Delayed autoregulation of the Ca^{2+} signals resulting from capacitative Ca^{2+} entry in bovine pulmonary artery endothelial cells

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1. In calf pulmonary artery endothelial (CPAE) cells loaded with fura-2, the effects of ATP on Ca^{2+} entry were mediated entirely by the ability of P_{2U} purinoceptors to stimulate $InsP_3$ formation, empty intracellular Ca^{2+} stores and thereby activate capacitative Ca^{2+} entry.

2. Restoration of extracellular Ca^{2+} to cells with empty intracellular stores evoked transient increases in cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) which then declined to an elevated plateau. These overshoots in $[Ca^{2+}]_i$ were not a consequence of store refilling nor of desensitization of the capacitative pathway. Similar responses were recorded from cells in which Ca^{2+} uptake into mitochondria had been inhibited by microinjection of Ruthenium Red. The amplitudes of the capacitative Ca^{2+} signals decreased at lower extracellular $[Ca^{2+}]$, but $[Ca^{2+}]_i$ invariably overshot before slowly declining to an elevated plateau. Even modest increases in $[Ca^{2+}]_i$ therefore caused a delayed attenuation of the Ca^{2+} signal evoked by capacitative Ca^{2+} entry.

3. Modest pre-elevation of $[Ca^{2+}]_i$ inhibited the ability of subsequent capacitative Ca^{2+} entry to further increase $[Ca^{2+}]_i$. The onset of the inhibition was slow (half-time ($t_{½}$), ~100 s) and more tightly correlated with the preceding peak $[Ca^{2+}]_i$ than with the $[Ca^{2+}]_i$ immediately preceding Ca^{2+} entry. Recovery was also slow and complete only after $[Ca^{2+}]_i$ had returned to its basal level for 320 ± 3 s.

4. In thapsigargin-treated cells loaded with mag-fura-2, the peak $[Ca^{2+}]_i$ that followed restoration of extracellular Ca^{2+} was accompanied by an abrupt ~2·5-fold decrease in the rate of Mn^{2+} entry, which then continued indefinitely at the reduced rate, demonstrating a rapid partial inactivation of the capacitative pathway.

5. The half-time for Ca^{2+} removal from the cytosol was significantly slower during the rising ($t_{½} = 22 \pm 2{\cdot}5$ s) than during the falling ($t_{½} = 7{\cdot}1 \pm 0{\cdot}7$ s) phase of the Ca^{2+} overshoot evoked by addition of extracellular Ca^{2+} to thapsigargin-treated cells.

6. We conclude that an increase in $[Ca^{2+}]_i$ rapidly inhibits the capacitative pathway and more slowly activates mechanisms that remove Ca^{2+} from the cytosol. Reversal of either or both of these regulatory mechanisms can occur only a considerable time after $[Ca^{2+}]_i$ has been completely restored to its resting level. These mechanisms are likely to protect cells from excessive increases in $[Ca^{2+}]_i$ and contribute to oscillatory changes in $[Ca^{2+}]_i$.

The large electrochemical gradients for Ca^{2+} that exist across both the plasma membrane and the membranes of intracellular stores allow rapid increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in response to stimuli that open Ca^{2+} channels within these membranes. A common feature of these Ca^{2+} channels is their inhibition by increased $[Ca^{2+}]_i$: voltage-gated Ca^{2+} channels, receptor-operated cation channels, the capacitative Ca^{2+} entry pathway (Zweifach & Lewis, 1995*a*, *b*) and the intracellular inositol 1,4,5-trisphosphate ($InsP_3$) (Taylor & Traynor, 1995) and ryanodine receptors are all subject to such feedback regulation. The regulation of Ca^{2+} channels by $[Ca^{2+}]_i$ both prevents excessive, and potentially toxic, increases in $[Ca^{2+}]_i$ and may be an important element of the mechanisms responsible for generating the oscillatory changes in $[Ca^{2+}]_i$ evoked by many extracellular stimuli.

The receptors for $InsP_3$ are Ca^{2+} channels that are co-operatively opened by $InsP_3$, allowing Ca^{2+} to leak into the cytosol from the lumen of the intracellular Ca^{2+} stores. The Ca^{2+} entry across the plasma membrane that almost

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invariably accompanies Ca^{2+} release, via the capacitative Ca^{2+} entry pathway, is now widely believed to be stimulated by a signal generated by empty Ca^{2+} stores (Putney & Bird, 1993; Berridge, 1995). The electrophysiological characteristics of the current (I_{crac}) activated by empty stores have been established (Penner, Fasolato & Hoth, 1993) and the channel itself has been proposed to be encoded by the *trp* genes (Zhu *et al.* 1996), but the nature of the signal that passes between empty stores and the capacitative Ca^{2+} entry pathway remains unknown. It has been proposed that empty stores stimulate synthesis, modification or intracellular redistribution of a soluble activator of the capacitative pathway, or that they regulate, via small G proteins, translocation of the capacitative Ca^{2+} channel to the plasma membrane (Penner *et al.* 1993; Putney & Bird, 1993). It has also been suggested that the empty stores communicate directly with the plasma membrane Ca^{2+} channel through protein–protein interactions (Berridge, 1995).

Endothelial cells form the innermost lining of blood vessels and respond to both mechanical stimulation and to paracrine and circulating hormones. ATP, released from platelets after vascular injury, stimulates phosphoinositide hydrolysis in endothelial cells leading to both $InsP_3$-dependent Ca^{2+} mobilization and capacitative Ca^{2+} entry (Schilling & Elliott, 1992). In the present study, we have examined the effects of ATP on Ca^{2+} entry in calf pulmonary artery endothelial (CPAE) cells and the role of cytosolic Ca^{2+} in autoregulating the Ca^{2+} signals that result from capacitative Ca^{2+} entry.

METHODS

Cell culture and loading of cells with fluorescent Ca^{2+} indicators

Calf pulmonary artery endothelial cells (passage 30) were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and used for up to ten passages after receipt. They were grown at 37 °C in minimal essential medium equilibrated with 95% air–5% CO_2 and supplemented with fetal calf serum (20%, v/v), penicillin (100 units ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and L-glutamine (2 mM). The cells were passaged weekly and plated onto either round (no. 2, 22 mm diameter) or rectangular (no. 1.5, 9 mm × 22 mm) glass coverslips. Cells were used after 6–9 days in culture when they were confluent.

For fura-2 loading, cells on coverslips were washed twice with modified Krebs medium (composition (mM): 135 NaCl, 5·9 KCl, 1·5 $CaCl_2$, 1·2 $MgCl_2$, 11·6 Hepes, 11·5 glucose, pH 7·3) and then incubated for 1 h at 22 °C in the same medium supplemented with fura-2 AM (2 μM), bovine serum albumin (1 mg ml^{-1}) and Pluronic F-127 (20 $\mu g\ ml^{-1}$). After loading, the cells were washed twice and incubated for 1–3 h in modified Krebs medium to allow hydrolysis of the cytosolic fura-2 AM to fura-2. Using methods described previously (Byron & Taylor, 1993), we established that with this loading protocol 78 ± 3% ($n = 3$) of the dye was cytosolic. Similar methods were used to load cells with mag-fura-2 by incubation with mag-fura-2 AM (2 μM, 1 h).

In experiments designed to establish the role of mitochondrial Ca^{2+} uptake, CPAE cells were loaded with fura-2 by pressure injection using an Eppendorf model 5242 automatic microinjector. The pipette solution contained analytical grade water (MilliQ Plus 185; Millipore) and fura-2 pentapotassium salt (2·5 mM) with or without Ruthenium Red (1 mM). Measurements of $[Ca^{2+}]_i$, using exactly the methods described above, were then performed within 5–30 min. In order to estimate the microinjection volume, the fluorescence of microdroplets, each comparable in size to a CPAE cell and containing known concentrations of fura-2, was compared with CPAE cells that had been microinjected with the same stock solution of fura-2. The results suggested that the contents of the injection pipette were diluted 600- to 1000-fold after microinjection into a CPAE cell, giving final intracellular concentrations of $> 2{\cdot}5\ \mu$M for fura-2 and $> 1\ \mu$M for Ruthenium Red.

Measurements of $[Ca^{2+}]_i$

Fura-2 fluorescence was measured in cell populations using either a Perkin Elmer LS50B or an Hitachi F-4500 fluorescence spectrometer. Rectangular coverslips were mounted vertically in a 4·5 ml optical methacrylate cuvette and perfused from the bottom with media at 22 °C using a peristaltic pump (~15 ml min^{-1}); the half-time for mixing within the cuvette was ~16 s. In some experiments, when very rapid additions were needed, reagents were injected directly into the cuvette during continued perfusion to give a mixing time of 2–4 s. Excitation wavelengths ($\lambda_{ex} = 340$, 360 or 380 nm) were rapidly switched (0·1–1 s $cycle^{-1}$) using either a rotating filter wheel or monochromators. Emitted light was collected at 510 nm. For both single cell and population measurements, autofluorescence at each excitation wavelength was determined by addition of ionomycin (1 μM) and $MnCl_2$ (1 mM) to quench fura-2 fluorescence. After correction for autofluorescence, the fluorescence ratio ($F_{340/380}$) was used to calculate $[Ca^{2+}]_i$.

For analysis of Ca^{2+} signals in single cells, round coverslips held in a perfusion chamber containing modified Krebs medium were mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Media were perfused over the cells at 22 °C and removed by constant aspiration from the top of the chamber. This perfusion system allowed media to be completely exchanged within 10 s. The cells were illuminated using a xenon arc lamp (100 W) mounted behind a rotating filter wheel. Narrow bandwidth filters mounted in the wheel were alternately placed in the light path, allowing rapid switching (1–5 s $cycle^{-1}$) between appropriate excitation wavelengths. Fluorescence emission at each excitation wavelength was collected by an intensified CCD video camera (Photonic Science, Tunbridge Wells, UK) after passing through a 400 nm dichroic mirror and a 480 nm high-pass barrier filter. Fluorescence signals were stored, corrected for autofluorescence, and processed using IonVision 3 software (Improvision, Coventry, UK) (Hargreaves, Lummis & Taylor, 1994).

Throughout the text we refer to the changes in $[Ca^{2+}]_i$ that follow enhanced Ca^{2+} entry as 'Ca^{2+} entry signals' as they reflect the balance between Ca^{2+} entry to, and removal from, the cytosol rather than Ca^{2+} entry *per se*.

Calibration of fluorescence signals to $[Ca^{2+}]_i$

Media containing known free Ca^{2+} concentrations were either obtained from Molecular Probes (100 mM KCl, 10 mM Mops, 10 mM EGTA, pH 7·2; $CaCl_2$ and $MgCl_2$ were added to give appropriate concentrations of free Ca^{2+} and 1 mM free Mg^{2+}) (Haughland, 1992) or prepared as reported previously (Byron & Taylor, 1995). Fura-2 pentapotassium salt (1 μM) was added to the Ca^{2+} standard solutions and the fluorescence ($F_{340/380}$) ratios were used to construct calibration curves for each instrument (Byron & Taylor, 1995).

Measurements of Mn^{2+} entry

Rates of Mn^{2+} entry were determined by recording the quench of either fura-2 ($\lambda_{ex} = 358$ nm) or mag-fura-2 ($\lambda_{ex} = 347$ nm) fluorescence at their isosbestic wavelengths for Ca^{2+} in either nominally Ca^{2+}-free modified Krebs medium supplemented with 50 μM $MnCl_2$ or in modified Krebs medium supplemented with 100 μM $MnCl_2$. In some experiments with mag-fura-2, the ratio of fluorescence at $\lambda_{ex} = 327$ and that at $\lambda_{ex} = 365$ nm ($F_{327/365}$) was used to monitor $[Ca^{2+}]_i$ while simultaneously recording Mn^{2+} entry ($\lambda_{ex} = 347$ nm). Rates of Mn^{2+} entry (measured in arbitrary fluorescence units s^{-1}) were calculated from the slopes of the lines fitted by least-squares linear regression to the fluorescence quench data.

All results are reported as means ± S.E.M. of n independent experiments.

Materials

Fetal calf serum was from Advanced Protein Products (Brierley Hill, West Midlands, UK). Cell culture media were from Gibco BRL (Paisley, UK). Fluorescent indicators, Pluronic F-127 and Ca^{2+} calibration kits were from Molecular Probes (Leiden, Netherlands). ATP was from Boehringer. Puriss grade EGTA and $CaCO_3$ were from Fluka. Thapsigargin was from Alamone Laboratories (Jerusalem, Israel). Ionomycin, okadaic acid and SKF-96365 were from Calbiochem. UTP, α,β-methylene ATP, ADP and suramin were from Sigma. Ruthenium red was from Fluka. All other reagents were of analytical grade.

