Cell Type-specific Modes of Feedback Regulation of Capacitative Calcium Entry

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The Ca\(^{2+}\)-ATPase inhibitor, thapsigargin, activated Ca\(^{2+}\) entry into pancreatic acinar cells, a process known as capacitative calcium entry. In cells loaded with the calcimeter chelator BAPTA, the transient Ca\(^{2+}\) release was blunted and the rise of [Ca\(^{2+}\)] in readdition of Ca\(^{2+}\) was slowed. However, the steady-state [Ca\(^{2+}\)], due to Ca\(^{2+}\) entry was substantially augmented compared with control cells. This indicates that [Ca\(^{2+}\)], exerts a negative feedback on Ca\(^{2+}\) entry from a compartment buffered by BAPTA and separated from the bulk of cytoplasmic Ca\(^{2+}\). This interaction probably occurs close to the calcium channel where [Ca\(^{2+}\)] is higher than in the bulk of the cytoplasm. In support of this interpretation, the slower Ca\(^{2+}\) chelator, EGTA, also blunted the release of Ca\(^{2+}\) and slowed the rise of the sustained [Ca\(^{2+}\)], phase but failed to augment steady-state [Ca\(^{2+}\)].

In contrast, Ca\(^{2+}\) entry in NIH 3T3 cells was characterized by a transient rise of [Ca\(^{2+}\)], that decays to near prestimulus levels. This decay in Ca\(^{2+}\) entry also results from negative feedback by Ca\(^{2+}\) because the decrease in Ca\(^{2+}\) entry was reversed by incubation in a Ca\(^{2+}\)-deficient medium. However, unlike its effects in acinar cells, BAPTA neither augmented steady-state [Ca\(^{2+}\)], nor prevented the inactivation of entry. Rather, in BAPTA-loaded cells, [Ca\(^{2+}\)], failed to increase substantially suggesting that negative regulation by Ca\(^{2+}\) may occur at a site distinct from the cytoplasmic compartment and inaccessible to cytoplasmic BAPTA. These two distinct types of feedback behavior may indicate subtypes of store-operated calcium channels expressed in different cells or a single type of channel which is differentially regulated in a cell type-specific manner.

Many hormones and neurotransmitters initiate cellular activation through the hydrolysis of phosphatidylinositol (4,5)-biphosphate generating the intracellular messenger inositol 1,4,5-triphosphate ((1,4,5)IP\(_3\)), which mobilizes Ca\(^{2+}\) from intracellular stores (1). This entry of Ca\(^{2+}\) is presumed to occur through Ca\(^{2+}\) channels in the plasma membrane, termed “store-operated channels” (5, 6), although the existence of such channels has not been proven unequivocally. Electrophysiological studies have described inward Ca\(^{2+}\) currents (I\(_{\text{CRAC}}\)) that are activated by intracellular Ca\(^{2+}\) pool depletion (7–10) and which may reflect the activity of these store-operated channels.

The underlying mechanisms that link internal Ca\(^{2+}\) pool depletion to the activation of Ca\(^{2+}\) entry are not yet understood. However, recent data indicate that this mode of Ca\(^{2+}\) entry can be regulated by Ca\(^{2+}\)-itself. Several studies have demonstrated that intracellular Ca\(^{2+}\) can feed back to inhibit Ca\(^{2+}\) influx current on two different time scales. Rapid inactivation of the inward current associated with capacitative calcium entry occurs on a millisecond time scale (8) and is believed to involve an action of cytoplasmic Ca\(^{2+}\) close to the mouth of the Ca\(^{2+}\) channel, 3–4 nm from the mouth of the pore (10). There is also a relatively slow inactivation process in Jurkat cells occurring over tens of seconds, but which also appears to be Ca\(^{2+}\)-dependent (11).

