Amplification of the thapsigargin-evoked increase in the cytosolic free Ca^{2+} concentration by acetylcholine in acutely isolated mouse submandibular acinar cells

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The intracellular Ca^{2+} concentration was measured in single, acutely isolated, mouse submandibular acinar cells loaded with fura-2 AM. All experiments were performed in the absence of extracellular Ca^{2+} in order to eliminate Ca^{2+} influx. The microsomal ATPase inhibitor, thapsigargin, was used to release Ca^{2+} from intracellular stores and simultaneously prevent re-uptake into the stores. Sequential application of thapsigargin (2 μM) and the Ca^{2+} ionophore ionomycin (500 nM) indicated that thapsigargin was able to mobilize practically all intracellular Ca^{2+}. Furthermore, in comparison with results obtained following inhibition of the plasma membrane Ca^{2+}-ATPase by La^{3+} (2 mM), it may be shown that slowly unloading the intracellular Ca^{2+} stores using thapsigargin does not normally cause a massive, cytotoxic, increase in the cytosolic Ca^{2+} concentration, because Ca^{2+} is rapidly extruded from the cell across the plasma membrane. Application of a submaximal dose of acetylcholine (500 nM) during the rising phase of the response to thapsigargin caused a 3–4-fold increase in the amplitude of the rise in the cytosolic Ca^{2+} concentration without any significant alteration of the time course of the response. As thapsigargin alone is capable of mobilizing all releasable Ca^{2+}, this increase in amplitude is most likely the result of inhibition of the Ca^{2+} extrusion process by acetylcholine.

INTRODUCTION

Salivary fluid and electrolyte secretion is driven by Cl^- efflux from the acinar cells through Ca^{2+}-activated Cl^- channels [1]. Control of secretion is therefore regulated by an increase in the intracellular free calcium concentration ($[Ca^{2+}]_i$), which follows activation of a receptor by an agonist and subsequent Ins(1,4,5)P_3 production [1]. There is a general consensus that Ca^{2+} is first released from intracellular pools by Ins(1,4,5)P_3 and that depletion of these intracellular pools is a trigger for Ca^{2+} influx [2].

Assuming that intracellular Ca^{2+} buffering remains constant, $[Ca^{2+}]_i$ cannot increase unless Ca^{2+} is released from stores or enters the cell via the plasma membrane. Most previous studies of Ca^{2+} signalling have focused on the effect that Ca^{2+} release from stores or Ca^{2+} influx has on the intracellular Ca^{2+} signal. However, the impact that Ca^{2+} release or Ca^{2+} influx can have on $[Ca^{2+}]_i$ is also governed by the processes whereby Ca^{2+} is removed from the cytoplasm. Ca^{2+} release from stores may have no detectable impact on $[Ca^{2+}]_i$ if Ca^{2+} is immediately taken back into the stores or expelled from the cell. We have shown previously that the 'resting' Ca^{2+} concentration in unstimulated exocrine acinar cells remains constant despite Ca^{2+} release from intracellular stores because this release is balanced by the re-uptake process [3]. This 'invisible' Ca^{2+} release under unstimulated conditions may be revealed using the microsomal Ca^{2+}-ATPase inhibitor thapsigargin, which does not itself cause Ca^{2+} release from stores [4], to prevent re-uptake [3].

Changes in $[Ca^{2+}]_i$ therefore depend on the balance between four processes: Ca^{2+} release from stores, Ca^{2+} influx, Ca^{2+} re-uptake into stores and Ca^{2+} extrusion. The contribution of Ca^{2+} influx to changes in $[Ca^{2+}]_i$ was eliminated from our experiments by removing Ca^{2+} from the extracellular medium. Similarly, the effect of Ca^{2+} re-uptake into stores was removed using thapsigargin. Thus we are able to isolate and study the relationship that Ca^{2+} release from stores and Ca^{2+} extrusion have in determining the magnitude and time course of changes in $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Adult male CD1 mice were killed by cervical dislocation, and submandibular cells were isolated by collagenase (Worthington Diagnostic) digestion in extracellular media containing 1 mM Ca^{2+}, as described previously [5]. Cells were loaded with fura-2 by a 10–20 min incubation in the presence of 1 μM fura-2 AM (Molecular Probes). A dilute cell suspension was placed into a perfusion chamber on the stage of a Nikon diaphot inverted microscope, and the cells were allowed to adhere to the glass bottom of the chamber.

