Role of the Endoplasmic Reticulum in Shaping Calcium Dynamics in Human Lens Cells

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PURPOSE. Localized cortical cataracts in the human lens have been shown to involve a selective increase in calcium with no change in sodium content. Recent studies in the rat lens in vitro have shown that the store-operated channel is highly selective for calcium over sodium, and therefore this channel was characterized further in human lens cells.

METHODS. Human primary cultures were initiated from epithelial explants and passaged onto coverslips. After incorporating Fura-2, agonist- or thapsigargin-induced changes in cytosolic calcium were monitored and calibrated using fluorometric digital imaging techniques.

RESULTS. Histamine and adenosine triphosphate (ATP; 10 μM) induced a large transient increase in cytosolic calcium followed by a maintained lower plateau phase in the continued presence of the calcium-signaling agonist. The second phase was abolished by removing external calcium and represented the contribution from the store-operated influx. The store-operated pathway was blocked by inorganic agents such as zinc and nickel (100 μM) but was insensitive to the voltage-sensitive calcium channel blocker, nifedipine (1 mM). Depolarizing the membrane voltage by raising the external potassium (75 mM) also blocked the influx. Similar results were obtained if the store was first emptied directly using thapsigargin (1 μM), and with this agent it was also possible to observe the very slow activation and inactivation kinetics (>10 seconds) of the channel. Addition of manganese to the bathing medium initiated a quench of Fura-2 isobestic fluorescence that was enhanced 2.9 ± 0.3-fold after 10 μM ATP addition. There was a delay of 82 ± 16 seconds between initiation of the calcium spike and the Mn2+ quench rate, indicating the presence of a delayed entry pathway. In the resting state, removal of, or increasing extracellular calcium concentration 10-fold did not perturb the level of cytosolic Ca2+. Similar maneuvers performed after agonist- or thapsigargin-induced store depletion of intracellular stores brought about dramatic changes in cytosolic Ca2+ consistent with the activation of a Ca2+ entry pathway. Lower concentrations of agonist induced oscillations of Ca2+ that continued for a short time in Ca-free solution. No increase in Mn2+ quench rate was associated with oscillations. A 100-μM zinc- and KCl-induced blockade of Ca2+ entry had no effect on the form of agonist-induced oscillations. Inhibition of Ca2+ influx by zinc (100 μM) converted a sustained Ca2+ response to a train of repetitive Ca2+ spikes.

CONCLUSIONS. Human lens cells normally have very low Ca2+ permeability. Depletion of intracellular stores by agonists or thapsigargin initiates a Ca2+ entry pathway that is not required for the Ca2+ oscillations induced by low concentrations of agonist. This potentially provides a signal transduction mechanism with minimal risk of Ca2+ overload to the lens, whereas overactivation of the store-operated channel is a possible way of increasing calcium in the lens and could explain the distribution found in localized cataracts. (Invest Ophthalmol Vis Sci. 2001;42:1009–1017)

Cytosolic calcium is a key regulator of a number of cellular processes that are involved in both the normal physiology of the lens and the formation of cataract. In particular, it has long been appreciated that there is an association between cortical cataract and increased levels of lens calcium, and recent evidence suggests that lens opacification results from the consequent activation of calcium-sensitive proteases. At the lower levels of free calcium concentration that are found in normal transparent lenses, activation of membrane receptors initiate calcium signals in lens epithelial cells. For example, it has been shown that lens cells possess a number of G-protein-coupled receptors and membrane receptor tyrosine kinases that are coupled to the release of calcium from intracellular stores. Mobilization of lens cell calcium leads to the complex modulation of different ion channels and cell growth, and furthermore unscheduled activation of muscarinic receptors in the lens also appears to give rise to cortical cataract.

As is the case in other nonexcitable and excitable cell types, calcium release induced by maximal concentrations of agonist applied to lens cells leads to a biphasic calcium response, with the second phase being sustained by an influx of calcium. It is generally accepted that the main mechanism for activation of this calcium influx component, termed capacitative calcium entry (CCE), is depletion of intracellular calcium stores, which somehow opens plasma membrane channels. Direct electrophysiological measurements of a calcium-selective current, Icalc, which is activated by depletion of the intracellular calcium store, have been made and its selectivity and activation and inactivation properties characterized. Recent progress has been made in the nature of the signal that activates calcium influx after calcium store depletion, and considerable effort has been invested in studying the role that influx plays in shaping intracellular calcium signals.

