Activation of store-operated calcium influx at resting InsP_3 levels by sensitization of the InsP_3 receptor in rat basophilic leukaemia cells

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1. Patch clamp and fura-2 AM measurements were performed to study the effects of sensitizing the inositol 1,4,5-trisphosphate (InsP_3) receptor to InsP_3 on the activation of Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) in rat basophilic leukaemia (RBL) cells.

2. The sensitizing agent thimerosal (1 μM) triggered Ca^{2+} release, and this was followed by Ca^{2+} influx. With no added InsP_3 in the patch pipette, thimerosal activated I_{CRAC}; this was prevented by heparin. I_{CRAC} activated by thimerosal was very similar to that evoked by InsP_3 or ionomycin.

3. Dialysing cells either for short (30 s) or long (600 s) periods of time prior to application of thimerosal did not affect the subsequent activation of I_{CRAC}, even though no InsP_3 was included in the patch pipette.

4. These results suggest that sensitizing the InsP_3 receptor can result in large Ca^{2+} influx in the presence of resting InsP_3, and that stores closer to the membrane may contribute more to activation of I_{CRAC} than stores further away.

In many electrically non-excitable cells, an elevation in the levels of the second messenger InsP_3 produces Ca^{2+} release followed by Ca^{2+} entry into the cell (Berridge, 1993). The main mechanism of Ca^{2+} entry now appears to be the capacitive Ca^{2+} influx, originally postulated by Putney (1986), in which the Ca^{2+} content of the InsP_3 stores controls the influx pathway such that depletion of stores activates Ca^{2+} influx. Using patch clamp experiments, it has been directly demonstrated that depletion of stores activates a Ca^{2+} current, termed I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current; Hoth & Penner, 1992). I_{CRAC} has subsequently been observed in a variety of non-excitable cells (Fasolato, Innocenti & Pozzan, 1994).

To activate I_{CRAC}, extreme conditions are routinely employed. These include dialysing cells with high InsP_3 concentrations, with high concentrations of Ca^{2+} chelators, and applying large doses of Ca^{2+} ionophores or thapsigargin to deplete the stores (e.g. Hoth & Penner, 1993).

The sulfhydryl-containing organic compound thimerosal reacts with thiol groups of cysteine amino acids and has been shown to increase the sensitivity of the InsP_3 receptor for InsP_3 (Missiaen, Taylor & Berridge, 1991; Bootman, Taylor & Berridge, 1992; Poitras, Bernier, Servant, Richard, Boulay & Guillemette, 1993; Hilly, Pietri-Rouxel, Coquil, Guy & Mauger, 1993). We have therefore taken advantage of this and examined whether increasing the sensitivity of the InsP_3 receptor can result in activation of I_{CRAC} under conditions where cytosolic Ca^{2+} is buffered to physiological levels. Our results demonstrate that, in the presence of resting InsP_3, thimerosal application results in full activation of I_{CRAC}. Regulation of the InsP_3 receptor might therefore be an important but hitherto unexplored way of controlling Ca^{2+} influx into a non-excitable cell.

METHODS

Rat basophilic leukaemia cells (RBL-1) were purchased from ATCC cell lines, Rockville, MD, USA, and were cultured as previously described (Fasolato, Hoth & Penner, 1993). Patch clamp experiments were conducted in the tight-seal whole-cell configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) at room temperature (20–25 °C) in standard saline solution containing (mM): NaCl, 140; KCl, 2·8; $CaCl_2$, 10; $MgCl_2$, 2; CsCl, 10; glucose, 11; Hepes–NaOH, 10; pH 7·2. CsCl was present to block the inwardly rectifying K^+ channel (Parekh & Penner, 1995). Sylgard-coated, fire-polished patch pipettes had resistances of 2–3 MΩ after filling with the standard intracellular solution which contained (mM): potassium glutamate, 145; NaCl, 8; $MgCl_2$, 1; MgATP, 2; EGTA, 10; Hepes–KOH, 10; pH 7·2. Ca^{2+} was clamped to 60 nM by either varying the EGTA : Ca-EGTA ratio or applying $CaCl_2$ to the internal solution and titrating the pH back to 7·2 with KOH. The amount of $CaCl_2$ added was calculated from a laboratory-written computer program. High-resolution current