The external bathing solution contained (in mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 0.1 EGTA, 10 glucose, 10 Hepes, pH 7.4. Thapsigargin was dissolved in DMSO, which was present in the final solution at a concentration of 0.1 % (v/v). DMSO alone was found to be without effect at a concentration of 1 % (results not shown). Cells were superfused continuously at 0.5 ml/min from one of several parallel superfusion pipettes. The solution bathing the cell could be changed in 1–2 s. All experiments were carried out at 24 $\pm$ 2 °C. Measurements were made at 400 $\times$ or 1000 $\times$ magnification on single cells, either completely isolated or part of a small (2–8) cell clump.

The ratio of UV light emitted at 510 nm following excitation at 340 nm to that emitted following excitation at 380 nm was measured using a Cairn spectrophotometer (excitation was at 96 Hz; data were averaged on-line and collected at 4 Hz). $[Ca^{2+}]_i$ was calculated from this ratio using the Grynkiewiez equation and custom written software. [Copies of the kinetic model software are available from P. M. Smith (Petesmif@liv.ac.uk); requires MS. Windows 3.1 and an SVGA monitor.]

Abbreviations used: ACh, acetylcholine; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration.

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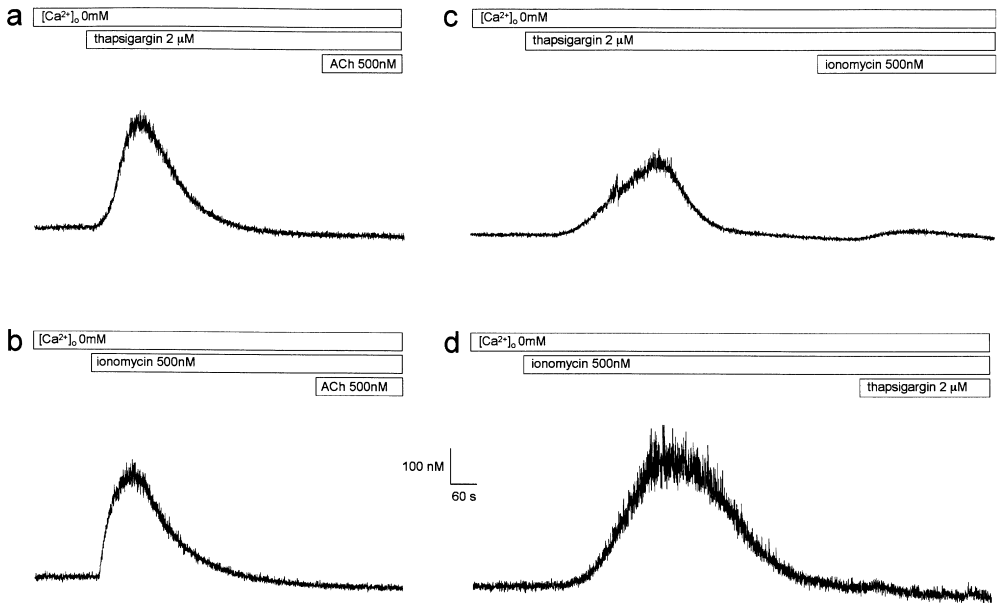

Figure 1 Thapsigargin mobilizes all releasable Ca^{2+}

(**a**) Application of 500 nM ACh following the thapsigargin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_0 = 0$), showing no further increase in $[Ca^{2+}]_i$. (**b**) Application of 500 nM ACh following the ionomycin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}, showing no further increase in $[Ca^{2+}]_i$. (**c**) Application of 500 nM ionomycin following the thapsigargin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}, showing a small additional increase in $[Ca^{2+}]_i$. (**d**) Application of 2 μM thapsigargin following the ionomycin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}, showing no further increase in $[Ca^{2+}]_i$.