In lens cells and other cell types, low, physiologically relevant concentrations of agonist induce repetitive calcium spikes, or calcium oscillations, that are more complex than the sustained signals seen after stimulation with maximal agonist concentrations. However, unlike many other cell types, lens cell calcium oscillations persist in the absence of extracellular calcium and this simple observation questions the nature and relative roles of calcium entry in each type of response. The purpose of our study was to characterize the route involved in calcium influx in human lens epithelial cell (HLEC) calcium signaling using a combination of fluorescence calcium imaging techniques, manganese fluorescence quench studies, and calcium influx blocking maneuvers. The present data indicate that, on calcium mobilization induced by a maximal concentration of agonist, there is a pronounced delay before a CCE pathway is activated. Calcium influx is inhibited by zinc and...
membrane potential depolarization and is relatively insensitive to nickel and nifedipine. Moreover, in human lens cells, calcium oscillations do not appear to require activation of the CCE pathway.

METHODS

HLEC Culture

HLECs were cultured as previously described by Riach et al. Briefly, lenses were obtained from the Bristol and East Anglian Eye Banks and placed in Eagles’ minimal essential medium (EMEM). The capsules with attached epithelium were removed and cultured in plastic flasks in EMEM supplemented with 20% fetal calf serum (FCS) for 2 to 3 weeks. The capsules with lenses were obtained from the Bristol and East Anglian Eye Banks and were placed in Eagles’ minimal essential medium (EMEM). The capsules with attached epithelium were removed and cultured in plastic flasks in EMEM supplemented with 20% fetal calf serum (FCS) for 2 to 3 weeks. The cells were then trypsinized (0.1%) and washed with EMEM, and a 100-μl sample containing 2 × 10⁴ cells was placed on a thin glass coverslip and allowed to settle. The cells were then flooded with medium supplemented with 10% FCS and cultured for 1 to 7 days before use.

Cell Loading and Fura-2 Fluorescence Ratio Measurements

To load the cells with fluorescent dye, 5 μM Fura 2-AM was added to the medium bathing a coverslip for a period of 45 minutes at 35°C. The cells were then washed for 20 minutes with artificial aqueous humor (AAH) of the following composition (in millimolar): 140 NaCl, 5 KCl, 1 CaCl₂, 0.5 MgCl₂, 5 glucose, and 10 HEPES (pH 7.25). To obtain Ca-free solutions, calcium was omitted and 1 mM-EGTA added. Unless otherwise stated, all chemicals were from Sigma (Poole, UK). The coverslip formed the base of an experimental chamber that was placed on the stage of an epifluorescence microscope (Nikon, Melville, NY), and the cells were continuously perfused with AAH at 35°C. Control and experimental solutions were administered through a two-way tap, and turnover time for solutions in the chamber (<5 seconds) was kept at a constant rate. This allowed an accurate measurement of the latency and time course between such changes in the bath. The cells were illuminated at 340 and 375 nm, alternately, and the resultant emissions for each wavelength were recorded every second at 510 nm by a charge-coupled device camera (CCD; Photon Technology, Newark, NJ). In each preparation, regions of interest in the field of view were identified, corresponding to the central regions of five individual cells, and a running ratio value could then be computed for each region. Calibration of intensity ratios into calcium values was performed as stated in Riach et al. using an in situ calibration formula described in Grynkiewicz et al. Because the calibration procedure is a rigorous one, not all preparations survived full calibration, and in these cases the data are presented in ratio form.

Determination of Manganese Entry

Fura-2 binding of the divalent cation manganese quenches the emitted fluorescence, and the manganese quench rate of the fluorescence intensity at the calcium-insensitive wavelength for Fura-2 permits the time course and relative magnitude of calcium influx to be assessed. The isobestic point for Fura-2 was determined to be 357 nm in the present system, and the resultant fluorescence was independent of changes in intracellular calcium. In addition, the fluorescence intensity in response to a 375-nm excitation was monitored to observe the onset and kinetics of agonist-induced responses. Linear fits using least-squares regression analysis were performed 30 seconds before and 30 seconds after an observed increase in the 357-nm quench rate.