While the majority of evidence for negative feedback by Ca\(^{2+}\) has come from patch-clamp studies, a Ca\(^{2+}\)-dependent regulation of Ca\(^{2+}\) entry has also been proposed from steady-state measurements of [Ca\(^{2+}\)], with fluorescent indicators. Missiaen et al. (12) demonstrated that, in HeLa cells, Ca\(^{2+}\) entry inactivates with a time course of tens of seconds giving rise in some cells to slow [Ca\(^{2+}\)] oscillations. The relationship of this slow inactivation of Ca\(^{2+}\) entry to the [Ca\(^{2+}\)],-dependent inactivation of Ca\(^{2+}\) current is not known. In addition, it is not known whether these mechanisms are intrinsic to capacitative calcium entry in all instances or represent cell-specific modes of regulation. In this study, we have monitored thapsigargin-induced capacitative Ca\(^{2+}\) entry in intact cells with the fluorescent indicator, fura-2, and have perturbed intracellular Ca\(^{2+}\) gradients by loading or injecting the cells with Ca\(^{2+}\) chelators. Our findings confirm the existence of at least two important Ca\(^{2+}\)-dependent negative feedback mechanisms, one which involves rapid effects of cytoplasmic Ca\(^{2+}\) on Ca\(^{2+}\) entry channels and a second slower mechanism which appears to depend on Ca\(^{2+}\) acting at an extracellular site or at a site within the calcium channel. Our findings also indicate that these mechanisms do not represent an intrinsic property of capacitative calcium entry in all cell types but rather appear to be differentially expressed in a cell type-specific manner.

MATERIALS AND METHODS

Preparation of Rat Pancreatic Cells—Pancreatic acinar cells were prepared essentially as described previously (13). Briefly, excised pancreata from 75–100-g rats were digested by injection of a HEPES-buffered physiological saline solution (HPSS; composition, in mM: 140 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES, 10 glucose, 0.1% bovine serum albumin, pH 7.4), supplemented with 10 mM pyruvate and 0.02% soybean trypsin inhibitor (Sigma). After mincing the tissue, the acinar...
cells were liberated by incubation with collagenase (3 mg/10 ml; Sigma) for 10 min at 37°C. Following collagenase digestion, the pancreatic cells were washed and suspended in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. The isolated cells were then allowed to attach to glass coverslips coated with Matrigel (Collaborative Biomedical Products).

Preparation of Mouse Lacrimal Cells—Lacrimal acinar cells were prepared essentially as described previously (14). Briefly, lacrimal glands, excised from 6 mice (30–40 g), were finely minced, suspended in DMEM, and then incubated with trypsin (0.25 mg/10 ml; Sigma) for 1 min at 37°C. The trypsin was removed by centrifugation, followed by a 5-min incubation with soybean trypsin inhibitor (2 mg/10 ml; Sigma), in the presence of 2.5 mM EGTA at 37°C. Finally, acinar cells were isolated following incubation with collagenase (4 mg/10 ml; Boehringer Mannheim) for 10 min at 37°C. The digested tissue was then washed and suspended in DMEM, supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Subsequently, the isolated cells were allowed to attach to glass coverslips coated with Matrigel (Collaborative Biomedical Products).

NIH 3T3 Cell Culture—NIH 3T3 cells were maintained at 37°C and 5% CO₂ in DMEM containing 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 units/ml streptomycin. After 3 days culture, the cells were passed at a dilution of 1:10. In preparation for experiments, cells were plated on glass coverslips 2 days before use.

Cellular Loading of the Fluorescent 
Ca²⁺ Indicator fura-2—In all cases, cell-attached coverslips were mounted in a Teflon microscope chamber (Bionique) before dye loading. NIH 3T3 cells were incubated in DMEM containing 1 μM fura-2/AM (Molecular Probes) for 15 min at 37°C, whereas pancreatic and lacrimal cells were incubated in DMEM containing 1.6 μM fura-2/AM for 30 min at room temperature. After dye loading, cells were washed and bathed in HPSS and incubated at room temperature for an additional 20 min before Ca²⁺ measurements were made to ensure complete hydrolysis of cellular fura-2/AM. In some experiments (indicated below), following the fura-2 loading procedure, cells were further incubated with either BAPTA/AM (50–100 μM) and/or EGTA/AM (500 μM) for 20 min at room temperature.