RESULTS

ATP-evoked Ca^{2+} entry in CPAE cells

ATP evoked an increase in $[Ca^{2+}]_i$ in populations of CPAE cells by stimulating both release of intracellular Ca^{2+} stores and Ca^{2+} entry. Several lines of evidence indicate that the effects of ATP were mediated entirely by its interaction with P_2 purinoceptors that stimulate InsP_3 formation. UTP (50 μM), a selective agonist of P_{2U} receptors, and ADP (50 μM), a potent agonist of the P_{2Y} receptor which is less potent for P_{2U} receptors (Fredholm *et al.* 1994), mimicked the effects of ATP (50 μM) on both phases of the Ca^{2+} signal (Fig. 1*A*). UTP and ATP mobilized the same intracellular Ca^{2+} stores, as demonstrated by the ability of a maximally effective concentration of ATP (50 μM) in the absence of extracellular Ca^{2+} to abolish almost completely the response to a subsequent challenge with a maximally effective concentration of UTP (50 μM), and vice versa (not shown). A selective agonist of some P_{2X} receptors, α,β-methylene ATP (50 μM), which also causes desensitization of some P_{2X} receptors during prolonged exposure (Brake, Wagenbach & Julius, 1994), did not mimic the effects of ATP (50 μM). Nor did it, after a 400 s pre-incubation, significantly affect the increase in $[Ca^{2+}]_i$ evoked by ATP in the continued presence of α,β-methylene ATP (Fig. 1*B*). Finally, under conditions where it has been shown to be an antagonist of many P_2 receptors but not of P_{2U} receptors (Fredholm *et al.* 1994; Castro, Mateo, Tomé, Barbosa, Miras-Portugal & Rosário, 1995), suramin (100 μM) had no significant effect on the Ca^{2+} signal evoked by ATP (Fig. 1*C*). The peak increase in $[Ca^{2+}]_i$ evoked by ATP (50 μM) alone was 609 ± 108 nM ($n = 5$) and it was 550 ± 88 nM ($n = 4$) in the presence of suramin; the half-times ($t_{½}$) for the decay of the Ca^{2+} signals were also similar in the presence and absence of suramin (35 ± 3 and 36 ± 3 s, respectively). These results suggest that in CPAE cells, the ATP-evoked increase in $[Ca^{2+}]_i$ is mediated predominantly by P_{2U} receptors, although we cannot exclude a contribution from P_{2Y} receptors. The Ca^{2+} signals evoked by ATP in CPAE cells therefore result entirely from receptor-stimulated InsP_3 formation.

The *initial rates* of rise of $[Ca^{2+}]_i$ evoked by very rapid restoration of extracellular Ca^{2+} were similar in cells incubated in the continuous presence of ATP (27 ± 11 nM s^{-1}, $n = 4$), or in cells treated briefly with ATP (200 s) to empty their intracellular Ca^{2+} stores and then exposed to extracellular Ca^{2+} after a further 50 s (28 ± 11 nM s^{-1}, $n = 4$) or 650 s (30 ± 9 nM s^{-1}, $n = 3$ (Fig. 2*A*). In paired comparisons of the rates of increase in $[Ca^{2+}]_i$ after restoration of extracellular Ca^{2+} in the presence of ATP or 50 s after its removal, the rates differed by only 2·4 ± 7·0% ($n = 4$). The lesser durations of the $[Ca^{2+}]_i$ rises in the absence of ATP presumably reflect the inactivation of capacitative Ca^{2+} entry as, in the absence of further ATP-evoked InsP_3 formation, the intracellular stores refill after restoration of extracellular Ca^{2+}. Furthermore, addition of ATP (50 μM) after restoration of extracellular Ca^{2+} to cells that had been pre-incubated with thapsigargin (1 μM, 15 min) in the absence of extracellular Ca^{2+} failed to evoke a further increase in $[Ca^{2+}]_i$ (Fig. 2*B*). Instead, ATP caused a modest transient decrease in $[Ca^{2+}]_i$, consistent with previous reports of an ATP-stimulated Ca^{2+} efflux pathway in endothelial cells mediated by activation of protein kinase C (Wang, Du, Diglio, Tsang & Kuo, 1991). Rapid restoration of Ca^{2+} to the medium bathing cells that had been pre-incubated with thapsigargin (1 μM, 15 min) to empty their intracellular Ca^{2+} stores evoked similar rates of increase in $[Ca^{2+}]_i$ in the presence (48 ± 17 nM s^{-1}, $n = 3$) or absence (44 ± 16 nM s^{-1}, $n = 3$) of ATP (50 μM).

Treatment with thapsigargin increased the rate of Mn^{2+} entry by 39 ± 5-fold ($n = 6$) and that of Ba^{2+} by 33-fold (from 1·3 ± 0·6 to 40 ± 6 arbitrary units s^{-1}; $n = 4$). These rates of both Ba^{2+} and Mn^{2+} entry were similar in thapsigargin-treated cells in the presence or absence of ATP (50 μM): their respective rates of entry in the presence of ATP were 109 ± 21% ($n = 4$) and 104 ± 14% ($n = 3$) of their rates in its absence. These results suggest that empty intracellular stores, rather than a more direct consequence of receptor activation, are *wholly* responsible for ATP-stimulated Ca^{2+} entry in CPAE cells. We conclude that the stimulatory effects of ATP on Ca^{2+} entry result entirely from activation of the capacitative pathway consequent upon emptying of the intracellular stores by InsP_3.

Cytosolic Ca^{2+} modulates ATP-evoked Ca^{2+} entry signals

Restoration of extracellular Ca^{2+} to cells in which the stores had been emptied by ATP caused $[Ca^{2+}]_i$ to rise rapidly to a peak before declining to a sustained phase of elevated $[Ca^{2+}]_i$.

Such Ca^{2+} overshoots occurred despite the continued presence of both ATP and extracellular Ca^{2+} (Figs 2*A a* and 3). Similar overshoots in $[Ca^{2+}]_i$ were observed when extracellular Na^+ was replaced by *N*-methyl-D-glucamine (NMDG) or choline. In NMDG-containing medium, the peak and plateau levels of $[Ca^{2+}]_i$ were 88 ± 2 and $89 \pm 1\%$ ($n = 3$) of those observed in the presence of Na^+; the comparable results for choline-containing medium were 94 ± 3 and $99 \pm 1\%$ ($n = 3$) (not shown).

The Ca^{2+} entry signal evoked by ATP was more substantial when the Ca^{2+} release and Ca^{2+} entry phases were separated. Since ATP-evoked Ca^{2+} release was complete within 100 s (Figs 2*A* and 3*A b*), we assessed the effects of Ca^{2+} entry on Ca^{2+} signals by comparing the increase in $[Ca^{2+}]_i$ 100 s after addition of ATP in the presence of extracellular Ca^{2+} (Fig. 3*A a*) with that evoked 100 s after restoration of extracellular Ca^{2+} to cells that had first been stimulated with ATP in the absence of extracellular Ca^{2+} (Fig. 3*A b*). By integrating the area beneath records similar to those shown in Fig. 3*A* for the 850 s when both extracellular Ca^{2+} and ATP were present and subtracting the component ascribable to Ca^{2+} release (Fig. 3*A a*), we estimated the total $[Ca^{2+}]_i$ signal resulting from Ca^{2+} entry (shaded areas in the insets to Fig. 3*A*). These results confirm that the total $[Ca^{2+}]_i$ signal evoked by Ca^{2+} entry was greater (by $45 \pm 5\%$, $n = 3$) when the Ca^{2+} release and entry phases were separated, although the method may underestimate the discrepancy because Ca^{2+}-induced Ca^{2+} release may amplify the Ca^{2+} signal evoked by Ca^{2+} entry, but only in cells with replete

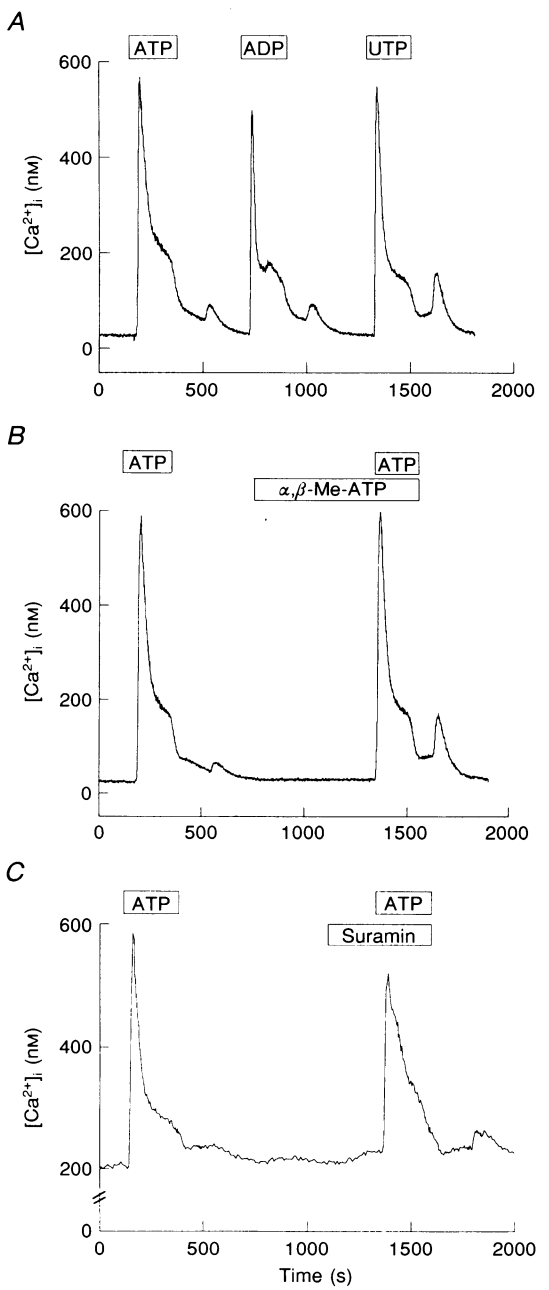

Figure 1. P_{2U} receptors mediate the effects of ATP on $[Ca^{2+}]_i$ in CPAE cells

Each trace, showing the responses of fura-2-loaded CPAE cells, is representative of at least 3 independent experiments. *A*, populations of cells were stimulated with 50 μM ATP, ADP or UTP for the periods shown. *B*, populations of cells were stimulated with ATP (50 μM) alone or in the presence of α,β-methylene ATP (50 μM). *C*, the response of a single cell to ATP (50 μM) in the presence or absence of suramin (100 μM) is shown. The trace is typical of at least 4 cells measured in 3 independent populations.

Ca^{2+} stores. The changes in $[Ca^{2+}]_i$ signal are unlikely to result directly from ATP-evoked changes in receptor sensitivity because repeated challenges with ATP or repeated restoration of extracellular Ca^{2+} in the continued presence of ATP evoked reproducible overshoots in $[Ca^{2+}]_i$ (Fig. 3*B*).

In most CPAE cell populations (~70%), removal of ATP in the continued presence of extracellular Ca^{2+} caused complex and reproducible changes in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ first rapidly declined to an elevated plateau which was followed, 83 ± 9 s ($n = 13$) after removal of ATP, by a small (39 ± 12 nM, $n = 13$) increase in $[Ca^{2+}]_i$ which then returned to its basal level (Figs 1*A* and B, 2*A a* and 3*A b*). Similar results were obtained from single cell measurements (Fig. 1*C*). These $[Ca^{2+}]_i$ overshoots were never observed when ATP was removed in the absence of extracellular Ca^{2+} (Fig. 2*A c*), nor when extracellular Ca^{2+} was removed in the continued presence of ATP (Fig. 3*B a*). The larger Ca^{2+} entry signals that occurred when Ca^{2+} entry and release were separated (Fig. 3*A*), the overshoots in $[Ca^{2+}]_i$ upon restoration of Ca^{2+} to cells stimulated with ATP (Figs 2*A* and 3), and the complex $[Ca^{2+}]_i$ changes that followed removal of ATP (Figs 1–3) suggest that the Ca^{2+} transport pathways responsible for these Ca^{2+} signals may be subject to control by Ca^{2+} that is relatively slow in both its onset and reversal. Subsequent experiments aimed to establish the mechanisms underlying this regulation.

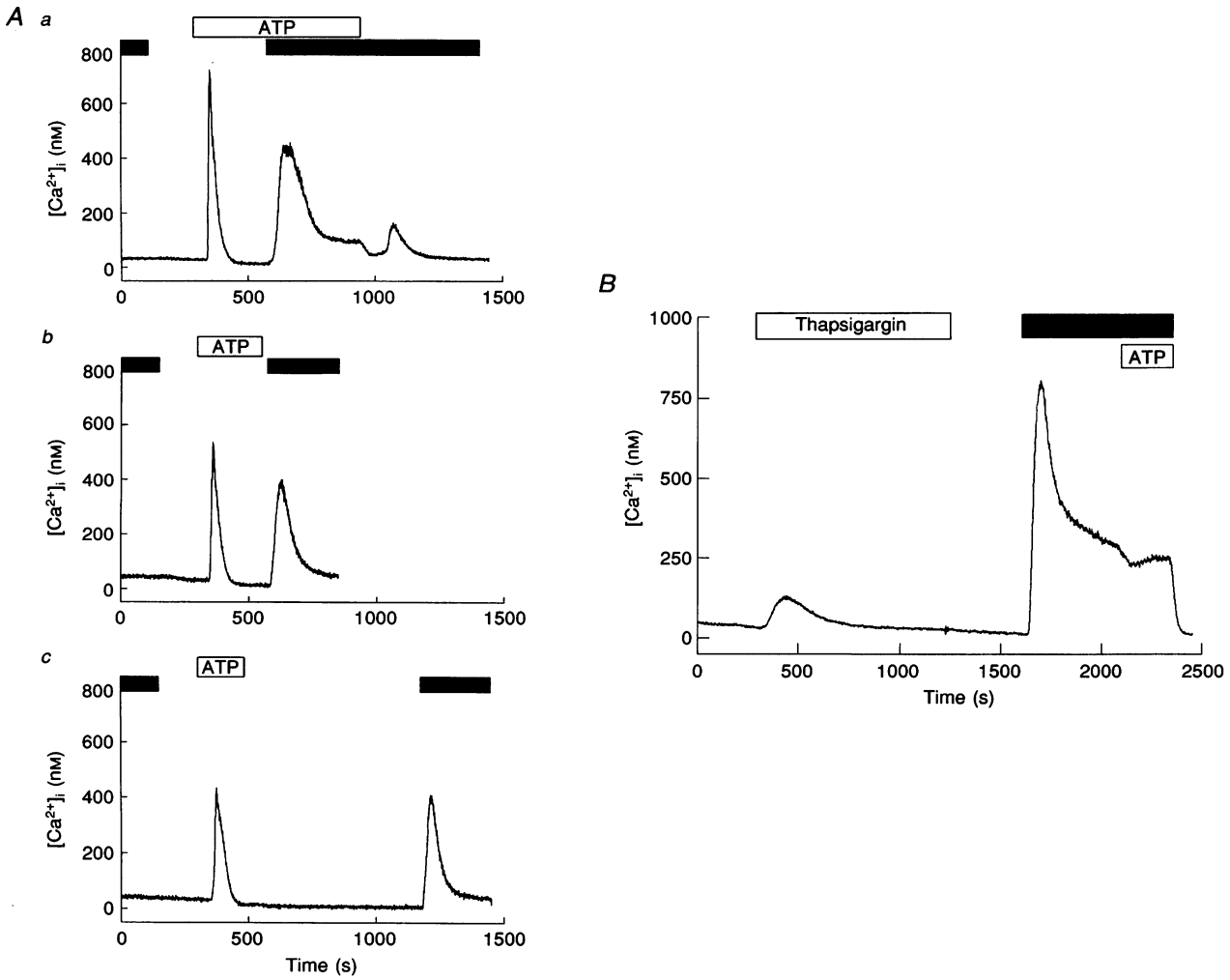

Figure 2. ATP-stimulated Ca^{2+} entry in CPAE cells results entirely from activation of capacitative Ca^{2+} entry

