

CCK-OP was a generous gift from the Squibb Institute. Soybean trypsin inhibitor, EGTA, carbachol, and atropine were from Sigma.
Purified collagenase (type CLSPA) was from Worthington. \(^{45}\)Ca was from DuPont-New England Nuclear. Fura 2/AM, Fluo 3/AM and BAPTA/AM were from Molecular Probes.

The incubation solution (solution A) contained 20 mM HEPES (at pH 7.4 or 8.4 with Tris), 120 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 10 mM glucose, 10 mM sodium pyruvate, 0.1% (wt/vol) bovine serum albumin, 0.01% (wt/vol) soybean trypsin inhibitor and with or without 1 mM CaCl\(_2\). When CaCl\(_2\) was omitted from the medium, Ca\(^{2+}\) concentration was approximately 10 \(\mu\)M. In some experiments 0.1 mM EGTA was added to nominally Ca\(^{2+}\)-free medium which reduced Ca\(^{2+}\) concentration to approximately 70 nm.

**Methods**

Preparation of Pancreatic Acini—Dispersed pancreatic acini were prepared from rats (75-150 g) by the procedures previously described (27, 28). The procedure involves two cycles of 10-15 min of digestion with collagenase of minced pancreas, wash of acini with solution A, and passage through a nylon mesh. The acini from one pancreas were suspended in 10-20 ml of solution A and either used immediately or kept on ice for up to 1 h before use.

Measurements of Free Cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) was measured by loading the acini with Fura 2 or Fluo 3. Acini from half a pancreas were suspended in 4 ml of solution A containing 1 mM CaCl\(_2\) and were incubated with 4 \(\mu\)M Fura 2/AM or 10 \(\mu\)M Fluo 3/AM for 30 min at 37°C. The cells were then washed twice with 35 ml of solution A and resuspended in 1.5-2 ml of the same solution. Then 100 \(\mu\)l of cell suspension was transferred to 1.9 ml of prewarmed medium, and fluorescence measurements were performed while the acini were continually stirred and maintained at 37°C. Fluorescence was measured with a Perkin-Elmer spectrofluorometer model LS-5B. For Fura 2 the excitation wavelengths were set at 340 or 385 nm and emission wavelength set at 500 nm for both [Ca\(^{2+}\)]\(_i\) and [Mn\(^{2+}\)], measurements. For Fluo 3 the excitation and emission wavelengths were set at 506 and 526 nm, respectively. The fluorescence signal of both dyes were calibrated by releasing the dyes from the cells with digitonin into medium containing 1-2 mM CaCl\(_2\) to obtain \(F_{max}\). Subsequently 10 mM EGTA and sufficient NaOH to elevate medium pH to above 8.5 were added to obtain \(F_{basal}\). [Ca\(^{2+}\)]\(_i\) was measured as described above using a 202 of 220 nm for the Fura 2-Ca (29) and 400 nm for the Fluo 3-Ca (30) dissociation constants.

\(^{45}\)Ca Fluxes—Acini suspended in a solution of desired composition were incubated with \(^{45}\)CaCl\(_2\) (about 10 \(\mu\)C/ml) at 37°C under continuous shaking. The indicated times, 0.2-m1 samples were transferred to 10 ml of ice-cold, Ca\(^{2+}\)-free solution A containing 1 mM LaCl\(_3\). The acini were collected by 30-s centrifugation at 150 x g and washed twice more with the same solution. The acini were then dissolved by heating at 60°C in 1 ml of 1 M NaOH for 20 min, and \(^{45}\)Ca was counted using standard liquid scintillation counting.

**RESULTS**

Plasma Membrane Permeability—The rate and extent of quenching of Fura 2 fluorescence by Mn\(^{2+}\) was used to determine the effect of agonists on plasma membrane permeability to Ca\(^{2+}\). Fig. 1 shows the entry of Mn\(^{2+}\) into pancreatic acini under different conditions. In the absence of any added agonist, there was a relatively slow quench of intracellular Fura 2. This shows that there is a basal plasma membrane Mn\(^{2+}\) permeability even in the absence of agonists.