Fluorescence Measurements—The fluorescence of fura-2-loaded cells was monitored with a photomultiplier-based detection system, mounted on a Nikon Diaphot inverted microscope equipped with a Nikon 40x (1.3 numeric aperture) Neofluor objective. The fluorescence light source was provided by a Deltascan D101 (Photon Technology International Ltd.), equipped with a light path changer and dual excitation monochromators. The light path changer enabled rapid interchange between two excitation wavelengths (340 nm and 380 nm), and a photomultiplier tube monitored the emission fluorescence at 510 nm, selected by a barrier filter (Omega Optical). All experiments were carried out at room temperature. Calibration and calculation of [Ca²⁺] was carried out as described previously (15).

Cell Microinjection—Pancreatic, lacrimal, and NIH 3T3 cells were microinjected with 2 μl fura-2 solution (in H₂O) via a glass micropipette attached to WPI PV830 Picopump (World Precision Instruments, New Haven, CT). In some experiments, the injection solution also contained 250 μM BAPTA or 250 μM EGTA.

Statistical Analysis—Statistical significance was determined by analysis of variance or by Student’s t test where appropriate. The level of significance (p) was 0.05.

RESULTS
Receptor-activated Ca²⁺ mobilization involves two phases: Ca²⁺ release from intracellular stores and extracellular Ca²⁺ entry (16). In the presence of 1.8 mM extracellular Ca²⁺, the addition of 2 μM thapsigargin to pancreatic acinar cells produced an elevation in [Ca²⁺], that was sustained or slowly falling and which remained above baseline for a period of at least 30 min (Fig. 1A). Addition of thapsigargin to fura-2-loaded pancreatic acinar cells incubated in a Ca²⁺-free medium caused a transient increase of [Ca²⁺], which was followed on restoration of external Ca²⁺ by an elevated and sustained [Ca²⁺], signal (Fig. 1A). This biphasic [Ca²⁺], signal induced by the Ca²⁺-ATPase inhibitor, thapsigargin, is diagnostic of cal-

Ca²⁺ entry linked to the depletion of intracellular calcium stores or capacitative calcium entry (4, 17).

In previous electrophysiological studies (8–10), a hallmark of the regulation by [Ca²⁺], of Ca²⁺ entry was the ability of intracellular Ca²⁺ chelators to augment inward Ca²⁺ current. In this study, we investigated the effects of intracellular BAPTA and EGTA on the thapsigargin-induced Ca²⁺ entry in pancreatic cells. In most experiments, the Ca²⁺ chelators were loaded into cells by incubation with their acetoxyethyl ester derivatives as described under “Materials and Methods.” In pancreatic acinar cells loaded with BAPTA (50 μM BAPTA/AM for 20 min), the transient release of Ca²⁺ (observed in control cells after the addition of thapsigargin in a calcium-free medium) was blunted (Fig. 1B). On restoring extracellular Ca²⁺, [Ca²⁺] rose at a rate of rise substantially slower than in control cells. The slower rate of rise in the presence of BAPTA is expected, since additional Ca²⁺ entry is required to overcome the additional Ca²⁺ buffering capacity of the cell before a steady-state elevation of [Ca²⁺] is observed. Unexpectedly, however, the resulting steady-state [Ca²⁺] due to Ca²⁺ entry was significantly higher in BAPTA-loaded cells compared to control cells (Fig. 1B).

This apparent augmentation of Ca²⁺ entry was found to depend on the species of intracellular Ca²⁺ buffer. Pancreatic acinar cells were loaded with even greater concentrations of the Ca²⁺ chelator, EGTA (500 μM EGTA/AM, 20 min), and treated with thapsigargin: however, steady-state [Ca²⁺] was not augmented (Fig. 1C). As observed with BAPTA-loaded cells, EGTA loading blunted the transient release of stored Ca²⁺ in the absence of external Ca²⁺, and, following the restoration of external Ca²⁺, the initial rate of Ca²⁺ entry was slower than in control cells (0.045 ± 0.015 versus 0.81 ± 0.11 nM/s, respective-ly). To ensure that the difference in effects of these two chelators was not a result of nonspecific inhibition of Ca²⁺ entry by EGTA, pancreatic cells were loaded with both BAPTA and EGTA (50 μM BAPTA/AM and 500 μM EGTA/AM, 20 min); these cells responded to thapsigargin with an augmented Ca²⁺ entry as if they had been loaded with BAPTA alone (Fig. 1C).