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Table 1. Effects of thimerosal, InsP_3 or ionomycin on activation of I_{CRAC}

Stimulus	Application time* (s)	Latency (s)	τ (s)	Amplitude −(pA pF^{-1})	n
Thimerosal (1 μM)	30	169 ± 31	28·7 ± 5·7	3·1 ± 0·4	6
	300	130 ± 24	21·6 ± 2·5	3·3 ± 0·8	3
	600	159 ± 42	29·9 ± 3·0	3·0 ± 0·3	8
InsP_3 (60 μM)	Break in	n.d.	19·4 ± 1·2	2·9 ± 0·2	19
Ionomycin (14 μM)	30	n.d.	17·6 ± 1·5	2·8 ± 0·7	4
	300	n.d.	14·0 ± 1·4	2·7 ± 0·3	8
	600	n.d.	18·8 ± 4·0	3·0 ± 0·4	5

* Time of application after obtaining the whole-cell configuration. τ, activation time constant. Amplitude, peak amplitude. n, no. of cells. n.d., not determined. Data are given as means ± S.E.M.

recordings were acquired by a computer-based patch clamp amplifier system (EPC-9, List Electronic). Capacitive currents were cancelled before each voltage ramp using the automatic capacitance compensation of the EPC-9. Series resistance (R_s) was between 5 and 9 MΩ. The Ca^{2+} current was analysed at −80 mV. Currents were filtered at 2·3 kHz and digitized at 100 μs. Ramps were given every 2 s (−100 to +100 mV in 50 ms) and cells were held at 0 mV between ramps. All currents were leak subtracted by averaging the ten ramps obtained just prior to thimerosal application, and then subtracting this from all subsequent traces. Extracellular solution changes were made by local pressure application from a wide-tipped micropipette placed within 20 μm of the cell.

For single-cell Ca^{2+} measurements, coverslips were incubated in normal saline solution (mM: NaCl, 140; KCl, 2·8; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 11; Hepes–NaOH, 10; pH 7·2) to which 5 μM fura-2 AM had been added. After 30 min, the coverslips were washed 5 times in normal saline solution and then incubated at 37 °C for 15 min. Single-cell Ca^{2+} levels were measured using a photomultiplier-based system as previously described (Neher, 1989). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated from the fluorescence ratio (360/390) as described (Neher, 1989).

InsP_3 was purchased from Amersham, fura-2 AM was from Molecular Probes and all other chemicals were from Sigma.

All results are means ± S.E.M.

RESULTS

Thimerosal mobilizes Ca^{2+} from internal stores in RBL cells

To see whether thimerosal was capable of releasing Ca^{2+} from intracellular stores in RBL cells, we applied 1 μM thimerosal to single fura-2 AM-loaded cells. Figure 1*A* shows the responses of three individual cells to application of thimerosal in Ca^{2+}-free solution. There is a small elevated baseline Ca^{2+} in between spikes, as seen in the upper and lower panels of Fig. 1*A*. A simple explanation for this is that there is a gradual increase in the sensitivity of InsP_3 receptors to InsP_3. As more receptors now start releasing Ca^{2+} from intracellular stores, there is a slow increase in basal Ca^{2+} which further sensitizes the InsP_3 receptors, resulting in regenerative release. The transient spikes would ride on top of the slightly elevated basal Ca^{2+} level. Figure 1*B* shows responses from three different cells to thimerosal applied in the presence of 1 mM extracellular Ca^{2+}. Prominent Ca^{2+} plateaux were now observed. Hence thimerosal can both release Ca^{2+} from internal stores and evoke Ca^{2+} influx in RBL cells, in agreement with the notion that it modifies the sensitivity of the InsP_3 receptor (Missiaen *et al.* 1991; Bootman *et al.* 1992; Poitras *et al.* 1993; Hilly *et al.* 1993). One interesting observation was the marked decrease in the delay before the onset of the Ca^{2+} signal in the presence of extracellular Ca^{2+} compared with that observed in its absence (compare panels *A* and *B* of Fig. 1). Although not investigated further, one possible explanation is that basal InsP_3 levels are higher in the presence of external Ca^{2+} than in its absence, thereby enabling more rapid Ca^{2+} release. Alternatively, the ability of thimerosal to permeate the cell membrane might be facilitated by external Ca^{2+}. Finally, it is likely that different InsP_3 stores will be depleted by thimerosal at different rates depending on their location, the local InsP_3 concentration, the density of the InsP_3 receptors and their affinity for InsP_3. Stores depleted first will activate capacitive Ca^{2+} influx and this extracellular Ca^{2+} contribution to the Ca^{2+} signal could overlap with the Ca^{2+} release phase of other, slower stores.