A, populations of cells were stimulated with ATP (50 μM) in the absence of extracellular Ca^{2+}. Extracellular Ca^{2+} (1·5 mM) was then rapidly restored for the periods shown by the filled bars. The results, typical of 3 independent experiments, indicate that initial rates of increase in $[Ca^{2+}]_i$ after rapid restoration of extracellular Ca^{2+} are unaffected by either the continued presence of ATP (*a*) or the interval since its removal (*b* and *c*). *B*, after pretreatment of populations of cells with thapsigargin (1 μM, 15 min) in the absence of extracellular Ca^{2+}, addition of ATP (50 μM) during sustained Ca^{2+} entry (medium $[Ca^{2+}] = 1·5$ mM, filled bar) evoked a transient decrease in $[Ca^{2+}]_i$. The traces shown (*A* and *B*) are each representative of 3 independent experiments.

Attenuation of capacitative Ca^{2+} entry signals by cytosolic Ca^{2+}

Since the Ca^{2+} entry evoked by ATP results entirely from activation of the capacitative pathway (Fig. 2), we used thapsigargin to empty the intracellular Ca^{2+} stores and investigate the behaviour of the capacitative Ca^{2+} entry pathway directly. The response of CPAE cells to addition of thapsigargin was similar to that of many other cells (Putney & Bird, 1993): an initial release of intracellular Ca^{2+} stores followed by a sustained elevation of $[Ca^{2+}]_i$ that rapidly reversed after removal of extracellular Ca^{2+} (not shown). This capacitative Ca^{2+} entry pathway was permeable to Mn^{2+} and Ba^{2+} (see above), and reversibly blocked by $LaCl_3$ (10 μM) (not shown). Restoration of extracellular Ca^{2+} to cells pretreated with thapsigargin in Ca^{2+}-free medium caused reproducible increases in $[Ca^{2+}]_i$, indicating that the capacitative Ca^{2+} entry pathway did not desensitize during even very prolonged activation ($\leqslant$100 min; Fig. 4*A*).

Restoration of extracellular Ca^{2+} to populations of cells pretreated with thapsigargin (1 μM, 15 min) evoked a substantial increase in $[Ca^{2+}]_i$ to 731 $\pm$ 30 nM ($n = 87$) above basal. $[Ca^{2+}]_i$ then declined to an elevated plateau, such that 400 s after addition of extracellular Ca^{2+}, $[Ca^{2+}]_i$ was 232 $\pm$ 10 nM ($n = 87$) and even 30 min later, $[Ca^{2+}]_i$ remained elevated at 122 $\pm$ 8 nM ($n = 3$) above its basal

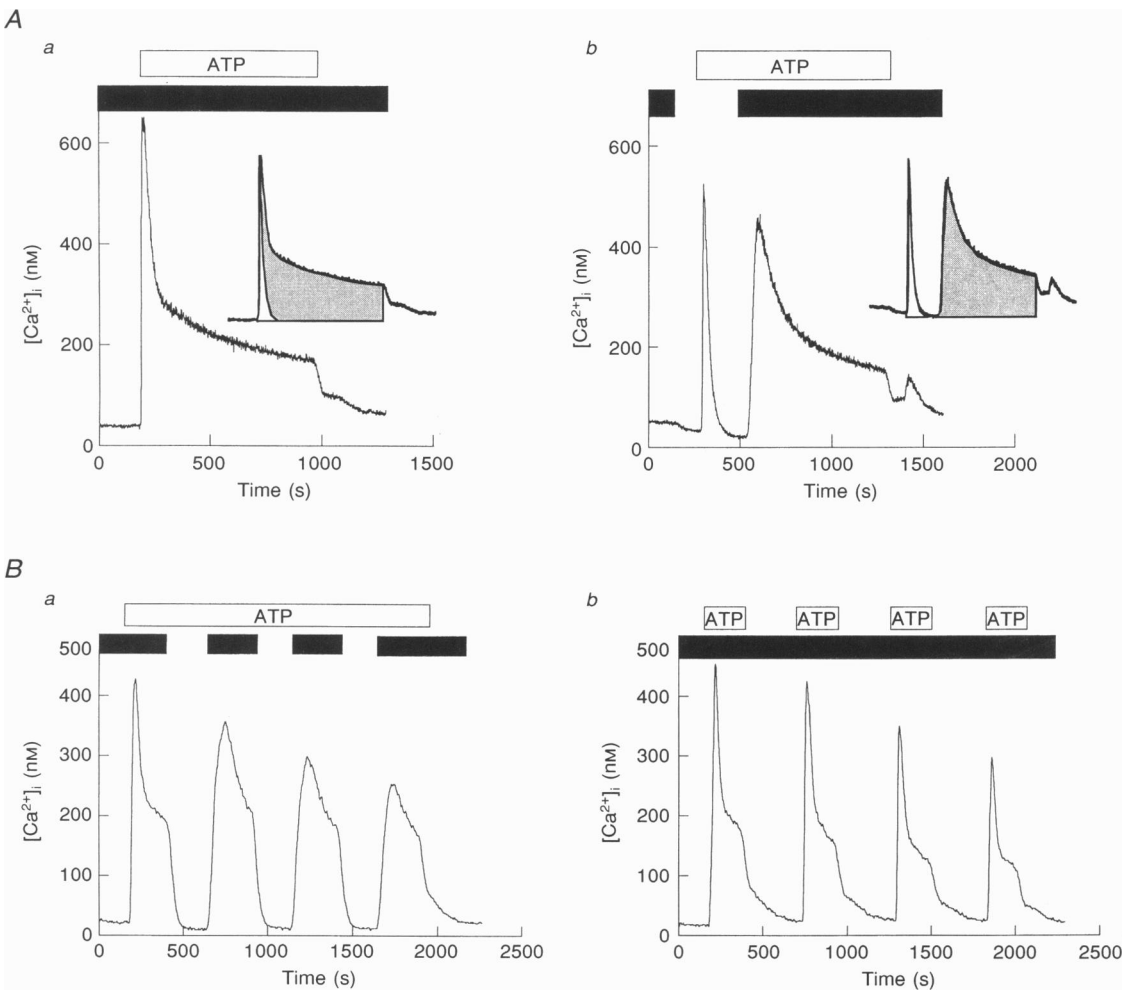

Figure 3. ATP-evoked Ca^{2+} entry evokes a greater increase in $[Ca^{2+}]_i$ when separated from Ca^{2+} release

A, the Ca^{2+} release and Ca^{2+} entry phases of the response to ATP were separated by addition or removal of extracellular Ca^{2+} (1·5 mM, filled bars) in the continued presence of ATP (50 μM). Since ATP-stimulated Ca^{2+} release is complete within 100 s, the extent of the Ca^{2+} entry signal was determined by integrating the area beneath each record when both ATP and extracellular Ca^{2+} were present, and substracting (in *a*) the component ascribable to Ca^{2+} release (the area is represented by shading in the insets). The results are typical of at least 3 independent experiments. *B*, populations of cells were incubated with ATP (50 μM) for the periods shown by open bars and extracellular Ca^{2+} (1·5 mM) was present for the periods shown by filled bars. The results, typical of at least 3 similar independent experiments, demonstrate that repeated restoration of Ca^{2+} in the continued presence of ATP (*a*) or repeated additions of ATP in the continued presence of extracellular Ca^{2+} (*b*) evoked reproducible increases in $[Ca^{2+}]_i$.

level (Fig. 4*B*). These results demonstrate that Ca^{2+} entry through the capacitative pathway causes a delayed decrease in the effectiveness with which continuing Ca^{2+} entry leads to an increase in $[Ca^{2+}]_i$. This phenomenon does not result from refilling of InsP_3-sensitive stores (which would be unlikely after thapsigargin treatment) and consequent inactivation of capacitative Ca^{2+} entry because similar complex Ca^{2+} signals were observed on readdition of extracellular Ca^{2+} in the presence of ATP (50 μM) (Fig. 4*C*). Furthermore, addition of ATP (50 μM) after rapid removal of extracellular Ca^{2+} by injection of Ca^{2+}-free modified Krebs medium to the cuvette during the plateau phase of the response to thapsigargin failed to evoke a rise in $[Ca^{2+}]_i$, confirming that the InsP_3-sensitive Ca^{2+} stores remained empty (not shown). Similar $[Ca^{2+}]_i$ overshoots were observed in media containing NMDG in place of Na^+, or in the presence of okadaic acid (30 nM), an inhibitor of protein phosphatases 1 and 2*A* (not shown).

The peak amplitudes of the Ca^{2+} signals were significantly greater in Ruthenium Red-injected cells (636 ± 111 nM, $n = 5$) relative to controls (376 ± 33 nM, $n = 3$), but the essential characteristics of the Ca^{2+} signals evoked by restoration of extracellular Ca^{2+} to thapsigargin-treated cells were similar (Fig. 5). In each, a transient $[Ca^{2+}]_i$ overshoot was followed by a decline ($t_{½} = 66 \pm 20$ s for Ruthenium Red-injected cells; $t_{½} = 92 \pm 8$ s for control cells) to an elevated plateau that was significantly higher in Ruthenium Red-injected cells (378 ± 50 nM *versus* 209 ± 19 nM).

The amplitudes of the $[Ca^{2+}]_i$ increases evoked by restoration of Ca^{2+} to the medium bathing thapsigargin-treated cells increased as the extracellular $[Ca^{2+}]$ was increased (from 200 μM to 1·5 mM), but in each case $[Ca^{2+}]_i$ overshot before slowly returning to an elevated plateau (Fig. 6*A*). Similar results were obtained when the amplitude of the increase in $[Ca^{2+}]_i$ was manipulated by addition of SKF-96365 (25 μM), an inhibitor of capacitative Ca^{2+} entry

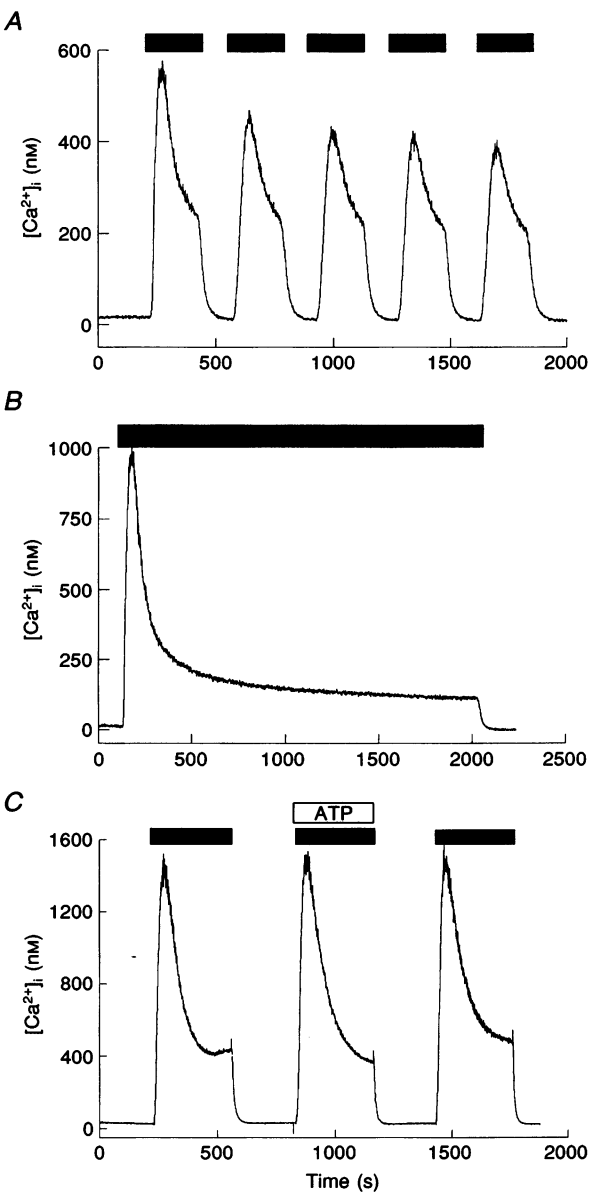

Figure 4. The capacitative Ca^{2+} entry pathway does not desensitize during prolonged activation

A, cells were pretreated with thapsigargin (1 μM, 15 min) in Ca^{2+}-free medium before periodic restoration of extracellular Ca^{2+} (1·5 mM) for intervals of 200 s (filled bars). The results demonstrate that even very sustained stimulation of the capacitative pathway does not cause it to desensitize. Similar results were obtained from at least 6 independent experiments in which Ca^{2+} was transiently restored at intervals of 30–100 min after store depletion. *B*, cells were treated as in *A* and then extracellular Ca^{2+} was restored for the period shown (filled bar) leading to a sustained elevation in $[Ca^{2+}]_i$. Similar results were obtained in at least 3 experiments. *C*, restoration of extracellular Ca^{2+} (1·5 mM, filled bars) to populations of cells in which the intracellular stores had been emptied by thapsigargin caused similar transient $[Ca^{2+}]_i$ overshoots in the absence or presence of ATP (50 μM, open bar). The results are typical of 4 independent experiments.