Following maximal carbachol stimulation in this Ca\(^{2+}\)-free medium, there was a rapid increase in [Ca\(^{2+}\)]\(_i\), due to release of intracellular Ca\(^{2+}\). Subsequently, the cells reduced [Ca\(^{2+}\)], to slightly below resting levels, due to Ca\(^{2+}\) export across the plasma membrane. When Mn\(^{2+}\) was added to these stimulated cells, there was a more rapid quenching of Fura 2 fluorescence. This can be interpreted as showing an increased rate of Ca\(^{2+}\) entry due to stimulation (Fig. 1b). A similar result was seen when atropine was used to terminate the stimulation. Thus, following stimulation by carbachol and inhibition by atropine (to initiate reloading), the addition of Mn\(^{2+}\) resulted in a larger rate of quench than in the absence of any treatment (Fig. 1c).

Table I summarizes the data from 12 similar experiments.

In the cells that had been stimulated, as compared with resting cells, the addition of digitonin gave a smaller quench of Fura 2 fluorescence, showing that the stimulated cells, whether treated subsequently with atropine or not, had a larger quantity of intracellular Mn\(^{2+}\). This is consistent with the enhanced rate of Mn\(^{2+}\) entry. Furthermore, this shows that the cells are not able to actively export Mn\(^{2+}\).

An increase of extracellular pH increases Ca\(^{2+}\) influx through agonist activated Ca\(^{2+}\) channels (31). The experiments in Fig. 2 were therefore carried out at pH 8.2. It can be seen that the rate of Mn\(^{2+}\) entry was enhanced in each of the experimental situations. The resting rate of Mn\(^{2+}\) induced quench was increased by 1.44-fold. The entry, post carbachol or carbachol and atropine treatments, was enhanced by approximately 3.8-fold. Furthermore, following stimulation, digitonin had no effect on Fura 2 fluorescence showing that the
In the absence of extracellular Ca\(^{2+}\), BAPTA reduced \([\text{Ca}^{2+}]\) by 72 ± 23\(^{\text{f}}\) resting cells is required for recovery of normal \([\text{Ca}^{2+}]\). Thus, the maintained Ca\(^{2+}\) entry noted above with Mn\(^{2+}\) in to interaction of the chelator with the dye. Since difference spectra are inappropriate for Fluo 3 and Mn\(^{2+}\) quench of its transient and an enhanced steady state \([\text{Ca}^{2+}]\). CCK-OP gave f 6 nM (n = 8) and \([\text{Ca}^{2+}]\) was restored with a sustained reduction of \([\text{Ca}^{2+}]\) by BAPTA (33). However, when cells were loaded with Fluo 3 in Ca\(^{2+}\) containing medium, there was only a transient decrease in the Fluo 3 signal showing that over the time period of the experiment in Fig. 3 data show that at the time of carbachol addition resting \([\text{Ca}^{2+}]\) had been restored. The fall in \([\text{Ca}^{2+}]\) induced by atropine inhibition, showing that the stores had been reloaded with Ca\(^{2+}\).

**Effects of Ca\(^{2+}\) Chelator on Fura 2 Signals**—If the cytosolic compartment is obligatory for Ca\(^{2+}\) reloading, then chelation of Ca\(^{2+}\) in this compartment should affect reloading. If the reloading is separate from the cytosolic compartment, either by direct communication or by vesicle shuttling, chelation of \([\text{Ca}^{2+}]\) by intracellular BAPTA should be without any effect. Fig. 3a shows the effect of carbachol, atropine, and CCK-OP added sequentially on \([\text{Ca}^{2+}]\), level of cells suspended in normal Ca\(^{2+}\) medium. Carbachol produced the usual \([\text{Ca}^{2+}]\), transient and an enhanced steady state \([\text{Ca}^{2+}]\). CCK-OP gave a second Ca\(^{2+}\) signal following atropine inhibition, showing that the stores had been reloaded with Ca\(^{2+}\).