Thimerosal activates I_{CRAC}

Since thimerosal application resulted in Ca^{2+} influx, we examined whether it was able to activate I_{CRAC} in whole-cell patch clamp recordings. Thimerosal (1 μM) was applied 30 s after obtaining the whole-cell configuration. After a variable latency (169 ± 31 s), an inward current developed with an activation time constant (τ) similar (only 1·4-fold slower) to I_{CRAC} activated by both a supramaximal concentration of InsP_3 and ionomycin (Fig. 2*A* and Table 1). This current was identified as I_{CRAC} on the basis of its strong inward rectification, voltage-independent gating, positive reversal potential (> +30 mV) and dependency on extracellular Ca^{2+} (lost when Ca^{2+} was reduced from 10 mM to 100 μM). In fact, I_{CRAC} activated by thimerosal was indistinguishable from I_{CRAC} activated by either InsP_3 or ionomycin (Table 1). The peak amplitude of I_{CRAC} evoked by 1 μM thimerosal was not significantly different from that evoked by 60 μM InsP_3 ($-3{\cdot}1 \pm 0{\cdot}4$ and $-2{\cdot}9 \pm 0{\cdot}2$ pA pF^{-1}, $n = 6$ and 19, respectively; Table 1).

To see whether thimerosal activated I_{CRAC} through the InsP_3 receptor, we dialysed cells with 1 mg ml^{-1} heparin, a competitive inhibitor of the InsP_3 receptor. Thimerosal application (1 μM), 30 s after obtaining the whole-cell configuration, now failed to activate I_{CRAC}, demonstrating that thimerosal was indeed acting through the InsP_3 receptor (Fig. 2*B*, 4 out of 4 cells). Dialysis of cells with 1 mg ml^{-1} heparin had no effect on the ability of ionomycin to activate I_{CRAC}, demonstrating that heparin was not interfering either with I_{CRAC} itself nor with its mechanism of activation.

Activation of I_{CRAC} by thimerosal is not lost when cells are extensively dialysed for a long time

As described in the previous section, applying thimerosal after 30 s of dialysis evoked I_{CRAC}, which had a peak amplitude of $-3{\cdot}1 \pm 0{\cdot}4$ pA pF^{-1} ($n = 6$; Fig. 2*C* and Table 1). If thimerosal was applied after 300 s of dialysis, I_{CRAC} was still activated (after a latency of 130 ± 24 s) and the current had a similar τ of activation and peak amplitude as I_{CRAC} activated after only 30 s of dialysis ($-3{\cdot}3 \pm 0{\cdot}8$ pA pF^{-1}, $n = 3$ cells; Fig. 2*C*). When cells were dialysed first for 600 s and then thimerosal applied, I_{CRAC} was still activated (after a latency of 159 ± 42 s, $n = 8$) and the current had a similar τ of activation and reached a similar peak amplitude to I_{CRAC} activated after 30 s of dialysis ($-3{\cdot}0 \pm 0{\cdot}3$ pA pF^{-1} for 600 s dialysis compared with $-3{\cdot}1 \pm 0{\cdot}4$ pA pF^{-1} for 30 s; Fig. 2*C* and Table 1). In fact, when thimerosal was applied after 600 s,