We assume that the loading of EGTA into the cytoplasm of the cells was at least comparable to BAPTA or likely even greater since EGTA was applied at a higher concentration than BAPTA. However, as this cannot be known for certain, we also carried out a series of experiments in which the pancreatic cells were microinjected with the free acid forms of these compounds (pipette concentration of BAPTA or EGTA was 250 mM, final cytoplasmic concentration of chelators is estimated as 2.5–5 mM (18)). As shown in Fig. 1D, the effects of the microinjected chelators were essentially the same as those observed using the acetoxyethyl ester loading. The summarized results from all experiments carried out with the protocols shown in Fig. 1 are given in Table I.

The different actions of BAPTA and EGTA are not likely due to differences in their affinities for Ca²⁺, since the Kᵣ for BAPTA (192 mM) is actually somewhat greater than for EGTA (67 mM). Rather, the differential effects of BAPTA and EGTA on Ca²⁺ entry probably result from the faster calcium binding kinetics of BAPTA (6 × 10⁻⁷ M⁻¹ s⁻¹ versus 1.5 × 10⁻⁶ M⁻¹ s⁻¹ for EGTA). The ability of BAPTA to enhance the steady-state Ca²⁺ entry would seem to reflect the rapid negative feedback on Ca²⁺ entry seen in earlier patch-clamp experiments (8, 10). Because this occurs in the face of an elevated level of Ca²⁺ in the cytoplasm, it must result from an action of BAPTA in a compartment separated to some degree from the bulk of cytoplasm. In all likelihood, this interaction would occur at a site close to the mouth of the calcium channel where the [Ca²⁺] is normally maintained higher than the bulk of the cytoplasm.
rapid entry through the channel. Consistent with this interpretation, the slower Ca\(^{2+}\) chelator, EGTA, mimicked the effect of BAPTA on the transient release of Ca\(^{2+}\) and on the rate of rise of the sustained [Ca\(^{2+}\)](i) phase, but EGTA failed to augment the steady-state [Ca\(^{2+}\)](i) level (Fig. 1, C and D). That is, the [Ca\(^{2+}\)](i) gradient which is responsible for the negative feedback is maintained by diffusion of Ca\(^{2+}\) through the channel at a rate too rapid to be buffered by the slower chelator, EGTA.

We next examined the effects of BAPTA loading on two other cell types to determine if a similar mode of negative feedback could be detected. We chose mouse lacrimal acinar cells and NIH 3T3 fibroblast cells, because the former display large, sustained elevations in [Ca\(^{2+}\)](i), due to capacitative calcium entry (15) and Fig. 2), while the latter exhibit a [Ca\(^{2+}\)](i) entry signal which approaches base-line with prolonged activation (Fig. 3). As seen in pancreatic acinar cells, in lacrimal acinar cells loaded with BAPTA (100 \(\mu\)M BAPTA/AM for 20 min), the thapsigargin-induced transient [Ca\(^{2+}\)](i), release was abolished, and the time to steady-state [Ca\(^{2+}\)](i), on addition of extracellular Ca\(^{2+}\) was also prolonged compared with controls. Also similar to pancreatic cells, BAPTA-loaded lacrimal cells had a significantly greater steady-state [Ca\(^{2+}\)](i) level, compared with control cells (Fig. 2 and Table II). Finally, loading lacrimal cells with the slower Ca\(^{2+}\) chelator, EGTA (500 \(\mu\)M EGTA/AM, for 20 min), abolished the thapsigargin-induced transient rise in
**Fig. 2.** Effect of BAPTA on thapsigargin-induced Ca\(^{2+}\) entry in single mouse lacrimal cells. Using a procedure similar to that described for pancreatic cells in Fig. 1, the effects of BAPTA and EGTA on thapsigargin-induced entry in lacrimal cells were examined. The concentration of BAPTA/AM was 100 \(\mu\text{M}\), the concentration of EGTA/AM was 500 \(\mu\text{M}\). The traces are representative of 11 (Control), 11 (BAPTA), or 4 (EGTA) independent experiments.