Fig. 3, b and c, show that BAPTA interacts with Fura 2. When recorded at excitation wavelengths of 340 (Fig. 3b) and 385 nm (Fig. 3c), BAPTA increased fluorescence at both 340 and 385 nm, the increase at the latter wavelength being greater. It has been suggested that these data are due to sustained reduction of \([\text{Ca}^{2+}]\), by BAPTA (33). However, when cells were loaded with Fluo 3 in Ca\(^{2+}\) containing medium, there was only a transient decrease in the Fluo 3 signal showing that over the time period of the experiment in Fig. 3, b and c, \([\text{Ca}^{2+}]\), was restored to normal levels. In Ca\(^{2+}\)-containing medium BAPTA loading decreased \([\text{Ca}^{2+}]\), by 32 ± 6 nM (n = 8) and \([\text{Ca}^{2+}]\), was restored with a \(t_{\alpha}\) of 2.5 min. In the absence of extracellular Ca\(^{2+}\), BAPTA reduced \([\text{Ca}^{2+}]\), by 72 ± 23 nM (n = 8) without recovery to resting levels. Thus, the maintained Ca\(^{2+}\) entry noted above with Mn\(^{2+}\) in resting cells is required for recovery of normal \([\text{Ca}^{2+}]\). The action of BAPTA on the Fura 2 signal is probably due in part to interaction of the chelator with the dye. Since difference spectra are inappropriate for Fluo 3 and Mn\(^{2+}\) quench of its fluorescence is less than that of Fura 2 (not shown), all further fluorescence experiments were done using Fura 2.

In six different experiments it was found that incubating acini with 50 \(\mu\text{M}\) BAPTA/AM for 15 min in the absence of Ca\(^{2+}\) or 20 min in the presence of Ca\(^{2+}\) in the incubation medium was sufficient to completely buffer changes in \([\text{Ca}^{2+}]\), upon stimulation with carbachol. At intermediate loading of BAPTA, the effect of cytosolic Ca\(^{2+}\) chelation on the \([\text{Ca}^{2+}]\), signal can be explored. Fig. 3, b and c, shows that incubating the acini with 50 \(\mu\text{M}\) BAPTA/AM for 7 min significantly reduced the \([\text{Ca}^{2+}]\), increase induced by carbachol. The Fluo 3 data show that at the time of carbachol addition resting \([\text{Ca}^{2+}]\), had been restored. The fall in \([\text{Ca}^{2+}]\), induced by atropine returned to base line but with a slower time course than that observed without BAPTA (Fig. 3 a-c). Further, there was only a small increment in \([\text{Ca}^{2+}]\), when CCK-OP was added (Fig. 3, b and c) in contrast to the equivalent release seen in the absence of BAPTA (Fig. 1a). This could be due to lower Ca\(^{2+}\) content of the stores or due to buffering of \([\text{Ca}^{2+}]\). To examine the various possibilities, studies were performed with \(^{40}\text{Ca}\).

**Effect of Ca\(^{2+}\) Chelation on \(^{40}\text{Ca}\) Response**—It has been shown that an efficient means of labeling the intracellular pool with \(^{40}\text{Ca}\) is to cycle the acini through an unloading phase with carbachol and then allow loading in the presence of atropine and \(^{40}\text{Ca}\) (18, 34). The result of such labeling is illustrated in Fig. 4, where the bottom curve shows direct uptake in the absence of cycling and the upper curve shows the effect of cycling. Addition of BAPTA/AM resulted in enhanced and parallel uptake of \(^{40}\text{Ca}\) into control and cycled cells. Hence, during BAPTA loading the acini incorporated Ca\(^{2+}\) from the extracellular medium to compensate for the increased Ca\(^{2+}\) buffering capacity of the cytosol. More importantly, loading with BAPTA does not appear to deplete the