At least four replicate experiments were performed on cells generated from different donor lenses, and the figures shown in the Results section represent qualitatively the responses from each protocol (i.e., 20 cells from four different preparations). When statistical data are presented, five cells were averaged in each preparation to arrive at the value for that preparation. The means from four different preparations are presented as means ± SEM (n = 4). The statistical significance of observed differences was assessed by Student’s unpaired t-test.

Ethics Committee approval was not required for these studies, because donor identification details were not released by the Eye Banks.

RESULTS

Time Course of Calcium Entry in a Nonoscillatory Response

To characterize the role of the calcium entry pathway in agonist-induced HLECs, calcium-signaling protocols based on the depletion of intracellular calcium stores were used. In the first instance, the characteristics of the calcium influx pathway associated with biphasic sustained calcium responses induced by maximal concentrations of adenosine triphosphate (ATP) or histamine were compared with calcium entry invoked by thapsigargin, the sarco–endoplasmic reticulum Ca–ATPase (SERCA) pump inhibitor. Figure 1A illustrates the relative contributions from intracellular and extracellular calcium to the formation of a maximal response induced by ATP. The more transient of the two superimposed responses was conducted in the absence of calcium from the bathing medium.
Initially, the two responses were directly comparable, which indicates that the first phase of the response was due to the release of calcium from intracellular stores. After 82 ± 16 seconds (n = 4) the two responses diverged, suggesting that at this point a very much delayed calcium influx pathway is activated. Moreover, responses to 30-second pulses of histamine conducted in the presence or absence of calcium from the bathing medium were found to be superimposable (see Riach et al.5). To verify the time course and extent of the delayed calcium influx component, manganese entry was used as a surrogate of calcium influx. 22–25 Addition of manganese (100 μM) to the control (1 mM Ca²⁺) extracellular medium induced a basal quench of the calcium-insensitive 357-nm fluorescence, indicating that there was a leak of manganese into the cytosol in the resting condition (Fig. 1B). Stimulation of HLECs with high concentrations of ATP elicited a maintained response that was depicted in an inverted fashion by the calcium-sensitive 375-nm fluorescence intensity (Fig. 1B). The validity of the chosen isosbestic point was confirmed by the absence of deviation of the 357-nm fluorescence intensity coincident with the onset of the calcium response registered by the 375-nm trace. There was no immediate increase in the manganese quench after formation of the calcium response, indicating that the degree of calcium entry remained inactive during the initiation of the ATP-induced calcium signal. Rather, there was a delay of 60 ± 16 seconds (n = 4) after the onset of the calcium response and an observed increase in manganese quench rate. This latency is not significantly different (P < 0.01) from the time taken for nonoscillatory superimposed ATP responses to deviate when the agonist was applied in the presence and absence of extracellular calcium (Fig. 1A). Lines of best fit applied to the rates before and after the agonist-induced increase in manganese quench indicate that the quench rate was stimulated 2.9 ± 0.3-fold (n = 4).

Capacitative Calcium Entry

The pronounced delay between initiation of the calcium signal and the onset of calcium entry suggests that, after membrane receptor activation, a signal is generated that activates the calcium entry pathway. In a number of nonexcitable cell types, the characteristics of the calcium entry pathway have been investigated using thapsigargin, which depletes intracellular calcium stores without a concomitant increase in inositol polyphosphates.23,27,28 Figure 2A clearly shows that under conditions in which the store was full, the calcium permeability was very low—so much so, that the cell could be exposed to a 10-fold increase in calcium concentration in the bathing medium with no appreciable change in cytosolic calcium. However, after thapsigargin-induced store depletion, similar levels of calcium supplementation induced pronounced changes in intracellular calcium superimposed on an elevated baseline.

Theoretically, these cytosolic calcium perturbations are due to the activation of a calcium influx pathway, a reduction in calcium-sequestering capacity, or both. We therefore adopted a much-used protocol in which the calcium store is depleted by either a maximal concentration of agonist or thapsigargin, in the absence of calcium from the bathing medium.28 Exposure to 10 μM histamine or thapsigargin under these conditions induced a transient increase in cytosolic Ca²⁺ (Fig. 2B). Reintroducing extracellular calcium induced a dramatic increase in cytosolic calcium, no matter whether the store had been depleted by histamine or thapsigargin (Fig. 2B). It is notable that
the kinetics associated with the cytosolic calcium increase were dependent on the mode of calcium store depletion (Figs. 2C, 2D).