Model

The relationship between Ca^{2+} release and Ca^{2+} efflux was modelled using simple kinetic equations based on the work of Goldbeter et al. [6] and Kuba [7]. Changes in $[Ca^{2+}]_i$ were calculated over time as the algebraic sum of four processes: Ca^{2+} influx, uptake of Ca^{2+} into intracellular stores, Ca^{2+} release from stores and Ca^{2+} efflux from the cell. Thus $[Ca^{2+}]_i$ = influx + (Ca^{2+} from store) − (Ca^{2+} to store) − efflux. The various parameters are defined as follows.

(a) Influx = 0, to simulate experiments performed in Ca^{2+}-free media.

$$\text{(b) Efflux} = \frac{V_{max} \cdot [Ca^{2+}]_i^{\,h}}{K_m^{\,h} + [Ca^{2+}]_i^{\,h}}$$

to simulate active transport of Ca^{2+} from the cell. V_{max} is the maximum rate of pumping, K_m is the affinity of the ATPase for Ca^{2+} and h is the Hill coefficient for Ca^{2+} binding to the ATPase.

$$\text{(c) From store} = (P_{IP3} + P_{leak})([Ca^{2+}]_s - [Ca^{2+}]_i)$$

to simulate passive efflux from an intracellular store, where P_{IP3} is the Ins(1,4,5)P_3- and Ca^{2+}-dependent Ca^{2+} permeability of the intracellular store and P_{leak} is the non-specific permeability of the store, where:

$$P_{IP3} = \frac{P_{max} \cdot [InsP_3]^h}{(K_{m,IP3}^{\,h} + [InsP_3]^h)\left(1 + \frac{K_{m,s}^{\,m}}{[Ca^{2+}]_s^{\,m}}\right)\left(1 + \frac{K_{m,i}^{\,L}}{[Ca^{2+}]_i^{\,L}}\right)}$$

P_{IP3} is determined by non-competitive activation by Ins(1,4,5)P_3, $[Ca^{2+}]_i$ and the Ca^{2+} concentration within the intracellular store ($[Ca^{2+}]_s$). P_{max} is the maximum permeability of the store. $K_{m,IP3}$, $K_{m,s}$ and $K_{m,i}$ represent the activity constants, and h, m and L the Hill coefficients, for Ins(1,4,5)P_3, $[Ca^{2+}]_s$ and $[Ca^{2+}]_i$ respectively.

$$\text{(d) To store} = \frac{V_{max} \cdot [Ca^{2+}]_i^{\,h}}{(K_{m,i}^{\,h} + [Ca^{2+}]_i^{\,h})\left(1 + \frac{[thap]^m}{K_{m,thap}^{\,m}}\right)}$$

to simulate the thapsigargin-inhibitable active transport of Ca^{2+} into the store. V_{max} is the maximum rate of pumping. $K_{m,i}$ and $K_{m,thap}$ are the affinity constants, and h and m the Hill coefficients, of the ATPase for Ca^{2+} and thapsigargin respectively. [thap] represents the concentration of thapsigargin.

The following values, in the physiological range [6–9], were used to establish a steady $[Ca^{2+}]_i$ of around 70 nM.

(a) Influx = 0

(b) Efflux: $V_{max} = 60\ \mu M/s$, $K_m = 2\ \mu M$, $h = 3$, $[Ca^{2+}]_i = 100$ nM.

(c) From store: $P_{max} = 500\ \mu M/s$, $K_{m,IP3} = 0.5\ \mu M$, $m = 2$, $[InsP_3] = 0.01\ \mu M$, $K_{m,i} = 1\ \mu M$, $L = 3$, $K_{m,s} = 1\ \mu M$, $m = 3$, $P_{leak} = 0.05\ \mu M/s$, $[Ca^{2+}]_s = 2\ \mu M$.