![FIG. 2. Effect of medium pH on Mn\(^{2+}\) influx. The experimental protocols were identical to those in Fig. 1 except that medium pH after addition of acini was 8.2. Resting [Ca\(^{2+}\)] was 135 ± 24 nM and carbachol increased [Ca\(^{2+}\)] to a peak of 1084 ± 103 nM (n = 4). Dig., digitonin.](image)

![FIG. 3. Effect of BAPTA on [Ca\(^{2+}\)]. Acini loaded with Fura 2 were suspended in solution A containing 2 mM CaCl\(_2\) and exposed to the indicated concentrations of carbachol, atropine, and CCK-OP (a). Acini were exposed to 50 \(\mu\text{M}\) BAPTA/AM while Fura 2 fluorescence was measured at excitation wavelengths of 340 (b) or 385 (c). After 7 min of incubation with BAPTA/AM, the acini were exposed to carbachol, atropine, or CCK-OP. Acini loaded with Fluo 3 were suspended in solution A containing 2 mM CaCl\(_2\) (d) or solution A containing 0.1 mM EGTA (e). BAPTA/AM 50 \(\mu\text{M}\) was then added where indicated in the figure.](image)
The internal stores completely reloaded with Ca2+ within 10 min of incubation at 37 °C in order to deplete the intracellular pool. Then at time zero CaCl2 (+45Ca) or a mixture of CaCl2 (+45Ca) and atropine (A) was added to yield a final concentration of 2 mM CaCl2 and 20 μM atropine and suspended to the respective groups. After 7.5 min of incubation at 37 °C a portion of control (O) or cycled (A) acini was transferred to tubes containing BAPTA/AM to yield a final concentration of 50 μM (O, A). At the indicated times samples were removed to determine 45Ca content. 45Ca uptake into control acini incubated for 25 min with 45Ca was taken as 100% control and 45Ca2+ uptake into the experimental groups were calculated as % of control. The figure shows the mean ± S. E. of three separate experiments.

Following 45Ca2+ labeling by the cycling procedure, BAPTA loading, and suspending the acini in 45Ca2+-free medium, there is a gradual loss of tracer Ca2+. In control acini, when no BAPTA is present, a steady state of counts is reached at about 80% of initial value, with a t½ of about 1.5 min. The addition of CCK-OP now gave a rapid release of the counts, with about 10% of initial 45Ca remaining associated with the acini 3 min after the addition of CCK-OP (Fig. 5). In case of the BAPTA containing acini, about 35% of the counts were lost initially with a t½ of about 3 min. However, following CCK-OP there was a marked reduction in the rate of 45Ca2+ loss as compared to the rate in the absence of BAPTA, although the final level was similar. This is consistent with buffering of [Ca2+]i by BAPTA and consequent reduction of plasma membrane Ca2+ pump activity accounting for the lower rate of loss of Ca2+ in the BAPTA-treated cells (Fig. 5).

To exclude further that the effect of BAPTA was due to a reduction in the size of the intracellular pool, and to provide evidence that BAPTA affected the rate, but not the magnitude of reloading, the rate of 45Ca uptake was studied. Acini were suspened in nominally Ca2+-free medium, stimulated with carbachol to deplete the internal Ca2+ pool, and loaded with BAPTA. Subsequent to BAPTA loading, Ca2+ reloading was initiated by the addition of a mixture of atropine and Ca2+ labeled with 45Ca. BAPTA increased the extent of Ca2+ uptake into unstimulated acini but decreased the rate of Ca2+ reloading (Fig. 6A). To estimate the effect of BAPTA on reloading rate, 45Ca uptake into control acini was subtracted from 45Ca uptake into cycled acini (Fig. 6B). It can be seen that BAPTA decreased the reloading rate by approximately 3-fold although the internal stores completely reloaded with Ca2+ within 10 min of incubation at 37 °C.