Close inspection of the kinetics of the cytosolic $[\text{Ca}^{2+}]$ increase obtained by exposure to increasing levels of external $\text{Ca}^{2+}$ in the presence of histamine revealed at least three components. A slow initial phase (Fig. 2C, open arrow) that lasted approximately 60 seconds, followed by a rapid increase that peaked within a further 20 seconds. This phase was in turn followed by a much slower recovery phase giving rise to a broad peak. The rates obtained for the first two phases were very similar for the three external calcium concentrations (Table 1). In contrast, when the store was depleted by thapsigargin, the initial slow phase was absent, and the residual rate and peak amplitude were very different, depending on the external calcium concentration applied (Table 1). The difference in the observed kinetics depended not only on the presence or absence of a functional SERCA pump, but also on the nature and activity of the calcium entry pathway(s) in each case. We therefore characterized the calcium entry pathway(s) activated in response to calcium store depletion induced by membrane receptor activation and thapsigargin, respectively.

**Sensitivity of the Calcium Influx Pathway to Inorganic Cations**

In the original characterization of the calcium release-activated current, $I_{\text{cra}}$, Hoth and Penner demonstrated a sensitivity to inorganic divalent cations. We therefore performed a comparative study of the sensitivity of the agonist- and thapsigargin-activated calcium influx pathways to nickel and zinc. Figure 3A illustrates that a maximal response to histamine was initially superimposable in the presence of zinc (100 μM), but the maintained elevated level was far more short-lived than the control. The behavior was similar to that exhibited in the calcium-free experiment shown in Figure 1A. In addition, we demonstrated a sensitivity of the calcium influx phase of the thapsigargin-induced response to zinc by adding the divalent cation during the sustained elevated level of cytosolic calcium (Fig. 3B). It is evident that zinc was more potent than nickel and reduced the sustained elevated level of cytosolic calcium to near the resting level. Divalent cations have been reported to alter Fura-2 fluorescence, but in a parallel series of experiments zinc and nickel did not alter the fluorescence intensity at the isosbestic point in the control or stimulated condition (data not shown). Hoth and Penner also observed a potent inhibition of $I_{\text{cra}}$ by zinc and a much reduced effect by nickel. However, divalent cations have also been shown to block voltage-operated calcium channels.

**Voltage Dependency of Calcium Influx Pathway**

A further series of experiments was therefore performed to assess the voltage dependency of the calcium influx pathway. Initially, it was determined that agonist-induced sustained calcium responses were not modified by concentrations of nifedipine known to block voltage-operated calcium channels in other tissues (Fig. 4A). Similarly, replacement of 75 mM Na$^+$ with K$^+$, previously shown to depolarize lens epithelial cells to the values expected to activate voltage-operated calcium channels, failed to induce an increase in intracellular calcium (Fig. 4B). Moreover, the sustained elevated baseline generated by thapsigargin-induced store depletion (Fig. 4C) was markedly reduced by increasing external K$^+$.

**Calcium Entry and Calcium Oscillations**

In addition to maintaining the elevated baseline associated with a maximal, nonoscillatory response, calcium entry has long been known to be increased during oscillatory responses in a number of cell types. Typically, low concentrations of agonist induced calcium oscillations in HLECs that persisted

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**Table 1. Rate of Increase of Cytosolic Calcium**

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<th>Histamine</th>
<th>Thapsigargin</th>
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<td></td>
<td>2* 5 10</td>
<td>2 5 10</td>
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<tr>
<td>Slow (initial)</td>
<td>0.6 ± 0.1</td>
<td>N/A</td>
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<td>Fast phase</td>
<td>9.6 ± 3.1</td>
<td>4.1 ± 1.1</td>
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Calcium influx rates after either histamine- or thapsigargin-induced calcium store depletion. The data are presented as mean nanomolar per second ± SEM for four preparations.

* Extracellular calcium concentration in millimolar.
Baseline calcium before adding thapsigargin. Calcium from the AAH medium. Depolarizing by KCl had no effect on depletion, also showing the relative effects of adding or removing calcium influx by high KCl (75 mM) after thapsigargin-induced store depletion, suggesting that the state of the calcium store is somehow coupled to the calcium influx pathway.