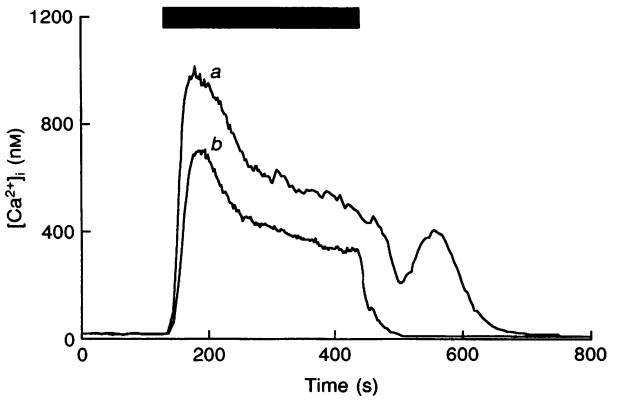

Figure 5. Mitochondrial Ca^{2+} uptake is not the sole cause of the complex Ca^{2+} signals that follow capacitative Ca^{2+} entry

Cells were microinjected with fura-2 (2·5 mM) with (*a*) or without (*b*) Ruthenium Red (1 mM) before incubation with thapsigargin (1 μM, 15 min) in Ca^{2+}-free medium. Extracellular Ca^{2+} (1·5 mM) was then restored for the periods shown by the filled bars. The results show the $[Ca^{2+}]_i$ of single cells. Each trace is representative of recordings from at least 5 cells from 3 (*a*) or 5 (*b*) independent coverslips.

(Merritt *et al.* 1990), to medium containing 1·5 mM Ca^{2+} rather than by changes in extracellular $[Ca^{2+}]$ (Fig. 6*B*). These results suggest that changes in $[Ca^{2+}]_i$ rather than changes in extracellular $[Ca^{2+}]$ *per se* modulate the Ca^{2+} signals, and that even modest increases in $[Ca^{2+}]_i$ cause a delayed attenuation of the Ca^{2+} signal evoked by capacitative Ca^{2+} entry. The influence of $[Ca^{2+}]_i$ on the subsequent ability of capacitative Ca^{2+} entry to evoke a further increase in $[Ca^{2+}]_i$ was directly examined by allowing $[Ca^{2+}]_i$ to reach an elevated plateau as a consequence of capacitative Ca^{2+} entry during a 400 s incubation of thapsigargin-treated cells in various extracellular Ca^{2+} concentrations (100–500 μM) (Fig. 7*A*). The results demonstrate that prior elevation of $[Ca^{2+}]_i$ caused a concentration-dependent inhibition of the peak $[Ca^{2+}]_i$ signal evoked by subsequently increasing the extracellular $[Ca^{2+}]$ to 1·5 mM, without affecting the plateau phase of the response (Fig. 7*B*). The correlation between $[Ca^{2+}]_i$ and inhibition of capacitative Ca^{2+} entry signals was higher when related to the preceding peak $[Ca^{2+}]_i$ (*a* in Fig. 7*A*; $r = 0{\cdot}89$, $P < 0{\cdot}001$) than when related to the $[Ca^{2+}]_i$ immediately preceding the assessment of capacitiative Ca^{2+} entry (*b* in Fig. 7*A*; $r = 0{\cdot}78$). These results are consistent with a long-lasting ability of transient increases in $[Ca^{2+}]_i$ to inhibit capacitative Ca^{2+} entry signals.

Time course of the effects of cytosolic Ca^{2+} on capacitative Ca^{2+} entry signals

The $[Ca^{2+}]_i$ of thapsigargin-treated cells was increased by raising the extracellular $[Ca^{2+}]$ from 0 to 500 μM, and at intervals thereafter the extracellular $[Ca^{2+}]$ was further

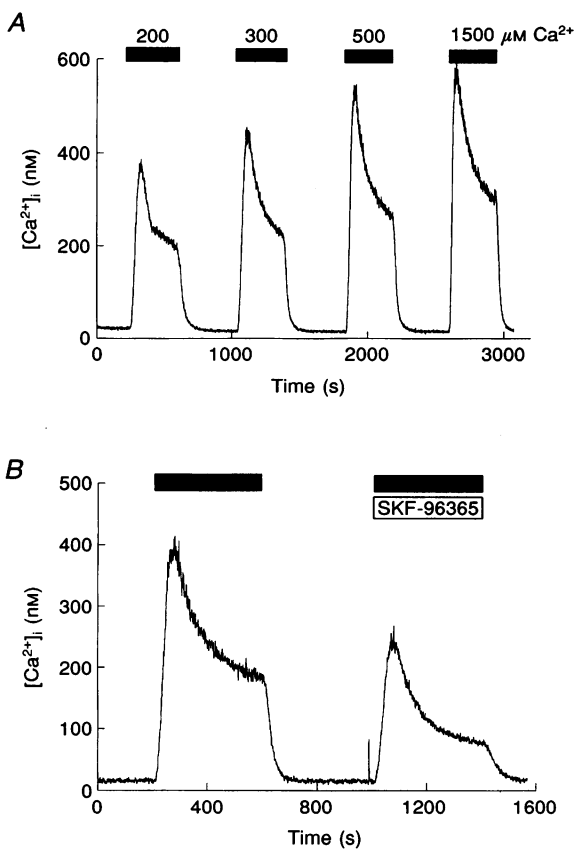

Figure 6. Modest increases in $[Ca^{2+}]_i$ attenuate the Ca^{2+} signals evoked by capacitative Ca^{2+} entry

A, populations of thapsigargin-treated cells were incubated in medium containing the indicated concentrations (μM) of Ca^{2+} for the periods of 400 s denoted by filled bars. The results, which are representative of 3 similar experiments, indicate that an overshoot in $[Ca^{2+}]_i$ occurs after each addition of extracellular Ca^{2+}. *B*, extracellular Ca^{2+} (1·5 mM, filled bars) was restored to thapsigargin-treated cells in the presence or absence of SKF-96365 (25 μM). Results are representative of 3 independent experiments.

rapidly increased to 1·5 mM to establish the extent to which the elevated $[Ca^{2+}]_i$ had inhibited the capacitative Ca^{2+} signal (Fig. 8*A*). The increase in $[Ca^{2+}]_i$ evoked by pre-incubating cells for 100 s in medium containing 500 μM Ca^{2+} caused 18 ± 6% ($n = 6$) inhibition of the subsequent peak increase in $[Ca^{2+}]_i$, and the inhibition reached its maximum (52 ± 3%, $n = 6$) after pre-incubation for 800 s (Fig. 8*B*). None of the pre-incubations affected the plateau phase of the response (Fig. 8*B*). These results demonstrate that the increase in $[Ca^{2+}]_i$ that follows restoration of extracellular Ca^{2+} causes a slow ($t_{½} = 182$ s) inhibition of the ability of further capacitative Ca^{2+} entry to increase $[Ca^{2+}]_i$. By correcting for the time taken for $[Ca^{2+}]_i$ to rise to a peak after restoration of extracellular Ca^{2+} (55 ± 5 s, $n = 17$; Fig. 8*A*), the inhibition triggered by the peak $[Ca^{2+}]_i$ was estimated to proceed with a $t_{½}$ of ~127 s. Another estimate of the time course of the inhibition is provided by the rate at which $[Ca^{2+}]_i$ declines from its peak to the steady-state plateau in medium containing 500 μM Ca^{2+} ($t_{½} = 83 \pm 10$ s, $n = 7$). These independent estimates of the rate at which $[Ca^{2+}]_i$ inhibits capacitative Ca^{2+} entry signals are in reasonable agreement and both are substantially slower than the rates of Ca^{2+} removal from the cytosol observed in Ca^{2+}-free media ($t_{½} = 7$ s; Fig. 14*A*), indicating that the time course of inhibition is not obscured by slow Ca^{2+} transport mechanisms.

Recovery from the inhibitory effects of elevated $[Ca^{2+}]_i$

In order to establish the time course of recovery from the inhibitory effects of elevated $[Ca^{2+}]_i$, thapsigargin-treated cells were incubated for 400 s in medium containing 1·5 mM Ca^{2+} to ensure inhibition of the capacitative Ca^{2+} signals. Extracellular Ca^{2+} was then rapidly removed and at intervals thereafter the extracellular $[Ca^{2+}]$ was again increased to 1·5 mM and the magnitude of the capacitative Ca^{2+} entry signals determined (Fig. 9*A*). After removal of extracellular Ca^{2+}, $[Ca^{2+}]_i$ rapidly returned to its basal level ($t_{½} \approx 7$ s), but complete recovery of the capacitative Ca^{2+} entry signals required removal of extracellular Ca^{2+} for 400 s (Fig. 9*B*). These results demonstrate that even after $[Ca^{2+}]_i$ has returned to its basal level, reversal of the inhibitory effects of increased $[Ca^{2+}]_i$ on capacitative Ca^{2+} entry signals is slow, requiring a further 320 ± 3 s ($n = 6$) to reach completion.

Recovery was further examined by incubating thapsigargin-treated cells for 400 s in medium containing 1·5 mM Ca^{2+} before reducing the extracellular $[Ca^{2+}]$ to between 100 and 500 μM for a further 400 s. The effects of the consequent changes in $[Ca^{2+}]_i$ on capacitative Ca^{2+} entry signals were assessed by then restoring the extracellular $[Ca^{2+}]$ to 1·5 mM. The results (Fig. 10) suggest that the lowest $[Ca^{2+}]_i$

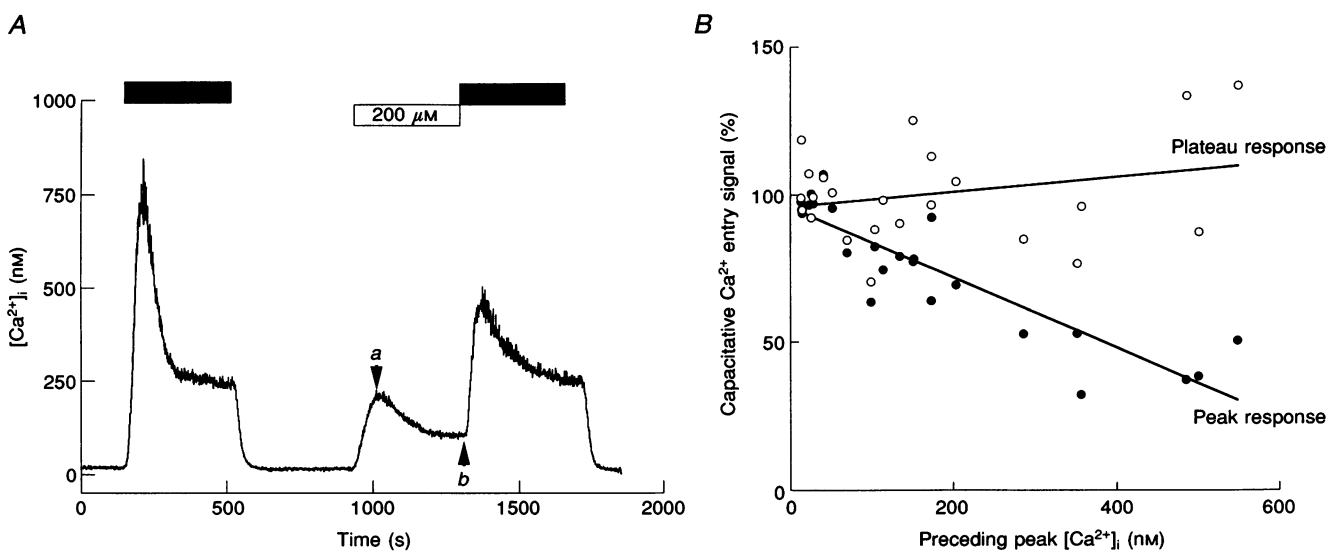

Figure 7. Cytosolic Ca^{2+} causes concentration-dependent inhibition of the Ca^{2+} signals evoked by capacitative Ca^{2+} entry

A, populations of thapsigargin-treated cells were incubated in Ca^{2+}-free medium before restoration of Ca^{2+} (1·5 mM, filled bar) for 400 s. Extracellular Ca^{2+} was then removed for a further 400 s before re-addition of Ca^{2+} (100–500 μM, open bar; 200 μM in this trace) for 400 s. After this interval, the extracellular $[Ca^{2+}]$ was again increased to 1·5 mM (filled bar) to assess the extent to which the capacitative Ca^{2+} entry signal was inhibited. Points *a* and *b* are referred to in the text: *a* denotes the peak $[Ca^{2+}]_i$ attained during the pre-incubation, and *b* denotes the $[Ca^{2+}]_i$ immediately preceding assessment of capacitative Ca^{2+} entry. *B*, the pooled results of 4–6 experiments at each extracellular $[Ca^{2+}]$ similar to those shown in *A* are plotted to show the relationships between the peak $[Ca^{2+}]_i$ attained during the pre-incubation (*a* in *A*) and the amplitudes of either the peak (●) or plateau (○) phases of the subsequent capacitative Ca^{2+} entry signal. Each response is plotted as a percentage of that recorded from the cells prior to elevation of $[Ca^{2+}]_i$ and the lines were fitted by least-squares regression.