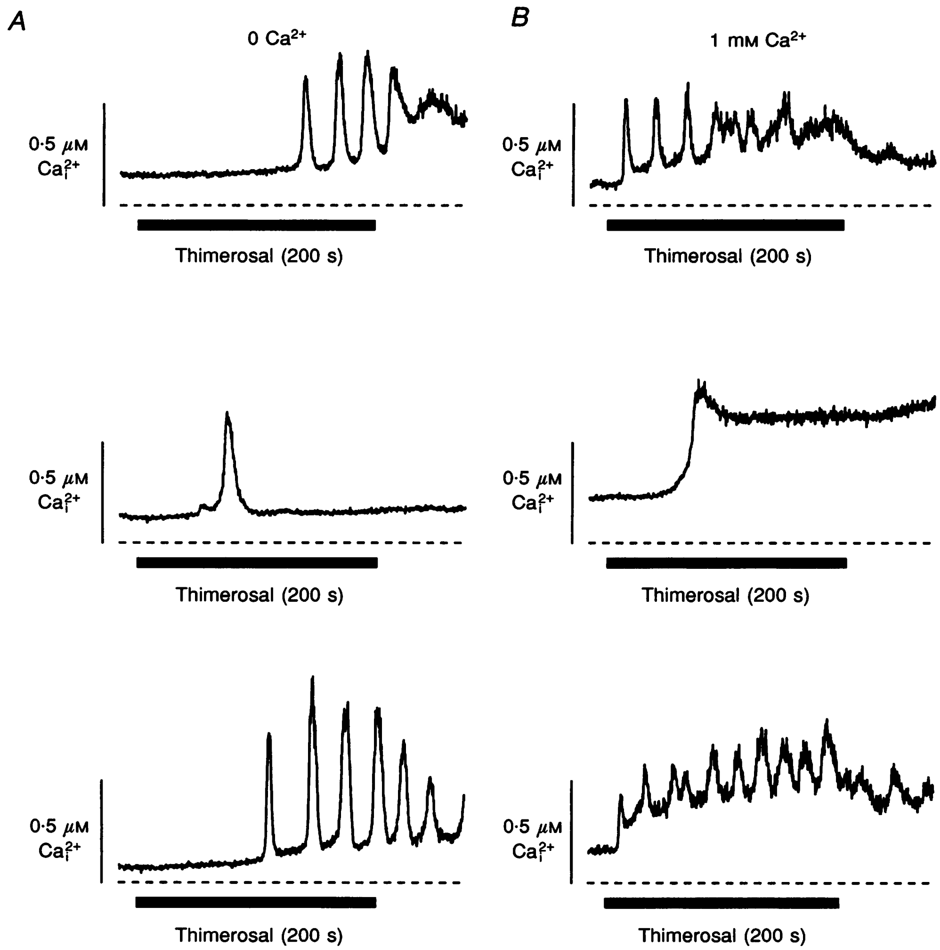

Figure 1. Thimerosal both releases Ca^{2+} from intracellular stores and causes Ca^{2+} influx in fura-2 AM-loaded single cells

A shows responses of 3 individual cells to application of 1 μM thimerosal for 200 s in nominally Ca^{2+}-free solution. In *B*, thimerosal was applied to 3 different cells in the presence of 1 mM external Ca^{2+}. In this case, prominent Ca^{2+} influx occurred.

there was a substantial latency of up to 300 s in some cells before the current activated. Hence, even after almost 900 s of dialysis, activation of I_{CRAC} was unaffected compared with activation of the current at much earlier times, despite thimerosal working through the InsP_3 receptor and there being no added InsP_3 in the patch pipette.