As is the case in other epithelial cell types there was a close temporal relationship between agonist-induced release of calcium from intracellular stores and a resultant influx of calcium from the medium bathing HLECs (Fig. 1). Maximal concentrations of agonist elicited a nonoscillatory response that consisted of two phases: release of calcium from intracellular stores that was independent of extracellular calcium (Fig. 1A), followed by an influx of calcium from the bathing medium.

The relative contributions of intracellular and extracellular calcium to the formation of agonist-induced nonoscillatory calcium signals in HLECs are shown in Figure 1. Because the first phases of the responses initiated in the presence and absence of extracellular calcium were superimposable (Fig. 1B), it is likely that this was attributable to the release of calcium from intracellular stores. However, the second phase was more short-lived in the absence of calcium from the bathing medium, implying that an influx of calcium contributes to the maintenance of the sustained elevated level that follows the peak of the response. Alternatively, the calcium-free environment may compromise the signal transduction pathway activated by the membrane receptor agonist in addition to electrochemically favoring the removal of calcium from the cell. Consistent with a role for calcium influx in the second phase of the response is the observation that after agonist-induced store depletion, the rate of Mn$^{2+}$ quench increased approximately threefold (Fig. 1B). Moreover, there was no significant difference between the apparent latency with and without a delay in the conformational coupling or generation of a capacitative influx factor could account for the magnitude of the delay between the formation of the calcium peak and the onset of calcium entry in HLECs.

**FIGURE 4.** Voltage dependence of store-operated calcium influx. (A) Superimposed paired ATP (10 μM)-induced calcium responses conducted in control (1 mM Ca$^{2+}$), calcium-free, and nifedipine (1 mM)-supplemented AAH. (B) Paired ATP-induced maximal calcium responses in control AAH and high-K$^+$ AAH. (C) Reduction of store-operated calcium influx by high KCl (75 mM) after thapsigargin-induced store depletion, also showing the relative effects of adding or removing calcium from the AAH medium. Depolarizing by KCl had no effect on baseline calcium before adding thapsigargin.

for several minutes in the absence of calcium from the bathing medium, albeit with reduced duration (Fig. 5A). Returning calcium to the bathing medium caused a small increase in the baseline and restoration of calcium spikes. Although these experimental conditions favored the loss of calcium from the cell, this behavior may indicate a dependence of the propagation of calcium oscillations on the activation of a calcium entry mechanism. There was, however, no observable increase in manganese quench rate on induction of a train of agonist-induced calcium oscillations (Fig. 5B). This contrasts with the marked increase in manganese quench rate associated with a maximal response (Fig. 1B). Nevertheless, it is possible that significant levels of calcium entry are required to sustain repetitive calcium spikes and this entry cannot be detected at lower levels by the manganese quench technique. We therefore investigated the effects of blocking calcium entry on the maintenance of calcium oscillations. High K$^+$-induced membrane depolarization and zinc, both potent blockers of calcium entry in human lens cells (Figs. 3, 4), had no observable effect on the form of calcium oscillations over a prolonged period (Figs. 5A, 5C, 5D, 5E, 6A, 6B). Of note, blocking calcium entry induced oscillations in cells where oscillatory behavior had been suppressed by a sustained entry of calcium (Fig. 7). Exposure to 10 μM zinc during the maintained phase induced slow oscillatory behavior from a lowered baseline.

**DISCUSSION**

Calcium influx in HLECs shares a number of characteristics associated with the capacitative entry pathway that has been characterized in a number of other systems. Calcium influx in HLECs does not require receptor occupancy, is insensitive to classic inhibitors of voltage-operated calcium channels, and is inhibited by membrane depolarization and some divalent cations.

As is the case in other epithelial cell types there was a close temporal relationship between agonist-induced release of calcium from intracellular stores and a resultant influx of calcium from the medium bathing HLECs (Fig. 1). Maximal concentrations of agonist elicited a nonoscillatory response that consisted of two phases: release of calcium from intracellular stores that was independent of extracellular calcium (Fig. 1A), followed by an influx of calcium from the bathing medium.