(d) To store: $V_{max} = 25\ \mu M/s$, $K_{m,i} = 1\ \mu M$, $h = 2$, $K_{m,thap} = 0.5\ \mu M$, $m = 2$, [thap] = 0 μM

RESULTS

Thapsigargin mobilizes all Ca^{2+} contained in intracellular stores

Figure 1(a) shows an example of the increase in $[Ca^{2+}]_i$ following exposure to thapsigargin (2 μM) in the absence of extracellular Ca^{2+}. Average data ($n = 28$) indicate that, following a lag of 46 ± 8 s, $[Ca^{2+}]_i$ rose by 156 ± 18 nM from 82 ± 4 nM to 237 ± 20 nM over a period of 216 ± 17 s, and then declined to 69 ± 7 nM, slightly below the baseline $[Ca^{2+}]_i$, during the next 374 ± 28 s. The Ca^{2+} ionophore ionomycin (500 nM), which displays a preference for endomembranes over the plasma membrane [10,11], caused a similar transient increase in $[Ca^{2+}]_i$

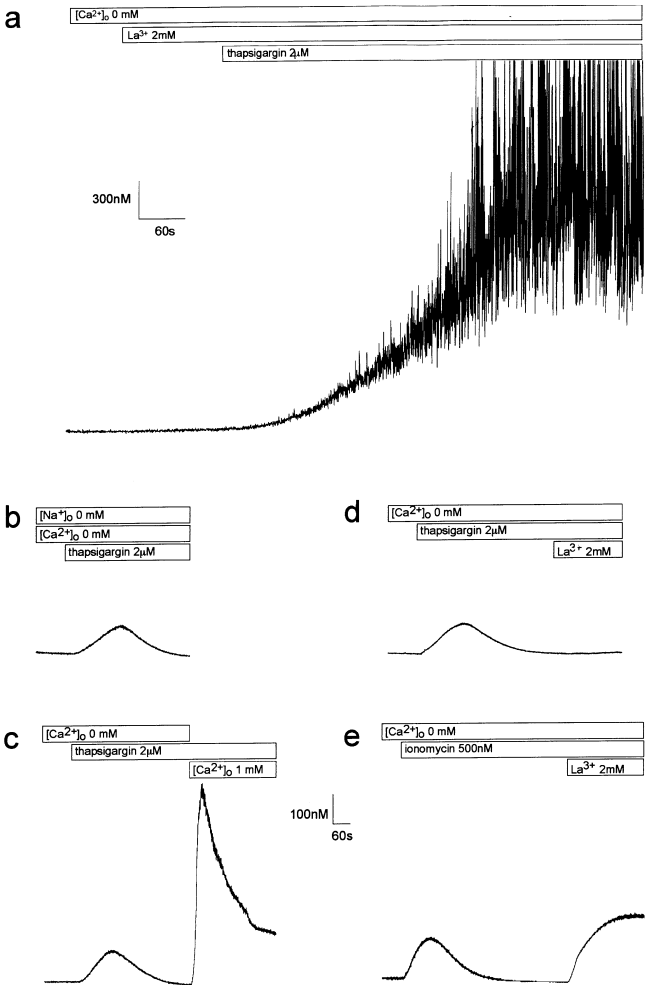

Figure 2 The amplitude and time course of the thapsigargin-evoked increase in $[Ca^{2+}]_i$ are greatly amplified when Ca^{2+} extrusion is prevented

(**a**) Large sustained increase in $[Ca^{2+}]_i$ following application of 2 μM thapsigargin in the presence of 2 mM La^{3+}. (**b**) Thapsigargin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o = 0$) and Na^+, showing no change in the amplitude or time course of the response (cf. Figures 1a, 1c and 2d). (**c**) Re-admission of extracellular Ca^{2+} following the thapsigargin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}, showing a large transient increase in $[Ca^{2+}]_i$ falling back to a sustained elevated level. (**d**) Application of 2 mM La^{3+} following the thapsigargin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}, showing no change in $[Ca^{2+}]_i$ (see the text for details). (**e**) Application of 2 mM La^{3+} following the ionomycin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+}, showing an apparent increase in $[Ca^{2+}]_i$ (see the text for details).

(Figure 1b). The peak of the transient following ionomycin treatment was 307 ± 66 nM ($n = 15$) above baseline. Following exposure of the cells to thapsigargin, ionomycin caused little or no further increase in $[Ca^{2+}]_i$ (Figure 1c); on average there was an increase in $[Ca^{2+}]_i$ of 17 ± 6 nM ($n = 5$). Thus ionomycin can cause little Ca^{2+} release following exposure of cells to thapsigargin, because thapsigargin has already mobilized essentially all of the Ca^{2+} contained within these cells. Thapsigargin caused no measurable change in $[Ca^{2+}]_i$ following pretreatment with ionomycin ($n = 4$; Figure 1d). The Ca^{2+} pool mobilized by either ionomycin or thapsigargin may be shown to be the same as that released by agonists, because subsequent treatment with acetylcholine (ACh; 500 nM) caused no further increase in $[Ca^{2+}]_i$ (Figures 1a and 1b).