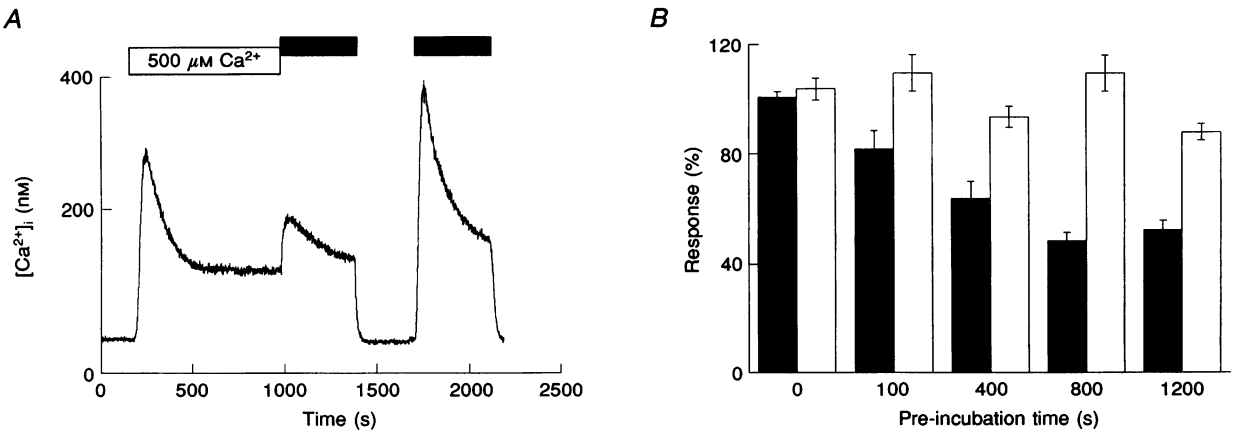

Figure 8. Time-dependent inhibition of capacitative Ca^{2+} entry signals by increased $[Ca^{2+}]_i$

A, populations of thapsigargin-treated cells were incubated in medium containing 500 μM Ca^{2+} for intervals of 100–1200 s (open bar, 800 s in this trace) before the extracellular $[Ca^{2+}]$ was increased to 1·5 mM for 400 s (filled bar). After a recovery period of 400 s in Ca^{2+}-free medium, the extracellular $[Ca^{2+}]$ was again increased to 1·5 mM to establish the maximal capacitative Ca^{2+} entry signal. *B*, results from experiments similar to those shown in *A* are summarized. The increases in $[Ca^{2+}]_i$ evoked by increasing the extracellular $[Ca^{2+}]$ to 1·5 mM after the indicated pre-incubation period in 500 μM Ca^{2+} are plotted as percentages of those evoked by increasing $[Ca^{2+}]$ in the medium from 0 to 1·5 mM. Filled columns denote the changes in the peak $[Ca^{2+}]_i$ and open columns denote the changes in the plateau level of $[Ca^{2+}]_i$. Results are means ± S.E.M. of 4–6 independent experiments.

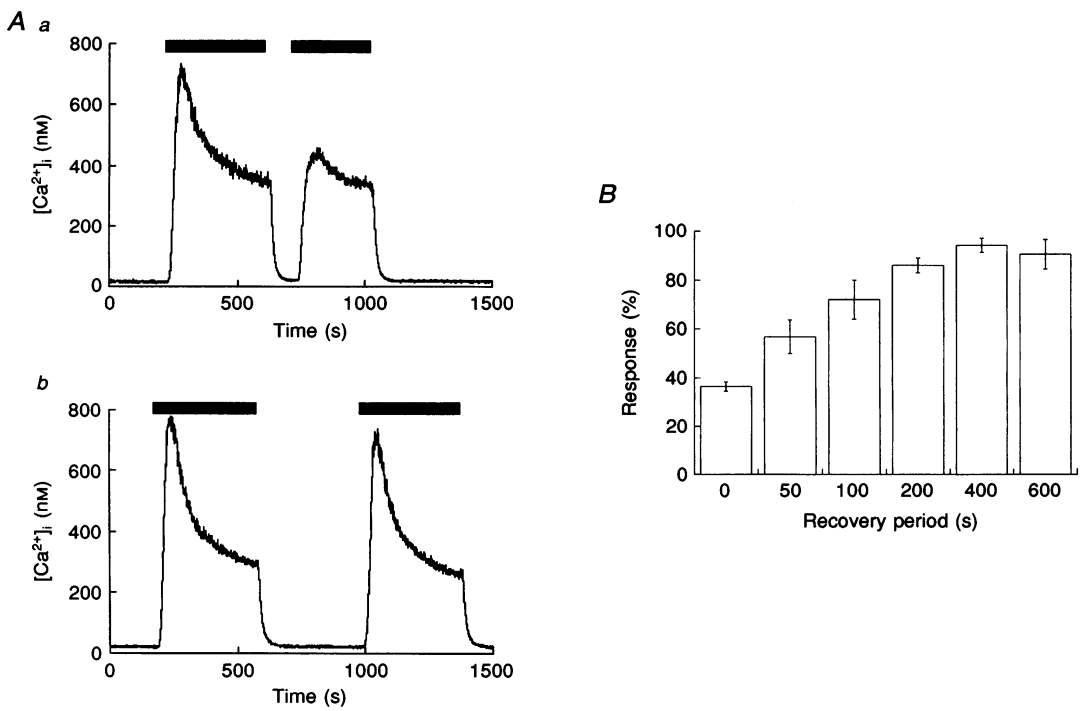

Figure 9. Slow recovery from the inhibitory effect of increased $[Ca^{2+}]_i$

A, populations of thapsigargin-treated cells were incubated with medium containing 1·5 mM Ca^{2+} for 400 s (filled bars) and then allowed to recover for intervals (50–600 s) before a second challenge with medium containing 1·5 mM Ca^{2+}. Two representative traces are shown in which the recovery periods were 100 s (*A a*) and 400 s (*A b*). *B*, results from experiments similar to those shown in *A* are plotted to show the peak amplitude of the second capacitative Ca^{2+} entry signal after the indicated periods of recovery from the first exposure to extracellular Ca^{2+}. The results (means ± S.E.M.) of 4–6 independent experiments show the amplitude of the second Ca^{2+} signal as a percentage of the first.

attained during the recovery period, rather than the $[Ca^{2+}]_i$ immediately preceding the capacitative Ca^{2+} entry signal, determines the extent to which the inhibition reverses. Recovery in medium containing 100 μM Ca^{2+}, for example, allowed $[Ca^{2+}]_i$ to transiently fall to 21 ± 2 nM ($n = 3$) before rising to a plateau of 83 ± 22 nM (Fig. 10*A*), whereas in medium containing 200 μM Ca^{2+}, $[Ca^{2+}]_i$ recovered almost monophasically to 39 ± 9 nM ($n = 4$) (Fig. 10*B*). Yet the capacitative Ca^{2+} entry signals recovered to $83 \pm 5\%$ and $67 \pm 6\%$ ($P < 0{\cdot}05$) of their control values during 400 s in medium containing 100 and 200 μM Ca^{2+}, respectively (Fig. 10). Further evidence that the lowest $[Ca^{2+}]_i$ attained during the recovery phase determines the extent of the recovery is supported by the more significant correlation between recovery of capacitative Ca^{2+} signals and the lowest $[Ca^{2+}]_i$ attained during the recovery period (*a* in Fig. 10*A*; $r = 0{\cdot}68$, $P < 0{\cdot}001$) than between the $[Ca^{2+}]_i$ immediately preceding the Ca^{2+} signal and recovery (*b* in Fig. 10*A*; $r = 0{\cdot}47$, $P < 0{\cdot}05$). Further insight into the recovery process is provided by the undershoots in $[Ca^{2+}]_i$ that follow a reduction in extracellular $[Ca^{2+}]$ (Fig. 10*A*). From the time course of the increase in $[Ca^{2+}]_i$ as it rises from the undershoot to an elevated plateau, the $t_{1/2}$ for recovery can be estimated at ~150 s.

Our results suggest that inhibitory regulation of capacitative Ca^{2+} entry signals by cytosolic Ca^{2+} is slow in both its onset (Figs 7 and 8) and recovery (Figs 9 and 10). The extent to which capacitative Ca^{2+} entry evokes an increase in $[Ca^{2+}]_i$ might therefore be influenced by prior changes in $[Ca^{2+}]_i$ and so endow the cell with a memory of preceding Ca^{2+} signals. This issue was addressed directly using the protocol depicted in Fig. 11. Thapsigargin-treated cells were exposed either to medium containing 100 μM Ca^{2+} for 600 s (Fig. 11*A*), or sequentially to media containing 1·5 mM (400 s) and then 200 μM (200 s) Ca^{2+} (Fig. 11*B*). With these treatments, $[Ca^{2+}]_i$ in the $\geqslant 100$ s preceding our assessment of the capacitative Ca^{2+} entry signals were not significantly different (difference $= 9 \pm 12\%$, $n = 6$), but the preceding peaks in $[Ca^{2+}]_i$ were $307 \pm 103\%$ larger in the cells pretreated with 1·5 mM Ca^{2+} (Fig. 11). However, the amplitude of the Ca^{2+} overshoot evoked by restoring extracellular Ca^{2+} to 1·5 mM was significantly lower by $34 \pm 6\%$ ($P < 0{\cdot}01$) in the cells that had previously had the greater increase in $[Ca^{2+}]_i$ (Fig. 11*B*). These results illustrate the long-lasting ability of a transient increase in $[Ca^{2+}]_i$ to inhibit the Ca^{2+} signals evoked by subsequent capacitative Ca^{2+} entry, despite an intervening period of reduced $[Ca^{2+}]_i$.

Rapid inhibition of capacitative Mn^{2+} entry by increased $[Ca^{2+}]_i$

Our results suggest that increases in $[Ca^{2+}]_i$ decrease the ability of capacitative Ca^{2+} entry to further increase $[Ca^{2+}]_i$. In subsequent experiments, we attempted to establish whether this attenuation of capacitative Ca^{2+} entry signals resulted from stimulation of Ca^{2+} removal from the cytosol or inhibition of the capacitative Ca^{2+} entry pathway.

Since the capacitative pathway is permeable to Mn^{2+} (see above), we used Mn^{2+} quenching of mag-fura-2 to assess the effects of $[Ca^{2+}]_i$ on unidirectional Mn^{2+} entry through the capacitative pathway. Mag-fura-2 was used in preference to

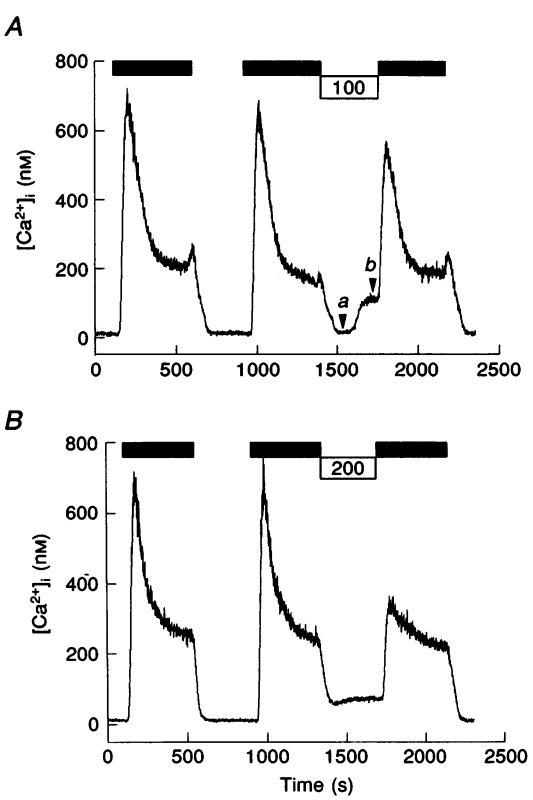

Figure 10. Recovery from the inhibitory effect of elevated $[Ca^{2+}]_i$ requires a substantial decrease in $[Ca^{2+}]_i$

Thapsigargin-treated cells were sequentially incubated with medium containing 1·5 mM Ca^{2+} (filled bars) between which the extracellular $[Ca^{2+}]$ was reduced to 0, 100 μM (*A*, open bar) or 200 μM (*B*, open bar). The results are each typical of 4 independent experiments. Points *a* and *b* are referred to in the text and respectively denote the lowest $[Ca^{2+}]_i$ attained during the recovery period and the $[Ca^{2+}]_i$ immediately preceding assessment of the capacitative Ca^{2+} signal.

fura-2 because it has much lower affinity for Ca^{2+} ($K_d^{Ca} = 50\ \mu M$) (Haughland, 1992). In cytosol, therefore, only a very small fraction of mag-fura-2 would be bound to Ca^{2+}, thereby eliminating any problems that might result from direct competition between cytosolic Ca^{2+} and Mn^{2+} for binding to the indicator. The characteristic pattern of an overshooting capacitative Ca^{2+} signal was similar whether recorded with mag-fura-2 (Fig. 12*A a*) or fura-2 (Figs 2–10). Mag-fura-2-loaded cells were pretreated with thapsigargin (1 μM, 15 min) before addition of medium containing $MnCl_2$ (100 μM) and $CaCl_2$ (1·5 mM).