The relative contributions of intracellular and extracellular calcium to the formation of agonist-induced nonoscillatory calcium signals in HLECs are shown in Figure 1. Because the first phases of the responses initiated in the presence and absence of extracellular calcium were superimposable (Fig. 1B), it is likely that this was attributable to the release of calcium from intracellular stores. However, the second phase was more short-lived in the absence of calcium from the bathing medium, implying that an influx of calcium contributes to the maintenance of the sustained elevated level that follows the peak of the response. Alternatively, the calcium-free environment may compromise the signal transduction pathway activated by the membrane receptor agonist in addition to electrochemically favoring the removal of calcium from the cell. Consistent with a role for calcium influx in the second phase of the response is the observation that after agonist-induced store depletion, the rate of Mn$^{2+}$ quench increased approximately threefold (Fig. 1B). Moreover, there was no significant difference between the apparent latency with and without a delay in the conformational coupling or generation of a capacitative influx factor could account for the magnitude of the delay between the formation of the calcium peak and the onset of calcium entry in HLECs.
Under resting conditions, it appears that HLECs exhibit a low permeability to calcium. This is inferred by the constant intracellular calcium concentration observed in response to changing the extracellular calcium concentration from negligible levels to 10 mM (Fig. 2A). This, perhaps, goes some way toward explaining why there does not appear to be the initiating phase provided by extracellular calcium in HLECs that is associated with spike formation in many other cell types. After thapsigargin-induced store depletion, the intracellular calcium concentration altered dramatically in response to increasing concentrations of extracellular calcium (Fig. 2A) or to removing calcium entirely from the bathing medium (Fig. 4C).

**Figure 5.** Dependency of calcium oscillations on calcium influx. (A) ATP (500 nM)-induced calcium oscillations are unaffected by increasing external K+ to 75 mM (see also C, D, and E). The oscillations also persist for a time in Ca-free medium. (B) There is no increase in the manganese quench rate of Fura-2 after the onset of ATP (500 nM)-induced calcium oscillations. (C) ATP-induced oscillations in control and high-potassium (75 mM KCl) media (mean of four spikes). (D) Comparison of ATP-induced oscillation amplitude (peak height minus baseline ratio) in control and 75 mM KCl. (E) Comparison of ATP-induced oscillation period (time in seconds between oscillations) in control and 75 mM KCl. Data are expressed as mean ± SEM of three preparations.
Furthermore, returning calcium to the extracellular medium after thapsigargin-induced store depletion induced a dramatic increase in intracellular calcium, revealing the activation of a calcium influx pathway that was not present before thapsigargin exposure (Fig. 2B). Such behavior after nonreceptor occupancy-induced store depletion is consistent with the presence of a CCE pathway. In the continued presence of thapsigargin, the amplitude and rate of calcium increase were dependent on the extracellular calcium load (Fig. 2B, Table 1). Saturation of the calcium regulatory mechanisms with an increasing intracellular calcium load resulted in an amplitude-modulated calcium signal. This contrasts with the situation when the calcium store was partially depleted, and the SERCA pumps on the calcium store were functional.

Figure 2B illustrates that a calcium-influx pathway was also activated after agonist-induced store depletion. Under these conditions successive calcium spikes initiated by an influx of calcium were biphasic and of constant amplitude (Fig. 2C, Table 1). It is likely that the first (slow) phase was a result of an influx of calcium from the bathing medium, and this moderate increase in cytosolic calcium served to enhance the release by InsP_3 receptors. This in turn induced a dramatic increase in cytosolic calcium, revealing the activation of a CCE pathway. The evidence primarily comes from observations of a nonoscillatory response (Fig. 1A). Over the longer term, the calcium-free protocol would invariably lead to loss of calcium from the cell and depletion of intracellular calcium stores. In other tissues it has been suggested that a stimulated Mn^{2+} entry pathway also contributes to maintained oscillations. The relative contribution of the calcium influx to the final cytosolic calcium level reached on exposure to either an agonist or thapsigargin can be investigated further by experiments designed to inhibit store-operated influx pathways. The inorganic divalent cations nickel and zinc are known blockers of store-operated calcium channels, and they inhibited the maintained elevated level associated with the nonoscillatory calcium signal (Fig. 3A) and thapsigargin-induced depletion of intracellular stores (Fig. 3B), but not the formation of the agonist-induced peak (Fig. 3A). Moreover, the relative potency with which nickel and zinc inhibited the respective pathways was qualitatively similar to that previously found for blocking L_{\text{Ca}}. In HLECs, the calcium entry pathway was not activated by membrane voltage depolarization, but rather the influx of calcium was stemmed by a diminution of the electrochemical gradient driving calcium into the cell (Fig. 4B). The role of calcium entry in forming a maintained, elevated response in HLECs (and other cell types) therefore seems to be clear cut. There is, however, more controversy surrounding the nature and role of store-operated calcium influx in forming calcium oscillations.