**Fig. 3.** Phases of [Ca\(^{2+}\)] change in single NIH 3T3 cells. For both traces, 2 \(\mu\text{M}\) thapsigargin was added where indicated. In the trace indicated Calcium Present (top), 1.8 mM extracellular Ca\(^{2+}\) was present until the extracellular medium was changed to a calcium-deficient medium (where indicated). In the trace indicated Calcium Present (bottom), Ca\(^{2+}\) was absent from the extracellular medium and restored to 1.8 mM where indicated.

**Table II**

| Conditions          | AM-loaded | Microinjected |
|---------------------|-----------|---------------|
| Control             | 382.5 ± 69.3 (11) | 346.7 ± 29.6 (3) |
| BAFTA               | 810.88 ± 88.5* (11) | 696.7 ± 77.9* (3) |
| EGTA                | 442.5 ± 38.1 (4) | Not done |

*Significantly greater than control.

The reversible and Ca\(^{2+}\)-dependent decline in steady-state [Ca\(^{2+}\)] could result from a Ca\(^{2+}\)-dependent modulation of either Ca\(^{2+}\) entry or Ca\(^{2+}\) efflux. To distinguish between these possibilities, we examined Ca\(^{2+}\)-dependent changes in the entry of Ba\(^{2+}\) as a surrogate for Ca\(^{2+}\) entry. Ba\(^{2+}\) is known to permeate the Ca\(^{2+}\) channels involved in capacitative Ca\(^{2+}\) entry (19, 20), but is a poor substrate for Ca\(^{2+}\)-ATPases (19–22). Fig. 6 depicts the experimental protocol: after the Ca\(^{2+}\) stores of NIH 3T3 cells were depleted by 2 \(\mu\text{M}\) thapsigargin, they were bathed in a medium containing 10 \(\mu\text{M}\) Ba\(^{2+}\) for 100 s and subsequently returned to a nominally Ca\(^{2+}\)-free medium; a second addition of 10 \(\mu\text{M}\) Ba\(^{2+}\) followed 400 s later. These additions of Ba\(^{2+}\) resulted in a rising fura-2 ratio indicative of Ba\(^{2+}\) entry, and, as shown in the inset to Fig. 6, in control cells the rate of Ba\(^{2+}\) entry during the second exposure was similar to that during the first. However, if during the period between the two test exposures to Ba\(^{2+}\) the cells were incubated in a Ca\(^{2+}\)-containing medium, then the rate of Ba\(^{2+}\) entry at the second exposure to Ba\(^{2+}\) was significantly reduced. This indicates that the exposure to Ba\(^{2+}\) inactivates divalent cation entry, and that the decline in steady-state [Ca\(^{2+}\)] in 3T3 cells results at least in part from inactivation of Ca\(^{2+}\) influx.

Although apparently much slower, such an inactivation could reflect the same, or a similar, mechanism to that seen with pancreatic and lacrimal cells. As with pancreatic and lacrimal cells, loading of 3T3 cells with BAPTA blunted the transient rise of [Ca\(^{2+}\)]. However, in contrast to pancreatic and

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**[Ca\(^{2+}\)]** Entry and Feedback Control

**Table II**

Effect of thapsigargin on the steady state Ca\(^{2+}\) entry level (nm) in mouse lacrimal cells loaded with or without additional Ca\(^{2+}\) buffers. The protocol was as for Table I. Data are means ± S.E. of (n) determinations.

| Conditions          | AM-loaded | Microinjected |
|---------------------|-----------|---------------|
| Control             | 382.5 ± 69.3 (11) | 346.7 ± 29.6 (3) |
| BAFTA               | 810.88 ± 88.5* (11) | 696.7 ± 77.9* (3) |
| EGTA                | 442.5 ± 38.1 (4) | Not done |