The preceding results using thimerosal demonstrate that the activation mechanism of I_{CRAC} does not wash out of the cell during extensive dialysis. The lack of washout was also evident when stores were depleted after various times of dialysis using ionomycin for depleting the stores. Administration of ionomycin after 30 s ($n = 4$), 300 s ($n = 8$) or 600 s ($n = 5$) of dialysis evoked identical currents with similar peak amplitudes and τ of activation (R_s was 3–6 MΩ for all experiments). These values are very similar to the peak I_{CRAC} observed after breaking into the cell with 60 μM InsP_3 ($-2{\cdot}9 \pm 0{\cdot}2$ pA pF^{-1}; Table 1) which activates within a few seconds and therefore when there is minimal washout. Hence lack of washout is observed independently of the method used to activate I_{CRAC}.

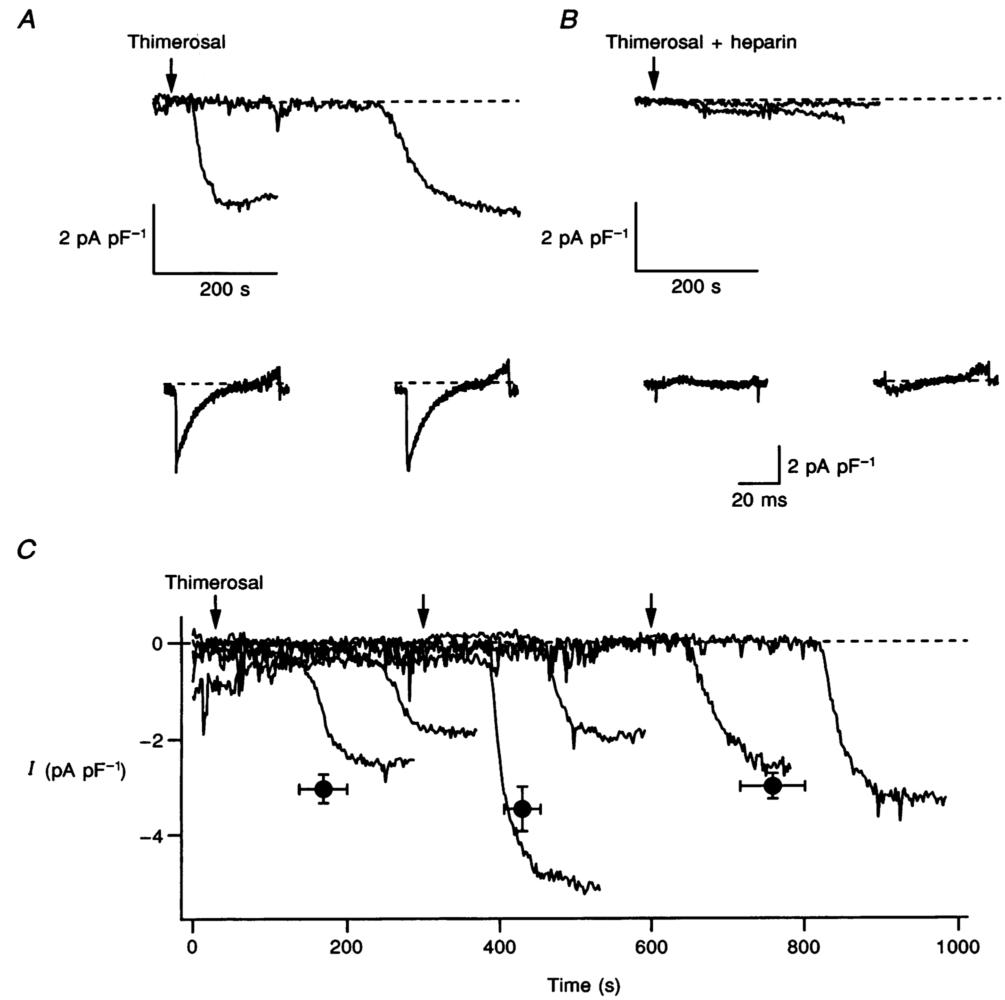

Figure 2. Thimerosal activates I_{CRAC} in whole-cell patch clamp recordings

A, 1 μM thimerosal was applied 30 s after obtaining the whole-cell configuration. The upper panel depicts the development of the inward current following thimerosal application for 2 different cells at −80 mV. The lower panel shows the currents (taken when I_{CRAC} had reached a peak) measured in voltage ramps (−100 to +100 mV in 50 ms). The ramp currents were identical to ramp currents in response to InsP_3 or ionomycin. In *B*, cells were dialysed with 1 mg ml^{-1} heparin. Under these conditions, thimerosal (1 μM) did not activate I_{CRAC}. The lower panel shows voltage ramps when the thimerosal-induced currents had peaked. In *C*, 1 μM thimerosal was applied at either 30, 300 or 600 s after obtaining the whole-cell mode (arrows), and development of I_{CRAC} at −80 mV is plotted as a function of time. Two cells for each application time are shown. Included in the plot is the mean amplitude of I_{CRAC} for all cells (●). The vertical standard error bars reflect the amplitudes whereas the horizontal bars correspond to the varying latencies of development of I_{CRAC} for each time of application.

DISCUSSION