Ca^{2+} efflux determines the magnitude and time course of the thapsigargin-evoked change in $[Ca^{2+}]_i$

The plasma membrane ATPase inhibitor La^{3+} [12] was used in order to determine the contribution that Ca^{2+} extrusion makes to the amplitude and time course of the change in $[Ca^{2+}]_i$ following Ca^{2+} release from stores. In the presence of La^{3+} there was no recovery of $[Ca^{2+}]_i$ following exposure to thapsigargin (Figure 2a). Furthermore, in the presence of La^{3+} the average increase in $[Ca^{2+}]_i$ following stimulation by thapsigargin was > 2000 nM. In 19 out of 30 experiments the fluorescence ratio in the presence of La^{3+} and thapsigargin approached the R_{max} (indicating saturation of fura-2 by Ca^{2+}); therefore the average increase in $[Ca^{2+}]_i$ could not be calculated. Comparison of these data with those shown in Figure 1 indicates that extrusion of Ca^{2+} across the plasma membrane is responsible not only for the recovery of $[Ca^{2+}]_i$ following Ca^{2+} release but also for greatly reducing the magnitude of the response.

Another possible mechanism for Ca^{2+} extrusion, in addition to primary active transport via the plasma membrane Ca^{2+}-ATPase, is via Na^+/Ca^{2+} exchange. The data in Figure 2(b) show that complete removal of extracellular Na^+, and therefore abolition of the Na^+ gradient which would drive Na^+/Ca^{2+} exchange, had no effect on the magnitude or time course of the thapsigargin response. These data support previous findings that Na^+/Ca^{2+} exchange does not have any significant role in Ca^{2+} extrusion in exocrine acinar cells [13].

Fura-2 has a greater affinity for La^{3+} [14] than for Ca^{2+}; furthermore, since both cations have similar excitation spectra [14], La^{3+} influx would be manifested as an apparent increase in $[Ca^{2+}]_i$. Therefore it was important to ensure that the data in Figure 2(a) were not due to La^{3+} entering the cells. It has previously been reported that La^{3+} does not enter exocrine cells [12], and the data in Figure 2 confirm this observation. There was no change in fluorescence following excitation at either 340 or 380 nm (results not shown), nor was there any apparent shift in $[Ca^{2+}]_i$ in the presence of La^{3+}, before application of thapsigargin (Figure 2a). However, it might be argued that the Ca^{2+} influx pathway is a likely means of La^{3+} entry and that this pathway is not active before thapsigargin stimulation. The data in Figure 2(c) show a large increase in $[Ca^{2+}]_i$ following re-admission of Ca^{2+} to the extracellular bathing solution after exposure to thapsigargin, indicating that the Ca^{2+} influx pathway was active following thapsigargin-induced emptying of the intracellular stores. La^{3+} caused no apparent change in $[Ca^{2+}]_i$ even under these conditions (Figure 2d). La^{3+} has previously been shown to enter keratinocytes following treatment with ionomycin [15]. Exposure to La^{3+} following prolonged exposure to ionomycin (500 nM) caused an apparent increase in $[Ca^{2+}]_i$ consistent with La^{3+} influx via ionomycin. The data in Figure 2(e) demonstrate that La^{3+} influx would have been readily detected had it occurred under the conditions shown in Figures 2(a), 2(c) and 2(d). Furthermore, La^{3+} influx following exposure to ionomycin indicates that in submandibular cells, unlike ECV304 cells [10], 500 nM ionomycin acts both at intracellular stores and at the plasma membrane.