### Figures

**Figure 4.** Effect of BAPTA on Ca2+ content of pancreatic acini. Acini were washed and suspended in nominally Ca2+-free solution A (O). A portion of the acini was stimulated with 0.2 mM carbachol for 5 min at 37 °C in order to deplete the internal stores of Ca2+ (■). Then at time zero CaCl2 (+45Ca) (O) or a mixture of CaCl2 (+45Ca) and atropine (A) to yield a final concentration of 2 mM CaCl2 and 20 μM atropine were added to the respective groups. After 7.5 min of incubation at 37 °C a portion of control (O) or cycled (A) acini was transferred to tubes containing BAPTA/AM to yield a final concentration of 50 μM (O, A). At the indicated times samples were removed to determine 45Ca content. 45Ca uptake into control acini incubated for 25 min with 45Ca was taken as 100% control and 45Ca2+ uptake into the experimental groups were calculated as % of control. The figure shows the mean ± S. E. of three separate experiments.

**Figure 5.** Effect of BAPTA on agonist-stimulated Ca2+ efflux. Acini suspended in nominally Ca2+-free solution A were stimulated with carbachol for 5 min at 37 °C. Then 2 mM CaCl2 (+45Ca) and 20 μM atropine were added. After 7.5 min of incubation at 37 °C a portion of the acini was transferred to a tube containing BAPTA/AM to a final concentration of 50 μM and the incubation continued for an additional 15 min. The "cycled" (O) and "cycled" BAPTA-loaded acini (A) were then collected by centrifugation and resuspended in solution A containing 2 mM of unlabeled CaCl2. After 5 min of incubation a portion of control from each group was stimulated with 10 nm CCK-OP (O, A). At the indicated times samples were removed to measure 45Ca2+ content in the acini. The initial 45Ca content of "cycled" acini was taken as 100% control and 45Ca content was calculated as % of control. The figure shows the mean ± S. E. of three separate experiments.

**Figure 6.** Effect of BAPTA on reloading. Acini were washed and suspended in nominally Ca2+-free solution A (O, A) and stimulated with 0.2 mM carbachol (Carb.) for 5 min at 37 °C (O, A). Then control (O) and carbachol-stimulated (A) acini were incubated with 50 μM BAPTA/AM for 15 min at 37 °C. At the end of the incubation with BAPTA/AM, 2 mM CaCl2 labeled with 45Ca (O, A) or 2 mM CaCl2 labeled with 45Ca and 20 μM atropine (Atro.) (O, A) were added to initiate Ca2+ uptake into unstimulated (O, A) and reloading into stimulated (O, A) acini (A). The figure shows the mean ± S. E. of three separate experiments. To illustrate the effect of BAPTA on reloading, 45Ca uptake into control acini was subtracted from 45Ca uptake of the respective "cycled" acini (B).
DISCUSSION

Non-excitable cells respond to Ca\(^{2+}\) mediated agonists in general by releasing intracellular Ca\(^{2+}\) and by increasing Ca\(^{2+}\) entry across the plasma membrane; the proportionality between these two pathways varies between cells and between agonists. Substantial evidence indicate that Ca\(^{2+}\) release from intracellular stores is mediated by the production of In-1,4,5-P\(_3\) in the cytosol (1). In contrast, there appear to be a variety of mechanisms involved in agonist stimulated Ca\(^{2+}\) entry into non-excitable cells. For example, In-1,4,5-P\(_3\) has been reported to induce Ca\(^{2+}\) entry in lymphocytes (35) and mast cells (36). In lacrimal acinar cells (37) both In-1,4,5-P\(_3\) and In-1,3,4,5-P\(_3\) appeared to be necessary for activation of Ca\(^{2+}\) entry. In hepatocytes, glucagon and epinephrine appear to induce two separable pathways of Ca\(^{2+}\) influx (38).