The data presented in this study demonstrate that the sulfhydryl agent thimerosal is able to activate I_{CRAC}. There are several possible mechanisms whereby thimerosal can achieve this. First, thimerosal has been reported to inhibit the Ca^{2+}-ATPase of the endoplasmic reticulum (Sayers, Brown, Michell & Michelangeli, 1993). By blocking this pump, stores will be depleted, thereby activating I_{CRAC}. This is unlikely to be the main action of thimerosal under our conditions because first, dialysing cells with 1 μM thapsigargin (a powerful Ca^{2+}-ATPase inhibitor) activated I_{CRAC} in only around 50% of the cells (A. B. Parekh & R. Penner, unpublished), whereas thimerosal was effective in all cells. Second, in Ca^{2+}-free solution, thimerosal evoked repetitive Ca^{2+} oscillations. However, we were never able to evoke such oscillations simply by blocking the Ca^{2+}-ATPase with thapsigargin (10 nM to 1 μM, not shown).

Another possibility could be that thimerosal increases InsP_3 levels by either stimulating phospholipase C or preventing InsP_3 breakdown. Although the effects of thimerosal on phospholipase C activity have not been investigated in RBL cells, thimerosal does not stimulate InsP_3 production in HeLa cells (Bootman *et al.* 1992) or gonadotrophs (Stojkilovic, Tomic, Kukuljan & Catt, 1994). Similarly, thimerosal has no effect on InsP_3 metabolism in cerebellar microsomes (Sayers *et al.* 1993).

Many channels are gated by the redox potential (Ruppersberg, Stocker, Pongs, Heinemann, Frank & Koenen, 1991), raising the possibility that thimerosal might directly reduce CRAC channels such that they become active. An argument against this direct mechanism is that heparin (which inhibits the InsP_3 receptor) and neomycin (which inhibits phospholipase C, data not shown) both prevented thimerosal from activating I_{CRAC}, thus demonstrating a requirement for InsP_3.

Numerous studies have documented that thimerosal increases the sensitivity of the InsP_3 receptor for InsP_3 (Missiaen *et al.* 1991; Bootman *et al.* 1992; Poitras *et al.* 1993; Hilly *et al.* 1993). Our findings that thimerosal required both InsP_3, and InsP_3 binding to the InsP_3 receptor in order to activate I_{CRAC} can most readily be explained by this mechanism. Thimerosal presumably alters the sensitivity, such that resting InsP_3 is able to deplete stores and thereby activate I_{CRAC}.