ACh amplifies the thapsigargin-evoked increase in $[Ca^{2+}]_i$

The data in Figure 3 show the change in $[Ca^{2+}]_i$ when ACh (500 nM) was applied during the rising phase of the response to thapsigargin. The response to thapsigargin alone is also shown on the same scale for comparison. In 15 out of 19 experiments there was a very rapid transient increase in $[Ca^{2+}]_i$ which occurred within seconds of applying ACh, followed by a much longer slower transient which closely mirrored the time course of the

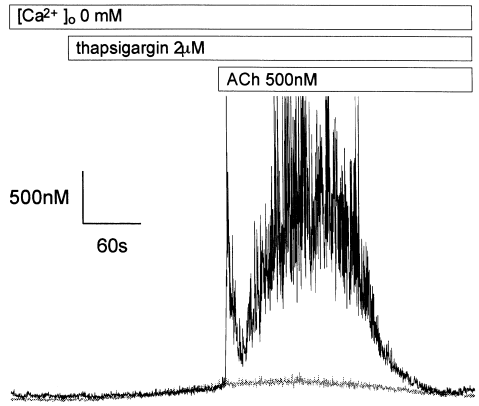

Figure 3 Amplification of the thapsigargin-evoked increase in $[Ca^{2+}]_i$ by ACh

Application of 500 nM ACh during the rising phase of the thapsigargin-evoked transient increase in $[Ca^{2+}]_i$ measured in the absence of extracellular Ca^{2+} (upper trace), showing a large amplification of the $[Ca^{2+}]_i$ transient compared with that evoked by thapsigargin alone (lower trace)

response to thapsigargin alone. The amplitude of the $[Ca^{2+}]_i$ transient was increased above control in 19 out of 19 experiments, including those which did not show an initial transient. On average, the duration of the response to thapsigargin was 590 ± 17 s ($n = 28$) and that of the response to thapsigargin plus ACh was 510 ± 32 s ($n = 19$). The peak increase in $[Ca^{2+}]_i$ caused by thapsigargin plus ACh was 628 ± 126 nM ($n = 19$). The data in Figure 1 show that thapsigargin is capable of releasing practically all the Ca^{2+} stored within the cell; therefore the greatly enhanced increase in $[Ca^{2+}]_i$ seen with ACh cannot be due to any additional release of Ca^{2+} from the stores. Application of ionomycin (500 nM) subsequent to exposure to thapsigargin and ACh produced a small transient similar to that shown in Figure 1(c) (results not shown). Ca^{2+} influx could not account for the enhancement of the peak of the $[Ca^{2+}]_i$ transient because there was no extracellular Ca^{2+} in these experiments.

The effect that Ca^{2+} release from stores has on the $[Ca^{2+}]_i$ is dependent on the rate at which Ca^{2+} is released from the stores and also on the rate at which Ca^{2+} is removed from the cytoplasm. One possible explanation for the ACh-induced amplification of the thapsigargin response is that it follows a more rapid emptying of the stores caused by ACh. Using the simple model outlined in the Materials and methods section, where an increased thapsigargin concentration causes a non-competitive inhibition of the intracellular store ATPase, we were able to mimic our observed response to thapsigargin (Figure 4a). We were also able to reproduce the initial transient increase in $[Ca^{2+}]_i$ seen by applying ACh during the rising phase of the thapsigargin response (Figure 4b) by increasing the intracellular Ins(1,4,5)P_3 concentration. However, we could not model the amplification of the thapsigargin response by ACh, despite using a wide range of parameters for ACh-induced Ca^{2+} release from the stores. An alternative expression governing Ca^{2+} release from stores, based on the bell-shaped Ca^{2+} response curve of the Ins(1,4,5)P_3-gated Ca^{2+} channel [8], was also employed. Using different parameter sets, this expression was also capable of mimicking the response to thapsigargin, but not, however, the ACh-induced increase in $[Ca^{2+}]_i$. In all cases, increasing the rate of Ca^{2+} efflux from the stores reduced the magnitude or the duration of the subsequent response to thapsigargin. Therefore we cannot account for the ACh-induced amplification of the thapsigargin-evoked increase