In control cells in the presence of extracellular Ca^{2+}, Mn^{2+} entry was undetectable with mag-fura-2. After thapsigargin treatment, Mn^{2+} entry during the rising phase of the Ca^{2+} signal caused the mag-fura-2 fluorescence ($\lambda_{ex} = 347$ nm) to decrease at a rate of 2·5 ± 0·4 arbitrary units s^{-1} ($n = 19$). Coincident with the peak $[Ca^{2+}]_i$, the rate of Mn^{2+} entry fell abruptly so that fluorescence then decreased at a rate of 0·97 ± 0·1 arbitrary units s^{-1}. In all experiments (19/25) in which restoration of extracellular Ca^{2+} to thapsigargin-treated cells evoked a readily detectable overshoot in $[Ca^{2+}]_i$, it was temporally correlated with an abrupt 2·5 ± 0·1-fold ($n = 19$) decrease in the rate of Mn^{2+} entry (Fig. 12*A*). The lesser, though still significantly stimulated, rate of Mn^{2+} entry then continued at a similar rate for at least a further 200–300 s, despite the substantial fall in $[Ca^{2+}]_i$ (Fig. 12*A*). When Mn^{2+} entry to thapsigargin-treated cells was measured in the absence of extracellular Ca^{2+}, fluorescence quenching (3·5 ± 0·4 arbitrary units s^{-1}, $n = 6$) was maintained throughout the incubation (150–200 s) (Fig. 12*B*). An abrupt decrease in the rate of capacitative Mn^{2+} entry therefore occurs only when accompanied by an increase in $[Ca^{2+}]_i$.

Our previous results (Fig. 8) suggested a delayed regulation of capacitative Ca^{2+} entry signals by $[Ca^{2+}]_i$, yet there is no detectable delay between the peak $[Ca^{2+}]_i$ and the consequent abrupt decrease in the rate of Mn^{2+} entry (Fig. 12*A*). Since capacitative Mn^{2+} entry continues, albeit at a reduced rate, after the peak $[Ca^{2+}]_i$, we examined whether a second, slower inhibition of the capacitative pathway by increased $[Ca^{2+}]_i$ might contribute to the delayed attenuation of capacitative Ca^{2+} entry signals. Capacitative Mn^{2+} entry was measured with mag-fura-2 during the sustained phase of capacitative Ca^{2+} entry by periodically restoring extracellular Mn^{2+} (Fig. 13). The rates of Mn^{2+} entry were similar during the slow phase immediately following restoration of extracellular Ca^{2+} and 500 s later (the difference was 2 ± 8%, $n = 4$). During 30 min of sustained Ca^{2+} entry, the rate of Mn^{2+} entry decreased minimally: the final rate of Mn^{2+} entry was 76 ± 3% ($n = 3$) of that recorded during the initial slow component (Fig. 13). These results demonstrate that within seconds of $[Ca^{2+}]_i$ reaching its peak, there is a substantial decrease in the rate of capacitative Mn^{2+} entry. Thereafter the rates of Mn^{2+}, and presumably Ca^{2+}, entry remain stable despite the fall in $[Ca^{2+}]_i$. Our evidence therefore suggests a rapid, but long-lasting, partial inhibition of the capacitative pathway by increased $[Ca^{2+}]_i$.

Delayed stimulation of Ca^{2+} recovery

The possibility that increased $[Ca^{2+}]_i$ might regulate Ca^{2+} recovery in addition to Ca^{2+} entry was addressed by

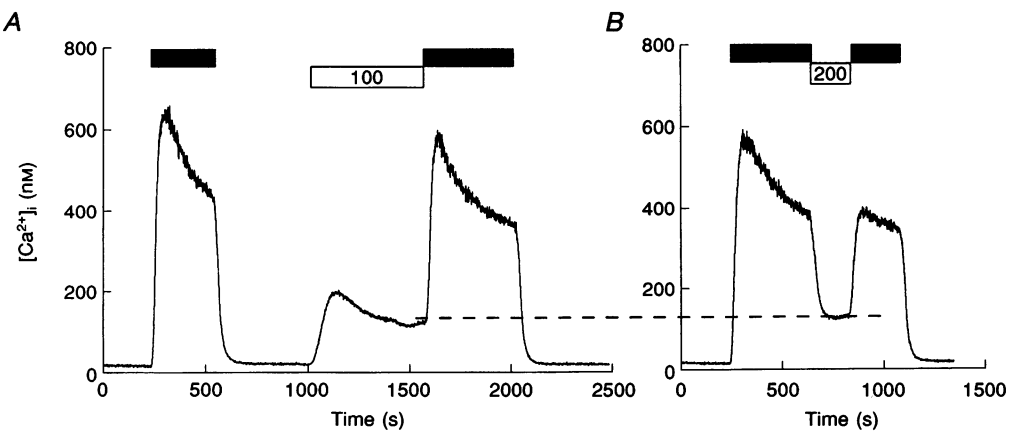

Figure 11. Capacitative Ca^{2+} entry signals are more sensitive to inhibition by $[Ca^{2+}]_i$ after a previous increase in $[Ca^{2+}]_i$

The magnitude of the overshoot in $[Ca^{2+}]_i$ evoked by incubation in medium containing 1·5 mM $[Ca^{2+}]$ (filled bars) was determined in populations of thapsigargin-treated cells after manipulation of intracellular $[Ca^{2+}]_i$ by pretreatment with either 100 μM Ca^{2+} for 600 s (*A*, open bar) or 1·5 mM Ca^{2+} for 400 s (*B*, filled bar) followed by 200 μM Ca^{2+} for 200 s (open bar). Although the two treatments evoked very different patterns of increase in $[Ca^{2+}]_i$ during the pretreatment period, the $[Ca^{2+}]_i$ in the $\geqslant 100$ s immediately preceding the assessment of the capacitative Ca^{2+} entry signals was similar in each experiment (dashed line). The traces shown are typical of 6 similar independent experiments.

recording the rate of decline of $[Ca^{2+}]_i$ after rapid removal of extracellular Ca^{2+} from thapsigargin-treated cells during both the rising phase of the capacitative Ca^{2+} entry signal and after the steady-state $[Ca^{2+}]_i$ had been attained. Our protocol ensured that the rates of Ca^{2+} removal from the cytosol were recorded from similar initial levels of $[Ca^{2+}]_i$ during the rising (327 ± 31 nM, $n = 7$) and falling (295 ± 24 nM, $n = 7$) phases of the response. The results demonstrate that under both conditions $[Ca^{2+}]_i$ declined mono-exponentially after removal of extracellular Ca^{2+}, but the rate of decline was significantly faster ($t_{½} = 7{\cdot}1 \pm 0{\cdot}7$ s, $n = 7$) during the declining phase of the capacitative Ca^{2+} signal than during its rising phase ($t_{½} = 22 \pm 2{\cdot}5$ s) (Fig. 14*A*). Rates of Ca^{2+} removal from the cytosol are therefore faster if preceded by a substantial increase in $[Ca^{2+}]_i$.

Our attempts to use La^{3+} to selectively inhibit plasma membrane Ca^{2+}-ATPases and more directly assess their role in allowing cells to recover from elevated $[Ca^{2+}]_i$ were frustrated because La^{3+} appeared to block capacitative Ca^{2+} entry more rapidly than it blocked Ca^{2+}-ATPases (Fig. 14*B*). La^{3+} (1–10 mM) did substantially slow the rate at which $[Ca^{2+}]_i$ recovered from the peak signal evoked by restoration of extracellular Ca^{2+} to thapsigargin-treated cells, but the effect was evident only ~30 s after La^{3+} addition (Fig. 14*B*). While these results are certainly consistent with a major role for plasma membrane Ca^{2+}-ATPases in mediating recovery from elevated $[Ca^{2+}]_i$, they also establish that La^{3+}, which is a more potent inhibitor of Ca^{2+} entry than of Ca^{2+}-ATPases, is of limited use in undertaking further analysis of the issue.

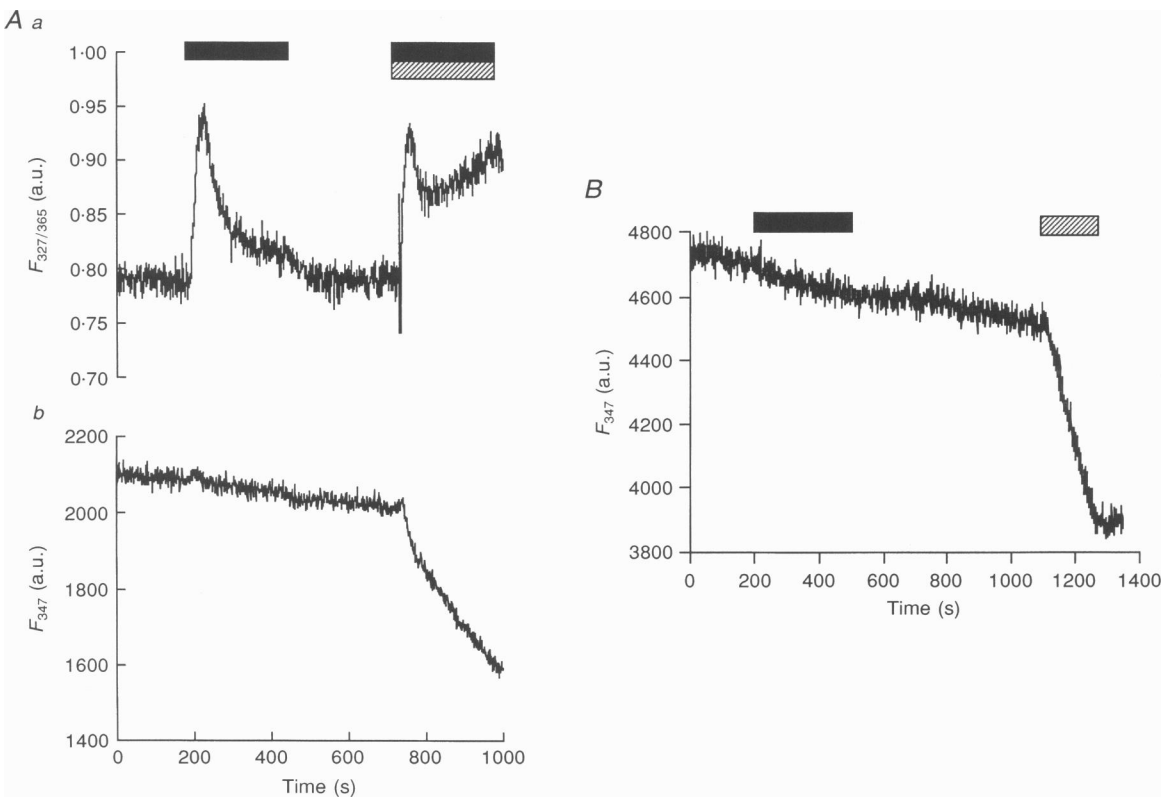

Figure 12. An increase in $[Ca^{2+}]_i$ abruptly attenuates capacitative Mn^{2+} entry

Populations of CPAE cells were loaded with mag-fura-2 and the ratio of fluorescence intensity at $\lambda_{ex} = 327$ and 365 nm ($F_{327/365}$) and the fluorescence at $\lambda_{ex} = 347$ nm (expressed in arbitrary units, a.u.) were simultaneously recorded to allow measurement of $[Ca^{2+}]_i$ and Mn^{2+} quenching of the cytosolic indicator. *A*, extracellular Ca^{2+} (1·5 mM, filled bar) was restored to thapsigargin-treated cells, resulting in a transient increase in $[Ca^{2+}]_i$ (*a*, $F_{327/365}$), but no change in the fluorescence recorded at $\lambda_{ex} = 347$ nm (*b*). Restoration of extracellular Ca^{2+} (1·5 mM) in the presence of Mn^{2+} (100 μM, hatched bar) gave a similar transient increase in Ca^{2+} in 19/25 experiments and in each case, the peak increase in $[Ca^{2+}]_i$ coincided with an abrupt decrease in the rate of Mn^{2+} entry (*b*). *B*, experiments identical to those shown in *A* were performed, but with Mn^{2+} (100 μM, hatched bar) added in the absence of extracellular Ca^{2+}. The results, typical of 6 similar experiments, show that capacitative Mn^{2+} entry continues at a uniformly enhanced rate after addition of Mn^{2+} in the absence of extracellular Ca^{2+}.

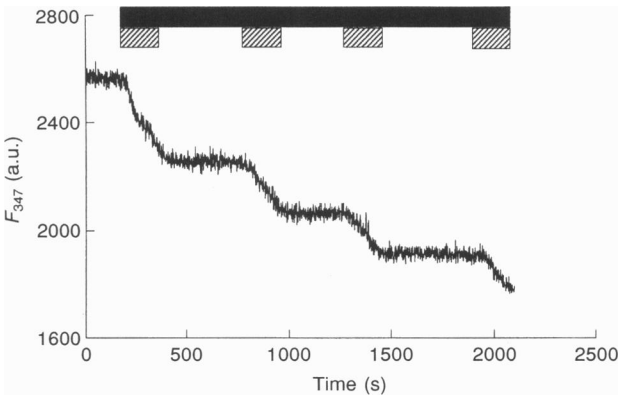

Figure 13. Prolonged capacitative Ca^{2+} entry does not further inhibit Mn^{2+} entry

Populations of mag-fura-2-loaded, thapsigargin-treated cells were incubated in medium containing extracellular Ca^{2+} (1·5 mM, filled bar) and periodically exposed to extracellular Mn^{2+} (100 μM, hatched bars), while recording the quench of fluorescence (λ_{ex} = 347 nm; arbitrary units, a.u.). The results demonstrate that after the first switch to a slow rate of fluorescence quench during the initial Ca^{2+} transient, the rate continues at the same slow rate during each of the subsequent challenges with extracellular Mn^{2+}.