InsP_3 has a lifetime of around 1 s in the cytosol (Kasai & Petersen, 1994), and would diffuse out of the cytosol into the pipette with a time constant of 30 s (with our average R_s of 4 MΩ; Pusch & Neher, 1988). It is therefore intriguing that thimerosal was able to activate I_{CRAC} through a process critically dependent on the presence of InsP_3, despite dialysing the cell for more than 600 s with a solution lacking InsP_3. In practically all studies where it has been investigated, thimerosal increases the sensitivity of the InsP_3 receptor only 2- to 5-fold (Bootman *et al.* 1992; Poitras *et al.* 1993; Hilly *et al.* 1993). We have found that an InsP_3 concentration of around 3 μM needs to be included in the patch pipette in order to activate I_{CRAC} (Parekh & Penner, 1995). If thimerosal increases the sensitivity of the InsP_3 receptor 5-fold in RBL cells (the largest increase we could find for other non-excitable cells), this would mean that a global concentration of around 600 nM InsP_3 would be required to fully activate I_{CRAC}, and this level would have to be supplied by basal InsP_3 turnover. It is not clear how, after extensive dialysis with solutions lacking InsP_3, resting InsP_3 can be of this order, especially since these levels can release substantial Ca^{2+} in permeabilized RBL cells (Meyer & Stryer, 1990).

One possible explanation for this apparent paradox is that thimerosal might induce a large shift in the sensitivity of the InsP_3 receptor, rather than the modest shift seen in other non-excitable cells. In this regard, the InsP_3 receptor in RBL cells would have to be unusual compared with other cell types. A more likely explanation would be that under our conditions, stores closer to the plasma membrane contribute to I_{CRAC} activation more than stores further away. Although InsP_3 is a global messenger and can reach a steady-state level rapidly in the cytosol, stores close to the membrane will experience higher InsP_3 levels than more distant stores, since InsP_3 is produced at the plasma membrane. Hence a modest increase in InsP_3 sensitivity by thimerosal might be sufficient for basal InsP_3 to deplete these proximal stores. Discrimination between these two possibilities will require detailed biochemical studies of the InsP_3 receptor in RBL cells.

The results obtained with thimerosal demonstrate that any factor that changes the affinity of the InsP_3 receptor is likely to have important effects on Ca^{2+} influx. Regulation of the InsP_3 receptor might be a very powerful way of indirectly controlling Ca^{2+} entry.

A previous study observed a loss of I_{CRAC} as a function of dialysis time in whole-cell patch clamp experiments and concluded that the activation mechanism of I_{CRAC} washed out of RBL cells with a τ of 250 s, for an R_s of 6·7 MΩ, when stores were depleted by either applying ionomycin or photolysing caged InsP_3 (Fasolato *et al.* 1993). In our experiments we did not see any washout of the activation of I_{CRAC} evoked by applying thimerosal at different times of dialysis. I_{CRAC} activated after 30 s dialysis was identical to that activated after 600 s dialysis (R_s values were 4·24 ± 0·18 and 3·96 ± 0·13 MΩ, respectively). In fact, these currents were identical to I_{CRAC} activated by including high InsP_3 in the pipette (60 μM; Parekh & Penner, 1995), which activates within a few seconds and hence when there is minimal dialysis. Similar results were obtained when stores were depleted using ionomycin. The reason why we did not see any washout may be due to the different recording conditions. In this study, we used EGTA as the Ca^{2+} chelator, whereas BAPTA was used in the aforementioned report (Fasolato *et al.* 1993). BAPTA pharmacologically prevents kinase-mediated inactivation of I_{CRAC} (Parekh & Penner, 1995), and it is conceivable that BAPTA somehow interferes with the activation mechanism. The other difference between our conditions and those of the previous report is that we used 2 mM ATP, as opposed to 0·5 mM (Fasolato *et al.* 1993). Since the activation of capacitive Ca^{2+} influx is very sensitive to ATP levels (Gambrerucci *et al.* 1994), it may be that higher global ATP is required in whole-cell recordings to sustain the current.

Our findings that the activation mechanism of I_{CRAC} does not wash out of the cell with time suggests that the activation mechanism is unlikely to encompass a freely diffusible, small molecule that pre-exists in the cytosol. Instead the activation mechanism is either a large molecule (a big protein), a small molecule that is bound to a large one or to an organelle, or a molecule compartmentalized within a store and released into the cytosol only after depletion of stores.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–325.