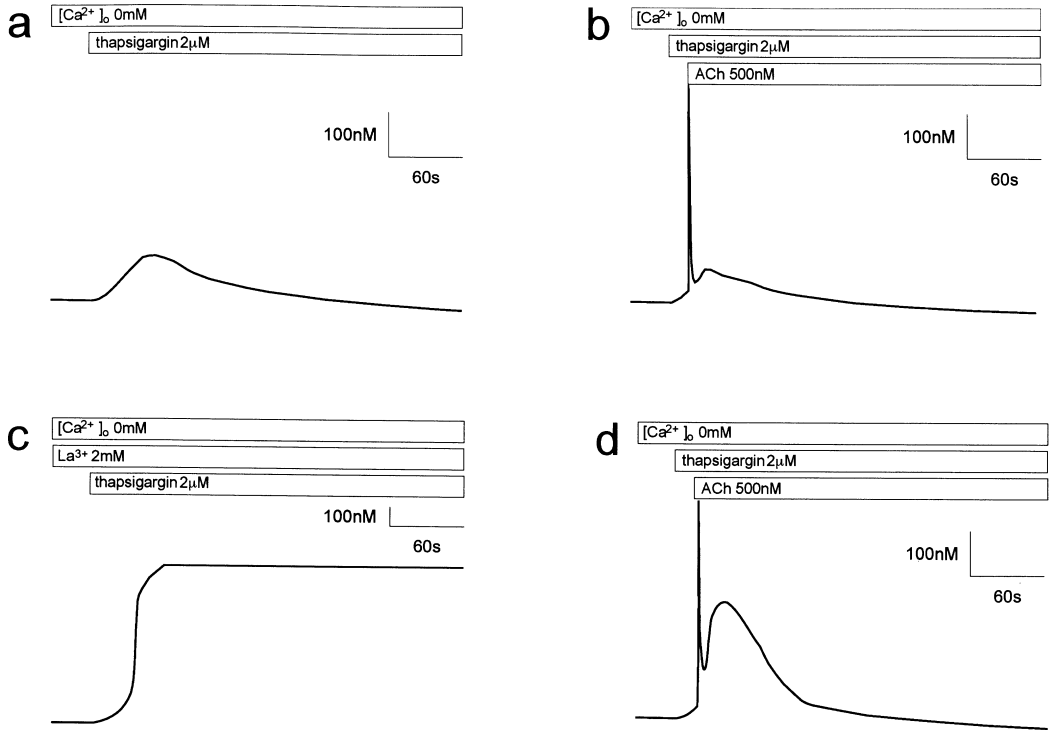

Figure 4 Output from the kinetic model outlined in the Materials and methods section

(**a**) Ramped increase in thapsigargin concentration from 0 to 2 μM over 60 s, starting at 30 s. (**b**) As (**a**), but with an increase in the InsP_3 concentration from 0.1 to 0.2 μM applied at 45 s. (**c**) As (**a**), but with the V_{max} for the plasma membrane Ca^{2+}-ATPase set to 0 at 0 s. (**d**) As (**b**), but with a transient reduction in the affinity of the Ca^{2+}-ATPase ramped from 2 to 6 μM between 45 and 60 s, returning to 2 μM over the next 60 s.

in $[Ca^{2+}]_i$ solely by invoking an ACh-induced increase in the rate of Ca^{2+} release from the stores. Figure 4(c) shows the response to thapsigargin modelled in the absence of Ca^{2+} extrusion, thus mimicking the effect of 2 mM La^{3+} and further demonstrating the importance of Ca^{2+} extrusion in determination of the amplitude and time course of the increase in $[Ca^{2+}]_i$ following Ca^{2+} release from the stores. Figure 4(d) shows how the ACh-induced amplification of the thapsigargin response could be modelled by incorporating a small, transient, ACh-dependent, decrease in the affinity of the plasma membrane Ca^{2+}-ATPase for Ca^{2+}. Thus our data can only be modelled if ACh causes a transient decrease in Ca^{2+} extrusion from the cells.

DISCUSSION

Our data show how the amplitude and time course of changes in $[Ca^{2+}]_i$ following Ca^{2+} release from intracellular stores may be modified by Ca^{2+} extrusion. The extent to which Ca^{2+} extrusion can limit changes in $[Ca^{2+}]$ is most clearly shown by preventing Ca^{2+} extrusion with La^{3+} and then unloading the Ca^{2+} stores using thapsigargin. The resultant increase in $[Ca^{2+}]_i$ is at least 10-fold greater than that produced by thapsigargin when Ca^{2+} extrusion is permitted. The inhibition of Ca^{2+} efflux, therefore, offers a mechanism whereby the effect of Ca^{2+} release from stores on $[Ca^{2+}]_i$ may be amplified. Thus, if a Ca^{2+}-mobilizing agonist simultaneously inhibited Ca^{2+} extrusion, release of a small amount of Ca^{2+} could be manifested as a large change in $[Ca^{2+}]_i$.