A property which must exist in all such cells, however, is a Ca\(^{2+}\) pathway for reloading of intracellular stores following discharge by In-1,4,5-P\(_3\). A major question that has arisen is the anatomical properties of the reloading pathway. One model has suggested direct communication between the medium and the In-1,4,5-P\(_3\) releasable pool (22, 25, 26). Another, more conventional model, view that uptake of Ca\(^{2+}\) occurs downhill across the plasma membrane into the cytosol and hence, by pump mediated uptake into the intracellular Ca\(^{2+}\) store of the endoplasmic reticulum or calciosome (12, 39). A third alternative is a vesicle cycling model whereby Ca\(^{2+}\) is transported into the pool by means of endosomal cycling between plasma membrane and endoplasmic reticulum. This latter suggestion arose from the observation that In-1,3,4,5-P\(_3\) was able to augment Ca\(^{2+}\) influx only in cells where the internal stores were depleted by In-1,4,5-P\(_3\) (37). It was hypothesized that In-1,3,4,5-P\(_3\) facilitated fusion between organelles containing the In-1,4,5-P\(_3\) activated channel (perhaps calciosomes) and a portion of the endoplasmic reticulum attached to the plasma membrane (40).

In both the direct communication model and in the cycling model, the loading of the endoplasmic reticulum should depend directly on the Ca\(^{2+}\) content of the medium. However, the extent of reloading appears constant over wide variations in medium Ca\(^{2+}\) concentration (0.5-2 mM) (9). In addition, during the reloading period, Ca\(^{2+}\) influx into the pool is at least 12-fold faster than Ca\(^{2+}\) efflux from the pool (18, 34). Furthermore, the direct pathway would seem to remove the need for a Ca\(^{2+}\) pump, given the normal constancy of extracellular Ca\(^{2+}\). In both the direct and the cycling model, additional regulatory systems would have to be involved, involving stimulation and inhibition of cycling or of direct communication. On the other hand, for the series model the maintained reloading at resting cytosolic Ca\(^{2+}\) level (9, 22-24) necessitates a determination of accessibility of the cytosolic pool during reloading and the effect of buffering the cytosolic Ca\(^{2+}\) pool on the properties of the intracellular stores.

Recently, the effect of the tumor promoter thapsigargin and methacholine on Ca\(^{2+}\) entry into parotid acinar cells were compared (41). It was concluded that these compounds activate Ca\(^{2+}\) entry by depletion of the intracellular Ca\(^{2+}\) pool through different mechanisms (41). However, the role of the thapsigargin-activated Ca\(^{2+}\) entry pathway in reloading was not defined, and these experiments did not investigate the role of the cytosolic Ca\(^{2+}\) pool in reloading. Here, we have examined both Mn\(^{2+}\) and BAPTA effects as indices of the role of the cytoplasmic Ca\(^{2+}\) pool in the reloading of the intracellular Ca\(^{2+}\) store.

In the pancreatic acini and in other cell types Mn\(^{2+}\) appears to act as a congener of Ca\(^{2+}\) in passive Ca\(^{2+}\) pathways (14, 32, 42). In the particular case of the pancreas, Mn\(^{2+}\) entry is stimulated by agonists such as carbachol or CCK and similar to Ca\(^{2+}\) (9), Mn\(^{2+}\) entry remains stimulated after atropine inhibition of carbachol stimulation. As for Ca\(^{2+}\) (31), Mn\(^{2+}\) entry is enhanced by elevation of medium pH. Inhibition of carbachol stimulation of In-1,4,5-P\(_3\) levels is achieved within 1-2 min following atropine (43, 44). Nevertheless, there is an increased rate of Mn\(^{2+}\) entry into the cytosolic pool which is maintained following atropine inhibition of the carbachol response. Recently, while these studies were in progress, similar observations were reported by studying the effect of agonist and antagonist on Mn\(^{2+}\) entry into umbilical-vein endothelial cells (14). In these cells, Ca\(^{2+}\) and Mn\(^{2+}\) entry during stimulation and reloading were significantly higher than those observed with pancreatic acinar cells. Thus, despite the differences in the absolute change in plasma membrane permeability, in both cell types Mn\(^{2+}\) enters the cytosolic pool during the reloading phase. These data are consistent with reloading of the stores by Ca\(^{2+}\) entry across plasma membrane, mixing of this Ca\(^{2+}\) with the cytosolic Ca\(^{2+}\) pool and uptake into the ER system by the Ca\(^{2+}\) pump. Mn\(^{2+}\) would act as a Ca\(^{2+}\) cogener in terms of entry across plasma membrane and mixing with the Ca\(^{2+}\) in the cytosol but is not incorporated into the store since the Ca\(^{2+}\) pump poorly transport Mn\(^{2+}\) (45). However, these data do not show directly the involvement of the cytosolic Ca\(^{2+}\) pool in reloading.