Bootman, M. D., Taylor, C. W. & Berridge, M. J. (1992). The thiol reagent, thimerosal, evokes Ca^{2+} spikes in HeLa cells by sensitizing the inositol 1,4,5-trisphosphate receptor. *Journal of Biological Chemistry* **267**, 25113–25119.

Fasolato, C., Hoth, M. & Penner, R. (1993). A GTP-dependent step in the activation mechanism of capacitative calcium influx. *Journal of Biological Chemistry* **268**, 20737–20740.

Fasolato, C., Innocenti, B. & Pozzan, T. (1994). Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? *Trends in Pharmacological Sciences* **15**, 77–83.

Gambrerucci, A., Innocenti, B., Fulceri, R., Banhegyi, G., Giunti, R., Pozzan, T. & Benedetti, A. (1994). Modulation of Ca^{2+} influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. *Journal of Biological Chemistry* **269**, 23597–23602.

Hamill, O., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

Hilly, M., Pietri-Rouxel, F., Coquil, J. F., Guy, M. & Mauger, J. P. (1993). Thiol reagents increase the affinity of the inositol 1,4,5-trisphosphate receptor. *Journal of Biological Chemistry* **268**, 16488–16494.

Hoth, M. & Penner, R. (1992). Depletion of intracellular stores activates a calcium current in mast cells. *Nature* **355**, 353–356.

Hoth, M. & Penner, R. (1993). Calcium release-activated calcium current in rat mast cells. *Journal of Physiology* **465**, 358–388.

Kasai, H. & Petersen, O. H. (1994). Spatial dynamics of second messengers: IP_3 and cAMP as long-range and associative messengers. *Trends in Neurosciences* **17**, 95–101.

Meyer, T. & Stryer, L. (1990). Transient calcium release by successive increments of inositol 1,4,5-trisphosphate. *Proceedings of the National Academy of Sciences of the USA* **87**, 3841–3845.

Missiaen, L., Taylor, C. W. & Berridge, M. J. (1991). Luminal Ca^{2+} promoting spontaneous Ca^{2+} release from inositol trisphosphate-sensitive stores in rat hepatocytes. *Nature* **352**, 241–244.

Neher, E. (1989). Combined fura-2 and patch-clamp measurements in rat peritoneal mast cells. In *Neuromuscular Junction*, ed. Sellin, L. C., Liberius, R. & Thesleff, S., pp. 65–76. Elsevier, Amsterdam.

Parekh, A. B. & Penner, R. (1995). Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. *Proceedings of the National Academy of Sciences of the USA* (in the Press).

Poitras, M., Bernier, S., Servant, M., Richard, D. E., Boulay, G. & Guillemette, G. (1993). The high affinity state of inositol 1,4,5-trisphosphate receptor is a functional state. *Journal of Biological Chemistry* **268**, 24078–24082.

Pusch, M. & Neher, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Archiv* **411**, 204–211.

Putney, J. W. Jr (1986). A model for receptor-regulated calcium entry. *Cell Calcium* **7**, 1–12.

Ruppersberg, J. P., Stocker, M., Pongs, O., Heinemann, S. H., Frank, R. & Koenen, M. (1991). Regulation of fast inactivation of cloned mammalian $I_K(A)$ channels by cysteine oxidation. *Nature* **352**, 711–714.

Sayers, L. G., Brown, G. R., Michell, R. H. & Michelangeli, F. (1993). The effects of thimerosal on calcium uptake and inositol 1,4,5-trisphosphate-induced calcium release in cerebellar microsomes. *Biochemical Journal* **289**, 883–887.

Stojkilovic, S. S., Tomic, M., Kukuljan, M. & Catt, K. J. (1994). Control of calcium spiking frequency in pituitary gonadotrophs by a single-pool cytoplasmic oscillator. *Molecular Pharmacology* **45**, 1013–1021.

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