Role of the Inositol 1,4,5-Trisphosphate Receptor in Ca\(^{2+}\) Feedback Inhibition of Calcium Release-activated Calcium Current (\(I_{\text{crac}}\))

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We examined the activation and regulation of calcium release-activated calcium current (\(I_{\text{crac}}\)) in RBL-1 cells in response to various Ca\(^{2+}\) store-depleting agents. With [Ca\(^{2+}\)], strongly buffered to 100 nm, \(I_{\text{crac}}\) was activated by ionomycin, thapsigargin, inositol 1,4,5-trisphosphate (IP\(_3\)), and two metabolically stable IP\(_3\) receptor agonists, adenophostin A and \(\alpha\)-glycerophosphate-d-myoinositol-4,5-bisphosphate (GPIP\(_2\)). With minimal [Ca\(^{2+}\)] buffering, with [Ca\(^{2+}\)] free to fluctuate \(I_{\text{crac}}\) was activated by ionomycin, thapsigargin, and by the potent IP\(_3\) receptor agonist, adenophostin A, but not by GPIP\(_2\) or IP\(_3\) itself. Likewise, when [Ca\(^{2+}\)] was strongly buffered to 500 nm, ionomycin, thapsigargin, and adenophostin A did and GPIP\(_2\) and IP\(_3\) did not activate detectable \(I_{\text{crac}}\). However, with minimal [Ca\(^{2+}\)] buffering, or with [Ca\(^{2+}\)] buffered to 500 nm, GPIP\(_2\) was able to fully activate detectable \(I_{\text{crac}}\) if uptake of Ca\(^{2+}\) intracellular stores was first inhibited. Our findings suggest that when IP\(_3\) activates the IP\(_3\) receptor, the resulting influx of Ca\(^{2+}\) quickly inactivates the receptor, and Ca\(^{2+}\) is re-accumulated at sites that regulate \(I_{\text{crac}}\). Adenophostin A, by virtue of its high receptor affinity, is resistant to this inactivation. Comparison of thapsigargin-releasable Ca\(^{2+}\) pools following activation by different IP\(_3\) receptor agonists indicates that the critical regulatory pool of Ca\(^{2+}\) may be very small in comparison to the total IP\(_3\)-sensitive component of the endoplasmic reticulum. These findings reveal new and important roles for IP\(_3\) receptors located on discrete IP\(_3\)-sensitive Ca\(^{2+}\) pools in calcium feedback regulation of \(I_{\text{crac}}\) and capacitative calcium entry.

Many extracellular stimuli act through cell surface receptors to promote generation of intracellular inositol 1,4,5-trisphosphate (IP\(_3\))^1 and consequently release intracellular Ca\(^{2+}\) stores (1). The release of stored Ca\(^{2+}\) is commonly accompanied by influx of Ca\(^{2+}\) from the extracellular space, through the “capacitative Ca\(^{2+}\)” pathway (2–4). Although depletion of intracellular stores and activation of capacitative calcium entry are inextricably linked, the underlying mechanism remains poorly defined (4).

Store-operated currents, which are proposed to mediate capacitative calcium entry, have been measured in various cell types (5). To date, “Ca\(^{2+}\)” release-activated Ca\(^{2+}\)” current (\(I_{\text{crac}}\)) is the best-defined member of the store-operated current family (6). \(I_{\text{crac}}\) measured in mast cells, T-lymphocytes, and rat basophilic leukemia (RBL-1) cells is both highly selective for Ca\(^{2+}\) as the permeant ion and strongly inhibited by Ca\(^{2+}\) feedback (5, 7–9). At least two forms of Ca\(^{2+}\)”-dependent inactivation of \(I_{\text{crac}}\) have been reported, termed fast and slow inactivation. Fast inactivation occurs on a sub-second time scale and is caused by increases in sub-plasmalemmal Ca\(^{2+}\) concentration in the vicinity of the Ca\(^{2+}\)” channel (7, 9). Slow inactivation, on the time scale of tens to hundreds of seconds, is caused by increases in the bulk cytosolic [Ca\(^{2+}\)] and is partly dependent on refilling of intracellular Ca\(^{2+}\)” stores (8). In order to maximize, or indeed detect, the typically small whole-cell CRAC currents, it is necessary to minimize these forms of Ca\(^{2+}\)” feedback.

First, the [Ca\(^{2+}\)] is tightly buffered to basal levels with high concentrations of Ca\(^{2+}\)” chelators such as BAPTA or EGTA. Second, the cell membrane potential is held at depolarized levels to decrease the driving force for Ca\(^{2+}\)” entry between current measurements. A major disadvantage of these conditions is the inability to measure simultaneous changes in [Ca\(^{2+}\)].

Recently, simultaneous measurements of [Ca\(^{2+}\)] and \(I_{\text{crac}}\) have been reported in RBL-1 cells, an immortalized mast cell line (10). In this study, the degree of Ca\(^{2+}\)” buffering was reduced from a level which effectively clamps [Ca\(^{2+}\)] to basal concentrations, to a minimal level, which permitted fluctuations of [Ca\(^{2+}\)]. Under these conditions, adenophostin A, an IP\(_3\) receptor agonist with 100-fold higher affinity for the IP\(_3\) receptor compared with the native ligand IP\(_3\) (11) induced Ca\(^{2+}\)” release and activation of \(I_{\text{crac}}\) (10). Several other agents that would be expected to deplete intracellular stores, including analogues of IP\(_3\) ([2,4,5]IP\(_3\), 3-deoxy-3-fluoro-IP\(_3\)), thapsigargin (a SERCA inhibitor), and ionomycin (a Ca\(^{2+}\)” ionophore), did not activate \(I_{\text{crac}}\) under these conditions. It was suggested that the unique ability of adenophostin A to activate \(I_{\text{crac}}\) was perhaps due to action at the level of Ca\(^{2+}\)”-dependent inactivation of the CRAC channel.