A second line of evidence suggesting that a series model is correct and that the cytosolic pool is used for reloading was obtained by using an intracytoplasmic Ca\(^{2+}\) buffer. BAPTA/AM does not act as a buffer outside the cell. However, following cellular uptake and release of BAPTA, the data presented above show that BAPTA acts as a buffer for cytosolic Ca\(^{2+}\). The chelator did not change the steady state level of [Ca\(^{2+}\)], as monitored by the fluorescence of Fluor 3. Nevertheless, the presence of BAPTA resulted in an attenuated, but normal time course for the initial carbachol-induced Ca\(^{2+}\) increase, but slowing of the atropine response and marked attenuation of the second release of intracellular Ca\(^{2+}\) by CCK. These data could be interpreted as an effect of cytosolic buffering on the reloading of the Ca\(^{2+}\) stores, consistent with a series model, not consistent with a communication or cycling model.

The presence of a changed cytosolic buffering capacity due to BAPTA makes quantitative comparisons with control conditions difficult when using another buffer, Fura 2, to monitor changes in [Ca\(^{2+}\)]. This difficulty was overcome using 45Ca\(^{2+}\) measurements. 45Ca\(^{2+}\) fluxes show that BAPTA did not affect the amount of Ca\(^{2+}\) stored in the releasable pool (Fig. 4) nor did BAPTA affect the amount of Ca\(^{2+}\) released by CCK-OP (Fig. 5). If BAPTA had been included in the pool and buffered Ca\(^{2+}\) in the intracellular store, both quantities should have changed. Thus, BAPTA/AM does not appear to enter the site of the Ca\(^{2+}\) storage directly, or if it does, it is not hydroyzed by esterases in the store, nor does a significant amount of free BAPTA accumulate in the site of Ca\(^{2+}\) storage. The presence of BAPTA in the cytosol of the pancreatic acinar cells however, slowed the rate of reloading of the intracellular Ca\(^{2+}\) store by about 3-fold. This is consistent with a buffering action of BAPTA on the Ca\(^{2+}\) being used for reloading the store. Since BAPTA is acting as a cytosolic buffer for Ca\(^{2+}\), it appears that these data also are consistent with the series model and exclude the direct or cycling models of the Ca\(^{2+}\) loading.

Since [Ca\(^{2+}\)], is maintained at or just below the resting level during Ca\(^{2+}\) reloading (9, 22-24), there must be a fine balance between the rate of Ca\(^{2+}\) entry into the cytoplasm and removal of Ca\(^{2+}\) from the cytoplasm by the pool Ca\(^{2+}\) pump. Furthermore, this reloading can occur long after termination of
stimulation (9, 13, 22). It is, therefore, likely that the regulatory mechanisms are built into the pathway itself, rather than being dependent on residual effects of agonists. This suggests a link between the plasma membrane Ca^{2+} pathway responsible for reloading the intracellular Ca^{2+} store, and the store itself or the Ca^{2+} pump involved in store replenishment. The most likely candidate for such a regulator would be the level of Ca^{2+} itself.

In summary, Mn^{2+} entry into the cytoplasm is enhanced and favor a model whereby reloading is achieved by entry into extracellular medium and an In-1,4,5-P3 releasable Ca^{2+} stores and uptake from the cytoplasm of the cell.

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