Repetitive intracellular spikes induced by low doses of agonists such as histamine and ATP (Fig. 5A) continued for a short period when calcium was removed from the bathing medium. Initially, intracellular calcium spike formation was thus independent of the presence of calcium in the bathing medium, which was similar to the calcium dependency of the first phase of a nonoscillatory response (Fig. 1A). Over the longer term, the calcium-free protocol would invariably lead to loss of calcium from the cell and depletion of intracellular calcium stores. In other tissues it has been suggested that a stimulated calcium entry pathway also contributes to maintained oscillations. The evidence primarily comes from observations of a stimulated Mn^{2+} quench during the oscillatory period. However, no appreciable increase in Mn^{2+} quench rate was

**Figure 6.** (A) Influence of 10 μM Zn^{2+} on ATP-induced oscillation amplitude. See legend to Figures 5D and 5E for details. (B) Influence of 10 μM Zn^{2+} on ATP-induced oscillation period. See legend to Figures 5D and 5E for details.

**Figure 7.** Initiation of slow oscillations in HLEC by Zn^{2+}. This cell was very sensitive to ATP (500 nM) and showed a maintained, elevated baseline at this relatively low concentration.
observed during calcium oscillations in HLECs (Fig. 5B), which suggests that minimal, if any, calcium (manganese) influx was activated.

Due to the problems associated with prolonged removal of calcium from the bathing medium and using manganese as a surrogate for calcium entry, we also studied the effects of blocking calcium entry on the formation of calcium oscillations. In keeping with the calcium-free and manganese observations, zinc and membrane depolarization, which had been shown to block store-operated calcium entry (Figs. 3, 4), had no effect on calcium oscillations in HLECs. Taken together, these observations suggest that the oscillatory machinery of HLECs is purely cytoplasmic. In other systems a role for calcium influx has been proposed to alter the formation of the intracellular calcium spike and/or play a role in the rate at which intracellular stores are refilled during the interpulse interval.

Shuttleworth and Thompson have dissected these relative roles in avian nasal gland cells and concluded that the calcium store is refilled during the downstroke of the calcium spike and that stores are already recharged before the interpulse interval. In their system the dependence of spike initiation on extracellular calcium was reflected in the immediate cessation of calcium oscillations after membrane depolarization. Presumably, lens cells are highly conservative with the calcium that is released from intracellular stores during the formation of a calcium spike and do not require the activation of calcium entry mechanisms to initiate spike formation or refill intracellular stores. A novel finding in the present study of oscillations was that blockade of the influx pathway could induce oscillations in a cell where the agonist (ATP) had elicited a high, sustained plateau (Fig. 7).

Calcium cell signaling in the lens may have evolved this way to provide a signal transduction mechanism that possesses an optimal signal-to-noise ratio, but also minimizes desensitization of target proteins, avoids excessive calcium pumping by adenine triphosphatases (ATPases), and avoids inappropriate activation of calcium-dependent proteases. It is thus not surprising that lens cells exhibited a low calcium permeability at rest and when stimulated with low levels of agonist. In addition, the electrical response of the lens to an agonist-induced release of calcium from intracellular stores is a membrane depolarization, which itself reduces the electrochemical gradient driving calcium into the lens. Furthermore, treatment of whole lenses with thapsigargin has recently been shown to activate an influx pathway that is highly selective for calcium over sodium and that serves to highlight the potential significance of an intact calcium store for the physiology and transparency of the whole organ. It is noteworthy that cataracts that involve an intact calcium store for the physiology and transparency of lenses with thapsigargin has recently been shown to activate a calcium current in mast cells. Nature. 1992;355:353–356.


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Acknowledgments

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