*Significantly greater than control.
The irreversible component of inhibited Ca\(^{2+}\) entry is dependent on Ca\(^{2+}\). NIH 3T3 cells were treated with thapsigargin and calcium entry was assessed by addition of 1.8 mM Ca\(^{2+}\) at \(t = 1,000\) s (black bar) and, at \(t = 1,500\) s, the extracellular medium was changed to a Ca\(^{2+}\)-deficient medium in which the cells remained for 500 s. A second challenge with 1.8 mM Ca\(^{2+}\) at \(t = 2,000\) s promoted an influx which was inhibited. In a second series of experiments, cells were treated with thapsigargin in the same manner but remained in the Ca\(^{2+}\)-deficient medium for 2,000 s (i.e., the first addition of Ca\(^{2+}\) at 1,000 s was omitted). Addition of 1.8 mM Ca\(^{2+}\) resulted in an influx which was greater than the response at 2,000 s in cells which had been pretreated with Ca\(^{2+}\) at 1,000 s and was not significantly different from the Ca\(^{2+}\) entry measured at 1,000 s.

lacrilal cells, BAPTA did not augment steady-state [Ca\(^{2+}\)], levels in 3T3 cells; rather, in BAPTA-loaded cells, [Ca\(^{2+}\)] was increased only marginally in the presence of thapsigargin and Ca\(^{2+}\) (Fig. 7, Table III). Microinjection of BAPTA (pipette concentration, 250 mM) into NIH 3T3 cells produced the same result as loading with BAPTA/AM (Table III).

**DISCUSSION**

The addition of a high concentration of a fast calcium buffer to the cytoplasm of acinar cells resulted in an elevated steady-state [Ca\(^{2+}\)], in response to thapsigargin. It is likely that this effect is due to the dissipation of a standing [Ca\(^{2+}\)], gradient in the cell that inhibits the activity of Ca\(^{2+}\) influx channels; the most likely locus for such a gradient would be the mouth of the channel where rapid inward flow of Ca\(^{2+}\) ions can maintain the concentration of Ca\(^{2+}\) higher than in the bulk of the cytoplasm. It is also likely that this is a manifestation of the same phenomenon described in patch-clamp studies of mast cells by Hoth and Penner (8) and Jurkat cells by Zweifach and Lewis (10). These investigators reported a rapid inactivation of inward calcium current in calcium store-depleted cells which was reversed by BAPTA but not by the slower chelator, EGTA. In principle, it would be possible for the results in the current study to be ascribed to a diminution of Ca\(^{2+}\) extrusion at the plasma membrane; however, a steady-state gradient due to active extrusion would produce a localized [Ca\(^{2+}\)] lower than the bulk of cytoplasm. Thus, the ability of BAPTA to dissipate standing Ca\(^{2+}\)-gradients would result, if anything, in an increase in the concentration of Ca\(^{2+}\) in the vicinity of Ca\(^{2+}\) extrusion sites, and this would be expected to increase rather than reduce the rate of Ca\(^{2+}\) efflux.

Zweifach and Lewis (11) have also described a slow inactivation of depletion-activated current in Jurkat cells. Because of the experimental protocol used by Zweifach and Lewis (11), the slow inactivation was due in part, but not wholly, to reuptake of Ca\(^{2+}\) into intracellular stores. Zweifach and Lewis (11) also reported that the slower calcium chelator, EGTA, diminished this inactivation leading them to conclude that the site of this feedback was not close to the mouth of calcium channel but was located somewhere within the major cytoplasmic compartment. In the present study, in NIH 3T3 cells, we observed a similarly slow inactivation of calcium entry that was dependent of the presence of Ca\(^{2+}\) in the incubation medium. However, in the presence or absence of excessive intracellular Ca\(^{2+}\) buffering, thapsigargin induced an elevated steady-state [Ca\(^{2+}\)], that was only about 20 nM greater than the baseline, prestimulus [Ca\(^{2+}\)], level. While one could envision microdomains of [Ca\(^{2+}\)], much higher than this level causing inactivation of calcium entry in control cells, this would not be expected to occur in the cells loaded with high concentrations of BAPTA. It is thus difficult to understand how such a minor elevation in [Ca\(^{2+}\)], could maintain almost complete inhibition of Ca\(^{2+}\) entry in the 3T3 cells. Rather, we conclude that the control by Ca\(^{2+}\) must occur at a site readily accessible to extracellular Ca\(^{2+}\), but inaccessible to cytoplasmic BAPTA. This could be either a site on the extracellular domain of the Ca\(^{2+}\) channel which is inhibited by extracellular Ca\(^{2+}\) when it is in the activated state or possibly a site within the channel pore inaccessible to BAPTA in the cytoplasm. Our results do not readily distinguish between these possibilities.