In order to determine whether ACh could amplify the intracellular Ca^{2+} signal by inhibiting Ca^{2+} efflux, we used thapsigargin to mobilize intracellular Ca^{2+} by emptying the intracellular stores and thus provide a Ca^{2+} signal of known time course and amplitude. ACh caused a 3–4-fold amplification of this signal, consistent with ACh-dependent inhibition of Ca^{2+} efflux. An alternative explanation, that the amplification of the thapsigargin response follows an ACh-dependent increase in the rate of Ca^{2+} efflux from the stores, is not supported by our data, because this mechanism would also shorten the time course of the response (i.e. increasing the rate of release from stores would cause a larger peak increase in $[Ca^{2+}]_i$, but for a shorter time). The time course of the amplified response to thapsigargin and ACh was very similar to that seen with thapsigargin alone. Unless ACh caused a radical (and rapidly reversible) change in Ca^{2+} buffering, or a massive reduction in cell volume (which was not observed), it is difficult to account for our observations by any mechanism other than a reduction in the rate of Ca^{2+} extrusion.

It has been shown previously that, as $[Ca^{2+}]_i$ increases, so does the rate of Ca^{2+} extrusion [13]. Our findings do not contradict these observations. A small, transient, reduction in the affinity of the Ca^{2+}-ATPase for Ca^{2+}, which is all that is required to model our data (see Figure 4d), would shift the relationship between $[Ca^{2+}]_i$ and Ca^{2+} extrusion to the right, but it would not prevent Ca^{2+} efflux rising as a function of $[Ca^{2+}]_i$.

The first step in the stimulus–secretion cascade triggered by ACh is activation of phospholipase C and cleavage of phosphatidylinositol 4,5-bisphosphate to give Ins(1,4,5)P_3 and diacylglycerol, both of which act as second messengers. Increased diacylglycerol activates protein kinase C. Although protein kinase C has previously been shown to interact with the plasma membrane Ca^{2+}-ATPase [9], it is unlikely that increased protein kinase C activity gives rise to our observations. In most previous studies, protein kinase C activation increased Ca^{2+}-ATPase activity [9,17,18] and attenuated rather than amplified Ca^{2+} signalling [19]. However, there are several possible mechanisms by which Ca^{2+}-ATPase activity could be reduced by inositol polyphosphates. In rat brain, PtdIns(4,5)P_2 has been shown to activate the Ca^{2+}-ATPase [20,21]. PtdIns(4,5)P_2 breakdown following ACh stimulation could therefore reduce Ca^{2+} pumping. Alternatively, Ins(1,4,5)P_3 has been shown to antagonize plasma membrane Ca^{2+}-ATPase activity in rat heart [22] and in human erythrocytes [23,24], possibly by inhibiting activation of the pump by calmodulin [23]. Thus increased Ins(1,4,5)P_3 following stimulation by an agonist such as ACh could serve both to release Ca^{2+} from intracellular stores and also to amplify the resultant increase in $[Ca^{2+}]_i$ by inhibiting Ca^{2+} pumping from the cell. Furthermore, the Ins(1,4,5)P_3 metabolite Ins(1,3,4,5)P_4 has also been shown to inhibit plasma membrane Ca^{2+}-ATPase activity in rat brain [25]. Together with our data, this observation could offer a novel mechanism to account for the synergism between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 which has been observed previously in exocrine acinar cells [26,27]

In many studies of Ca^{2+} signalling, great emphasis is placed on the relationship between the Ca^{2+} release process and increased $[Ca^{2+}]_i$, and relatively little on any role that Ca^{2+} removal mechanisms might have. Our findings indicate that amplification of the Ca^{2+} signal by inhibition of Ca^{2+} extrusion could represent an energetically efficient refinement to the Ca^{2+} signalling process, and therefore that the agonist-dependent regulation of Ca^{2+} extrusion could have an important role in stimulus–secretion coupling in exocrine acinar cells.

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