DISCUSSION

ATP-evoked Ca^{2+} entry in CPAE cells occurs entirely via the capacitative pathway

The Ca^{2+} entry that almost invariably accompanies activation of receptors linked to $InsP_3$ formation is commonly ascribed to activation of the capacitative Ca^{2+} entry pathway as a consequence of depletion of intracellular Ca^{2+} stores (Putney & Bird, 1993; Berridge, 1995). In CPAE cells too, depletion of the intracellular Ca^{2+} stores by incubation with thapsigargin activates a Ca^{2+} entry pathway (Fig. 4), which is also permeable to Mn^{2+} and Ba^{2+} and reversibly blocked by La^{3+} and SKF-96365. The signals responsible for stimulating capacitative Ca^{2+} entry have yet to be unequivocally identified (see Introduction). We have not addressed the issue further, other than to demonstrate firstly that okadaic acid-sensitive protein phosphatases appear not to be involved in CPAE cells, although they have been implicated in other cells (Parekh *et al.* 1993). Secondly, we have shown

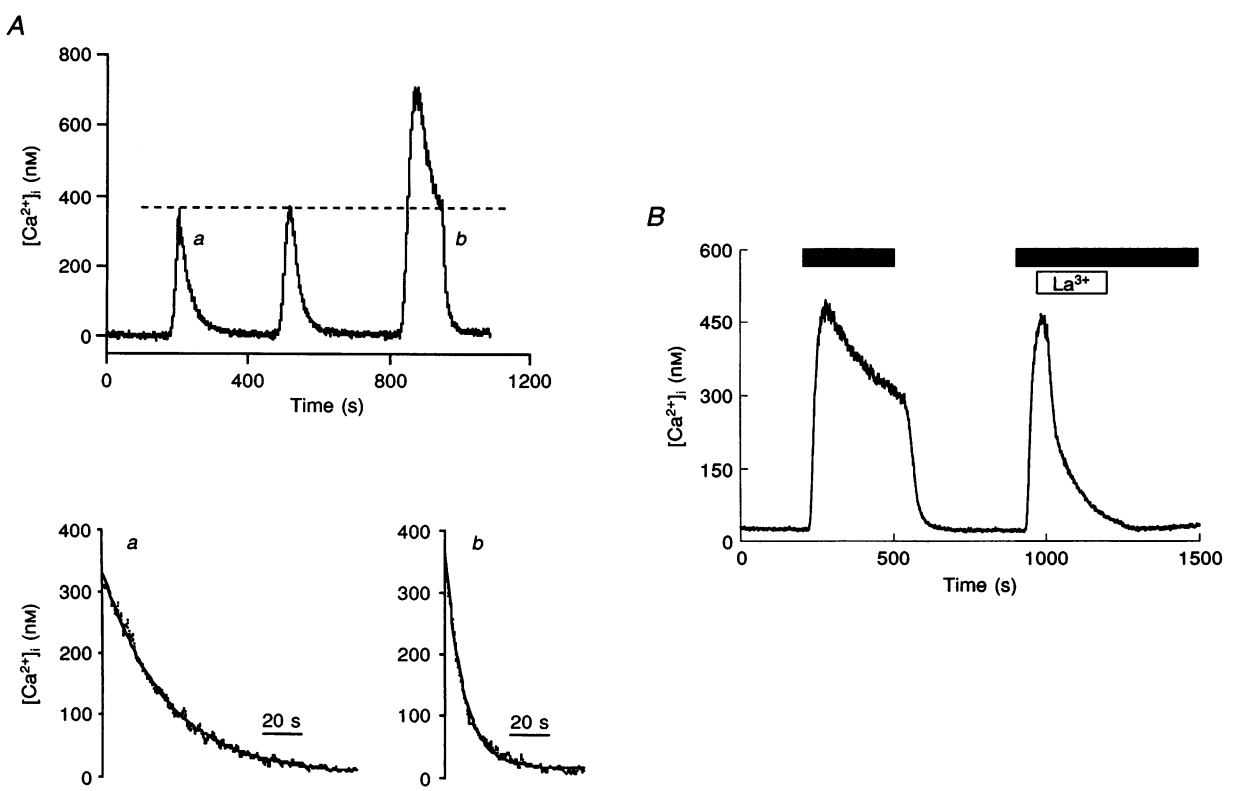

Figure 14. Increased $[Ca^{2+}]_i$ causes a sustained enhancement of the rate of recovery of $[Ca^{2+}]_i$

A, extracellular Ca^{2+} (1·5 mM) was restored to thapsigargin-treated cells and the rates of decrease of $[Ca^{2+}]_i$ were then determined after rapid removal of extracellular Ca^{2+} during either the rising phase of the capacitative signal (*a*) or over the same range of $[Ca^{2+}]_i$ on its falling phase (*b*). The lower panels illustrate examples of the mono-exponential curve fits used to establish the half-times for recovery. Results are typical of 7 similar experiments. *B*, extracellular Ca^{2+} (1·5 mM) was restored to thapsigargin-treated cells for the periods denoted by filled bars, and $LaCl_3$ (1 mM) was added for the period shown by an open bar. The results are typical of 3 experiments; similar results were obtained with 10 mM $LaCl_3$.

that, as with *Xenopus* oocytes (Petersen & Berridge, 1994), even during very prolonged activation of the capacitative pathway there is no appreciable decrease in the ability of the cells to generate the signal responsible for activation of the pathway (Fig. 4).

Although the capacitative pathway is widely expressed and certainly contributes to the Ca^{2+} entry evoked by receptors linked to InsP_3 formation, there is rather little evidence, beyond the shared characteristics of receptor-regulated and capacitative Ca^{2+} entry (Schilling & Elliott, 1992; Putney & Bird, 1993), to indicate whether it is the sole means whereby such receptors regulate Ca^{2+} entry. Very recently, experiments in which antisense methods were used to attenuate expression of endogenous trp proteins suggested that trp, and by inference the capacitative Ca^{2+} entry pathway, was the sole means whereby acetylcholine muscarinic receptors stimulated Ca^{2+} entry to mouse fibroblasts (Zhu *et al.* 1996). In parotid acinar cells, the sustained phase of the Ca^{2+} entry evoked by thaspigargin is not further increased by activation of muscarinic acetylcholine receptors (Takemura, Hughes, Thastrup & Putney, 1989), suggesting that capacitative Ca^{2+} entry may be the only Ca^{2+} entry pathway activated by these receptors. However, even that conclusion must be qualified because activation of many receptors, including the P_{2U} receptor of CPAE cells (Fig. 2*B*), also leads to enhanced Ca^{2+} efflux (Wang *et al.* 1991; Byron & Taylor, 1995), which might obscure any additional effect of a receptor on Ca^{2+} entry when only the steady-state $[Ca^{2+}]_i$ is recorded. In many cells, the same stimuli, possibly acting via the same receptors, evoke both capacitative Ca^{2+} entry via their ability to empty intracellular Ca^{2+} stores and activation of an additional Ca^{2+} entry pathway (Stauderman & Pruss, 1989; Byron & Taylor, 1995). In A7r5 smooth muscle cells, for example, Arg^8-vasopressin activates, via V_{1A} receptors, both the capacitative pathway and an additional Ca^{2+} entry pathway with different bivalent cation selectivity (Byron & Taylor, 1995). It is, therefore, important to establish the relative contributions of capacitative and non-capacitative Ca^{2+} entry pathways to the Ca^{2+} signals evoked by receptors linked to InsP_3 formation.

Several lines of evidence indicate that in CPAE cells, the Ca^{2+} entry evoked by ATP occurs entirely via the capacitative pathway. Firstly, neither the steady-state increase in $[Ca^{2+}]_i$ in thapsigargin-treated cells (Fig. 2*B*) nor the unidirectional rates of Ba^{2+} and Mn^{2+} entry were further increased by addition of ATP. Secondly, in cells pretreated with ATP to empty their intracellular Ca^{2+} stores, the initial rates of increase in $[Ca^{2+}]_i$ when extracellular Ca^{2+} was restored were similar in the continued presence of ATP or at various intervals after its removal (Fig. 2*A*). Finally, in thapsigargin-treated cells, the *initial* rates of increase in $[Ca^{2+}]_i$ when extracellular Ca^{2+} was restored were similar in the presence and absence of ATP. These results with CPAE cells provide the first conclusive demonstration that the ability of empty intracellular Ca^{2+} stores to activate the capacitative pathway is *wholly* responsible for the Ca^{2+} entry evoked by a receptor linked to InsP_3 formation. In primary cultures of bovine aortic endothelial cells, both ATP and inositol 1,3,4,5,-tetrakisphosphate (InsP_4) have been shown to activate channels that are similarly permeable to Mn^{2+}, Ca^{2+} and Ba^{2+} (Lückhoff & Clapham, 1992). However, since InsP_4 was ineffective at resting $[Ca^{2+}]_i$ and the relationship between the channels activated by ATP and InsP_4/Ca^{2+} is not established, the role of InsP_4 in mediating the effects of ATP on Ca^{2+} entry is unclear. Our results, suggesting that store depletion is the sole means whereby ATP evokes Ca^{2+} entry to endothelial cells, are difficult to reconcile with a physiological role for InsP_4 in mediating Ca^{2+} entry. It may be that the pathway is not expressed in the CPAE cell line, ATP may fail to evoke formation of sufficient InsP_4, the InsP_4-activated Ca^{2+} entry may be too small relative to that evoked by empty stores to be detected, or $[Ca^{2+}]_i$ may be too low to facilitate the effects of InsP_4.

Rapid inactivation of capacitative Ca^{2+} entry by increased $[Ca^{2+}]_i$

In a variety of cells, capacitative Ca^{2+} entry has been shown to be inhibited by increases in $[Ca^{2+}]_i$ (Hoth & Penner, 1992; McDonald, Premack & Gardner, 1993; Berridge, 1995). Electrophysiological analysis of the capacitative current (I_{crac}) in Jurkat T-lymphocytes have identified three mechanisms whereby increased $[Ca^{2+}]_i$ causes inactivation of I_{crac}. Ca^{2+} appears to bind to a site close to the mouth of the open channel to cause inactivation that is both initiated and then reversed within milliseconds of changing the rate of Ca^{2+} flux through the channel (Zweifach & Lewis, 1995*b*). As expected, and demonstrated in other measurements of the capacitative pathway (Putney & Bird, 1993; Byron & Taylor, 1995), cytosolic Ca^{2+} also inactivates the pathway by allowing the intracellular Ca^{2+} stores to refill over a period of seconds (Zweifach & Lewis, 1995*a*). Finally, another slow component of inactivation by increased $[Ca^{2+}]_i$, the mechanism of which is unknown, can be inhibited by okadaic acid and 1-norokadaone (Zweifach & Lewis, 1995*a*). In our experiments with CPAE cells, the intracellular Ca^{2+} stores were shown to remain empty throughout the period when Ca^{2+} was modulating the capacitative Ca^{2+} signals and okadaic acid failed to affect the Ca^{2+} overshoots evoked by restoration of extracellular Ca^{2+} to thapsigargin-treated cells. Neither of the two slow components of inactivation of I_{crac} shown to occur in Jurkat cells is therefore likely to contribute significantly to the attenuation of capacitative Ca^{2+} signals by increased $[Ca^{2+}]_i$ observed in CPAE cells.

Although our methods lack the temporal resolution of electrophysiological analyses, our results are consistent with rapid feedback inhibition of the capacitative pathway by increased $[Ca^{2+}]_i$ in CPAE cells. In the absence of extracellular Ca^{2+}, the rate of Mn^{2+} entry via the capacitative pathway was maintained (Fig. 12*B*). However, when Mn^{2+} entry was measured in the presence of extracellular Ca^{2+}, and therefore accompanied by an increase in $[Ca^{2+}]_i$, the initial rapid rate of Mn^{2+} entry abruptly decreased by more

than 2-fold as $[Ca^{2+}]_i$ reached its peak (Fig. 12*A*). The slower, though still stimulated, rate of capacitative Mn^{2+} entry was then maintained for at least 30 min (Fig. 13). We conclude that in CPAE cells, Ca^{2+} entering via the capacitative pathway causes rapid inhibition of further Ca^{2+} entry.