Regardless of the underlying mechanism, the lack of an effect of BAPTA on the steady-state [Ca\(^{2+}\)], and the transient nature of the capacitative Ca\(^{2+}\) entry in NIH 3T3 cells suggest that a mechanism of negative regulation, distinct from that
Inhibitory effect of external Ca\(^{2+}\) on Ba\(^{2+}\) influx. NIH 3T3 cells were treated with 2 μM thapsigargin in nominally Ca\(^{2+}\)-free medium. Ca\(^{2+}\) influx was assessed by adding 10 mM Ba\(^{2+}\) for 100 s as indicated to obtain a measure of Ba\(^{2+}\) influx. The extracellular medium was then either restored to a Ca\(^{2+}\)-deficient medium (control, dashed line) or changed to a medium containing 1.8 mM Ca\(^{2+}\) (solid line). Approximately 400 s later, the solution was changed to a Ca\(^{2+}\)-deficient medium containing 10 mM Ba\(^{2+}\). The second rate of Ba\(^{2+}\) influx was measured and expressed as a percentage of the first rate of Ba\(^{2+}\) influx in each tracing. The data averaged from 5–7 such experiments are summarized in the inset.

Effect of thapsigargin on the steady-state [Ca\(^{2+}\)]\(_i\) (nM) in NIH 3T3 cells loaded with or without BAPTA. The protocol was as for Table I. Data are means \(\pm\) S.E. of (n) determinations.

|          | AM-loaded | Micronjected |
|----------|------------|--------------|
| Control  | 18.29 \(\pm\) 3.9 (8) | 22.3 \(\pm\) 6.1 (6) |
| BAPTA    | 20.65 \(\pm\) 5.5 (8) | 14.4 \(\pm\) 4.1 (6) |

*Significantly greater than control.

observed in pancreatic and lacrimal cells, accounts for the reduced [Ca\(^{2+}\)]\(_i\) level. Thus, there appear to be at least two different mechanisms for regulation of Ca\(^{2+}\) entry by Ca\(^{2+}\), and, if one considers the Ca\(^{2+}\)-dependent irreversible inactivation shown by the results in Fig. 4D and Fig. 5, there may be three. It is the effect of BAPTA on Ca\(^{2+}\) entry that clearly distinguishes the action of Ca\(^{2+}\) in 3T3 cells from that in acinar cells. In the case of 3T3 cells, we considered that the slow rate of inactivation and recovery might indicate a process of Ca\(^{2+}\)-dependent covalent modification, perhaps by phosphorylation, of the channel or a protein regulating it. However, we have been unable to obtain pharmacological evidence for such regulation; pretreatment (15 min at room temperature) with the following modifiers of kinase or phosphatase activity failed to prevent the slow inactivation of Ca\(^{2+}\) entry in 3T3 cells (data not shown): phorbol 12-myristate 13-acetate (1.6 μM), staurosporine (0.1 μM), KN-62 (10 μM), chelerythrine chloride (1 μM), okadaic acid (1 μM), cyclosporin A (5 μg/ml).

The appearance of these distinct modes of Ca\(^{2+}\) entry regulation may indicate that the different cell types express distinct subtypes of capacitative calcium entry channels. Alternatively, the channels may be regulated differently because the cell types express different kinases or other Ca\(^{2+}\)-dependent regulatory proteins. Until the capacitative Ca\(^{2+}\) channel is isolated, cloned, and sequenced, these possibilities cannot be tested. Ca\(^{2+}\)-dependent inactivation of Ca\(^{2+}\) channels occurs in a variety of cells and is likely of physiological importance; this negative feedback loop will limit the rise of [Ca\(^{2+}\)]\(_i\) in activated cells and thus provide a mechanism to limit the amplitude of Ca\(^{2+}\) signals. Furthermore, it is possible that the distinct modes of regulation occurring in different cell types could be exploited to develop pharmacological agents with cell type-specific actions.

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