Delayed regulation of capacitative Ca^{2+} entry signals by $[Ca^{2+}]_i$

Although our results show that rapid feedback inhibition of the capacitative pathway by cytosolic Ca^{2+} occurs in CPAE cells, several lines of evidence indicate that capacitative Ca^{2+} entry signals, whether triggered by ATP-stimulated $InsP_3$ formation or thapsigargin, are also subject to *delayed* feedback inhibition by increased $[Ca^{2+}]_i$. Complex changes in $[Ca^{2+}]_i$ followed the removal of ATP from cells stimulated in the presence of extracellular Ca^{2+} (Figs 1–3). The Ca^{2+} entry signals evoked by ATP were larger when separated from the Ca^{2+} release phase of the response (Fig. 3). Restoration of extracellular Ca^{2+} to cells in which the stores were emptied by either ATP (Figs 2 and 3) or thapsigargin (Figs 4–11) caused $[Ca^{2+}]_i$ to transiently overshoot before falling to an elevated plateau, despite our demonstration that the stores remained completely empty (Fig. 4*C*). Similar overshoots in capacitative Ca^{2+} entry signals have been observed in other cells including *Xenopus* oocytes (Petersen & Berridge, 1994), HeLa cells and undifferentiated BC_3H1 cells (Missiaen, De Smedt, Parys, Oike & Casteels, 1994), parotid acinar cells (Foskett & Wong, 1994) and neutrophils (Foder, Scharff & Thastrup, 1989); they are not, however, observed in all cells (Missiaen *et al.* 1994), indicating that the underlying mechanism is not an intrinsic feature of a ubiquitous capacitative pathway. Previous reports of these Ca^{2+} overshoots suggested that they resulted entirely from attenuation of capacitative Ca^{2+} entry by increased $[Ca^{2+}]_i$ (Missiaen *et al.* 1994; Foskett & Wong, 1994), but such a mechanism is unlikely to provide the complete explanation (Foder *et al.* 1989; Scharff & Foder, 1994), and it is certainly unable to wholly explain the results in CPAE cells.

In CPAE cells, even modest capacitative Ca^{2+} entry caused overshoots in $[Ca^{2+}]_i$ (Fig. 6*A*) that resulted from regulation of the Ca^{2+} signals by cytosolic Ca^{2+} (Fig. 6*B*). The extent to which $[Ca^{2+}]_i$ inhibited the ability of capacitative Ca^{2+} entry to evoke a further increase in $[Ca^{2+}]_i$ was most tightly correlated with the preceding peak $[Ca^{2+}]_i$, and two independent methods of measuring the onset of the inhibition indicated that it was slow ($t_{½} = 83$–127 s). Furthermore, recovery from the inhibitory effects of elevated cytosolic Ca^{2+} appears to be determined by the lowest $[Ca^{2+}]_i$ attained during the recovery period (Fig. 10) and then it too proceeds slowly ($t_{½} \sim 150$ s). The slow onset and reversal of the ability of elevated $[Ca^{2+}]_i$ to inhibit the Ca^{2+} signals evoked by further capacitative Ca^{2+} entry cannot be mediated by the very rapid inhibition of the capacitative pathway by increased $[Ca^{2+}]_i$ (Fig. 11). Within seconds of $[Ca^{2+}]_i$ reaching its peak, unidirectional Mn^{2+} entry is inhibited (Fig. 12*A*), yet during the following 30 min, when capacitative Ca^{2+} signals are subject to increasing inhibition (Fig. 8), there is no further change in the rate of Mn^{2+} entry (Fig. 13). Inhibition of capacitative Ca^{2+} entry by increased $[Ca^{2+}]_i$ cannot therefore fully explain the effects of cytosolic Ca^{2+} on capacitative Ca^{2+} entry signals.

Since the capacitative pathway is permeable to Na^+ in the absence of extracellular Ca^{2+} (McDonald *et al.* 1993), Ca^{2+} overshoots could result from a diminished ability of cells to extrude Ca^{2+} via Na^+–Ca^{2+} exchange or from reversal of the exchanger, as Na^+ passing through the capacitative pathway during its prolonged activation in the absence of extracellular Ca^{2+} caused accumulation of intracellular Na^+. This mechanism is unlikely to contribute to the responses of CPAE cells because Na^+–Ca^{2+} exchange is not a major Ca^{2+} efflux pathway in endothelial cells (Sage, Van Breemen & Cannell, 1991; Schilling & Elliott, 1992) and we recorded similar Ca^{2+} overshoots when extracellular Na^+ was replaced by the impermeant cations, NMDG or choline.

By comparing the rates of Ca^{2+} removal from the cytosol during the rising phase of a Ca^{2+} overshoot and during its falling phase, we established that Ca^{2+} recovery was ~3-fold faster if preceded by a substantial increase in $[Ca^{2+}]_i$ (Fig. 14*A*). The nature of the pathway responsible for the recovery has not been identified: it is unlikely to be either Na^+–Ca^{2+} exchange across the plasma membrane (see above) or the Ca^{2+}-ATPases of the intracellular stores, because they were fully inhibited by thapsigargin.

Ca^{2+} uptake by mitochondria is believed to occur only when $[Ca^{2+}]_i$ reaches the micromolar range, but substantial evidence now suggests that mitochondria near open Ca^{2+} channels in either the plasma membrane (Friel & Tsien, 1994; Drummond & Fay, 1996) or membranes of the intracellular stores (Rizzuto, Brini, Murgia & Pozzan, 1993; Hajnóczky, Robb-Gaspers, Seitz & Thomas, 1995) may be exposed to such substantial increases in $[Ca^{2+}]_i$. Mitochondria might therefore rapidly sequester Ca^{2+} when $[Ca^{2+}]_i$ reaches its highest levels (Lawrie, Rizzuto, Pozzan & Simpson, 1996) and then slowly release it to the cytosol as $[Ca^{2+}]_i$ declines (Friel & Tsien, 1994) thereby prolonging the Ca^{2+} signals evoked by capacitative Ca^{2+} entry. Indeed, the long-lasting ability of smooth muscle cells to recover from an increase in $[Ca^{2+}]_i$ more rapidly after a prior elevation of $[Ca^{2+}]_i$ (Becker, Singer, Walsh & Fay, 1989) has been attributed to increased mitochondrial Ca^{2+} handling (Drummond & Fay, 1996). That explanation is, however, difficult to reconcile with three observations from CPAE cells. Firstly, similar Ca^{2+} overshoots, but of much lesser amplitude, were evoked by restoration of low concentrations of extracellular Ca^{2+} to thapsigargin-treated CPAE cells (Fig. 6*A*), suggesting that this pattern of response does not depend upon $[Ca^{2+}]_i$ exceeding a critical threshold. Secondly, Ruthenium Red at a concentration ($> 1\ \mu M$) far in excess of that previously shown to inhibit mitochondrial Ca^{2+} uptake ($K_i = 30$ nM; Reed & Bygrave, 1974) increased the amplitude of the capacitative Ca^{2+} entry signals without preventing the characteristic overshoot evoked by restoration of extra-

cellular Ca^{2+} (Fig. 5). Finally, the inhibitory effect of elevated $[Ca^{2+}]_i$ reverses only after $[Ca^{2+}]_i$ has returned to its basal level for 320 ± 3 s, suggesting that Ca^{2+} efflux from mitochondria is unlikely to be significant during this period, when capacitative Ca^{2+} entry signals are substantially inhibited (Figs 9 and 10). These results suggest that while mitochondria may sequester Ca^{2+} at the peak of the intracellular Ca^{2+} signal, they are unlikely to be responsible for the long-lasting effects of prior Ca^{2+} signals.

Perhaps the most likely target for the delayed effect of increased $[Ca^{2+}]_i$ on Ca^{2+} recovery is the plasma membrane Ca^{2+}-ATPase (Fig. 14). Although this explanation would be difficult to reconcile with the behaviour of pancreatic acinar cells in which increased $[Ca^{2+}]_i$ immediately stimulates plasma membrane Ca^{2+}-ATPases (Toescu & Petersen, 1995), there are within the large family of related proteins to which all plasma membrane Ca^{2+}-ATPases belong (Missiaen *et al.* 1993), many isoforms subject to different forms of regulation. Cytosolic Ca^{2+} is one of the regulators of these Ca^{2+}-ATPases and can mediate its effects via binding of Ca^{2+}–calmodulin, by phosphorylation or via limited proteolysis of the Ca^{2+}-ATPase by Ca^{2+}-activated proteases (Carafoli, 1991). In erythrocytes, for example, the transient cytosolic Ca^{2+} spike that follows Ca^{2+} entry has been clearly shown to result from delayed activation of the plasma membrane Ca^{2+}-ATPase by Ca^{2+}–calmodulin (Foder & Scharff, 1992). A similar mechanism, albeit with rather less convincing evidence (Scharff & Foder, 1994), has been proposed for neutrophils where Ca^{2+} overshoots similar to those observed in CPAE cells are evoked by capacitative Ca^{2+} entry (Foder *et al.* 1989).

Long-lasting consequences of transient increases in $[Ca^{2+}]_i$

Our results suggest that in CPAE cells, increases in $[Ca^{2+}]_i$ exert at least two inhibitory effects on the Ca^{2+} signals evoked by capacitative Ca^{2+} entry: a very rapid, though incomplete, inhibition of the capacitative pathway and a delayed activation of a mechanism, probably the plasma membrane Ca^{2+}-ATPase, responsible for removal of Ca^{2+} from the cytosol. Both effects are probably evoked by Ca^{2+} derived either from intracellular stores (Fig. 3) or Ca^{2+} entry pathways (Fig. 6). Complete recovery from the inhibitory effects of cytosolic Ca^{2+} on capacitative Ca^{2+} entry signals is slow and proceeds only after $[Ca^{2+}]_i$ has returned to its basal level (Figs 9 and 10). Practical problems have so far prevented us from unequivocally resolving whether this slow recovery is a characteristic of both the capacitative pathway and the Ca^{2+} removal mechanism or of just one of these processes. The necessity for $[Ca^{2+}]_i$ to be substantially reduced before recovery can proceed and the slow pace of that recovery are important because they endow cells with a memory of Ca^{2+} signals that may outlast the initial transient increase in $[Ca^{2+}]_i$ by hundreds of seconds (Fig. 11) (Becker *et al.* 1989). Delayed regulation of capacitative Ca^{2+} signals might thereby also contribute to the mechanisms needed to generate Ca^{2+} oscillations. Indeed, damped oscillatory changes in $[Ca^{2+}]_i$ have been recorded from some cells after thapsigargin treatment (Petersen & Berridge, 1994; Foskett & Wong, 1994; Missiaen *et al.* 1994). In our experiments with thapsigargin-treated CPAE cells, we often observed undershoots in $[Ca^{2+}]_i$ after decreases in extracellular $[Ca^{2+}]$ (Fig. 10*A*), and more rarely (4 from > 30 experiments) observed a sequence of damped oscillations in $[Ca^{2+}]_i$ after restoration of low concentrations of extracellular Ca^{2+} (Fig. 15).

Oscillatory changes in $[Ca^{2+}]_i$ are a common feature of Ca^{2+} signalling pathways which may serve to protect cells from excessively prolonged elevations of $[Ca^{2+}]_i$. Periodic discharge of intracellular Ca^{2+} stores or interplay between voltage-regulated Ca^{2+} channels and Ca^{2+}-regulated ion channels in the plasma membrane are each capable of generating such $[Ca^{2+}]_i$ oscillations. Neither mechanism can have been responsible for the Ca^{2+} oscillations observed in CPAE cells because they occurred under conditions where the endoplasmic reticulum remained completely depleted of Ca^{2+} (Fig. 15) and there is no evidence to suggest the existence of voltage-gated Ca^{2+} channels in endothelial cells (Schilling & Elliott, 1992). Feedback regulation of capacitative Ca^{2+} entry by cytosolic Ca^{2+} was previously proposed to underlie oscillatory changes in $[Ca^{2+}]_i$ in parotid acinar cells (Foskett & Wong, 1994). Our results suggest that a similar mechanism in combination with delayed regulation of Ca^{2+} extrusion mechanisms can generate oscillatory changes in $[Ca^{2+}]_i$ in CPAE cells.

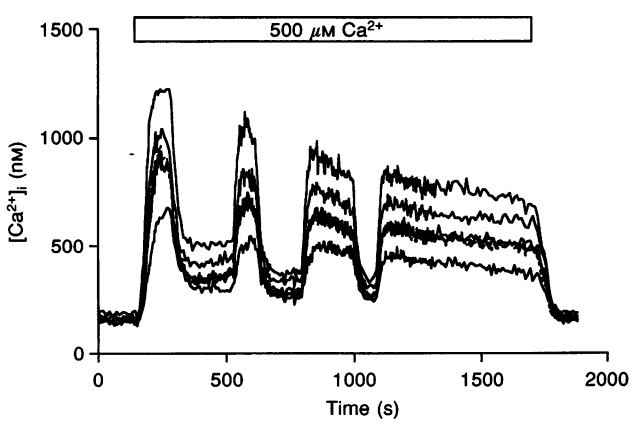

Figure 15. Damped Ca^{2+} oscillations in thapsigargin-treated cells

Restoration of 500 μM extracellular Ca^{2+} (open bar) to thapsigargin-treated cells evoked a series of damped oscillations in $[Ca^{2+}]_i$. The trace shows the synchronous changes in $[Ca^{2+}]_i$ recorded from 6 individual cells in a single field. Similar results were observed in only 4 from more than 30 independent experiments.

We conclude that in CPAE cells, where capacitative Ca^{2+} entry is the sole means whereby P_{2U} purinoceptors stimulate Ca^{2+} entry, increases in $[Ca^{2+}]_i$ cause rapid inhibition of the capacitative pathway and delayed stimulation of a mechanism responsible for removal of Ca^{2+} from the cytosol. At least one of these mechanisms, and probably both, recover from the effects of increased $[Ca^{2+}]_i$ only after $[Ca^{2+}]_i$ has reverted to its resting level and then only slowly. Such long-lasting effects of transient Ca^{2+} signals on Ca^{2+} regulatory mechanisms endow the cell with a memory of previous Ca^{2+} signals that may provide considerable opportunity for signal processing.

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