In the present study we investigated activation of \(I_{\text{crac}}\) in RBL-1 cells under conditions of weak Ca\(^{2+}\)” buffering, while simultaneously monitoring [Ca\(^{2+}\)]. We compared the ability of various store depletion agents and combinations of these agents to induce a rise in [Ca\(^{2+}\)] and to activate detectable \(I_{\text{crac}}\). We now find that adenophostin A, thapsigargin, and ionomycin all activate \(I_{\text{crac}}\) under low buffering conditions; however, IP\(_3\) and a stable analogue of IP\(_3\), \(\alpha\)-glycerophosphate-d-myoinositol-4,5-bisphosphate (GPIP\(_2\)), do not. This failure was not the result of Ca\(^{2+}\)” feedback on the CRAC channel. Rather, deactivation of the current appears due to inactivation of IP\(_3\) receptors and rapid refilling of critical Ca\(^{2+}\)” stores thereby diminishing the signal for activation of \(I_{\text{crac}}\). This work reveals a new mechanism that involves the IP\(_3\) receptor for Ca\(^{2+}\)”

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§The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; \(I_{\text{crac}}\), Ca\(^{2+}\)” release-activated Ca\(^{2+}\)” current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; GPIP\(_2\), \(\alpha\)-glycerophosphate-d-myoinositol-4,5-bisphosphate; HBSS, HEPES-buffered saline solution; 1These are limited to the Ca\(^{2+}\)”-dependent Ca\(^{2+}\)” current; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; GPIP\(_2\), \(\alpha\)-glycerophosphate-d-myoinositol-4,5-bisphosphate; HBSS, HEPES-buffered saline solution;
feedback on \( I_{\text{crac}} \). These \( I_{\text{crac}} \) receptors are likely located in spatially restricted regions within the endoplasmic reticulum and are closely coupled to activation and regulation of \( I_{\text{crac}} \).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Rat basophilic leukemia cells (RBL-1) were purchased from the ATCC (1578-CRL). Cells were cultured in Earle’s minimal essential medium with Earle’s salts, 10% fetal bovine serum, 2 mM \( L \)-glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin (37 °C, 5% \( CO_2 \)). For experiments, cells were passaged onto glass coverslips (number 1.5) and used 12-36 h after plating.

**Fura-2 Loading and Fluorescence Measurements**—Coverslips with attached cells were mounted in a Teflon chamber and incubated at room temperature for 25 min in HEPES-buffed saline solution (HBSS; in mM, 140 NaCl, 4.7 KCl, 1.8 CaCl\(_2\), 1.5 MgCl\(_2\), 10 glucose, and 10 HEPES, pH 7.2) containing 1 \( \mu \)M Fura-2 AM (Molecular Probes). Cells were then washed and bathed in HBSS for at least 10 min before \( Ca^{2+} \) measurements were made.

Fluorescence was monitored by placing the Teflon chamber with the coverslip of Fura-2-loaded cells onto the stage of a Nikon Diaphot microscope (40X Neofluor objective). Cells were excited by light (340 and 380 nm) from a Deltasonic D101 (Photon Technology International Ltd.) light source equipped with a light path chopper and dual excitation monochromators. Emitted fluorescence (510 nm) was collected by a photomultiplier tube (Omega). All experiments were conducted at room temperature (22 °C). Calibration of \([Ca^{2+}]_i\) was performed by reference to a look-up table created from \( Ca^{2+} \) standards supplied by Molecular Probes.

**Electrophysiology**—Patch clamp experiments were conducted in the standard whole-cell recording configuration (12). Patch pipette (2–4 megaohm, Corning glass, 7052) solutions contained (in mM) 140 cesium aspartate, 2 MgCl\(_2\), 10 HEPES, 1 MgATP, and either 10 BAPTA-Cs\(_4\) (with free \( Ca^{2+} \) set to 100 or 500 nm, calculated using MaxChelator software (version 6.60) or 0.1 BAPTA-C\(_4\) (with no \( Ca^{2+} \) added), pH 7.2. Fura-2 free acid (50 \( \mu \)M) was included in the pipette as indicated. Bath solution (HBSS) was as described above, except CaCl\(_2\) was increased to 10 mM for \( Ca^{2+} \)-free HBSS or omitted for nominally \( Ca^{2+} \)-free HBSS (10 mM MgCl\(_2\) was included in nominally \( Ca^{2+} \)-free HBSS). 0.2 mM EGTA was included where indicated.

In all experiments, upon forming the whole-cell configuration the cell membrane potential was held at +30 mV (to minimize \( Ca^{2+} \) entry and \( Ca^{2+} \)-dependent inactivation of CRAC channels). Periodically (every 5 s) the membrane potential was stepped to –100 mV (for 20 ms to assess \( I_{\text{crac}} \)), and then a voltage ramp to +60 mV, over a period of 160 ms, was applied. Currents are normalized to membrane capacitance. All voltages are corrected for a 10 mV liquid-junction potential. Membrane potentials are closely coupled to activation and regulation of \( I_{\text{crac}} \).

**Materials**—Adenophostin A was a gift from Drs. M. Takahashi and K. Tanzawa (Sankyo Co., Ltd., Tokyo, Japan). IP\(_3\) and ionomycin were from Calbiochem. Thapsigargin was from LC Laboratories, and GPIP\(_2\) (100 or 200 nm) from Roche Molecular Biochemicals. Cs\(_4\)BAPTA (1,2-bis(2-amino-9-methyl-9H-xanthen-6-yloxy)ethane-N,N,N',N'-tetraacetic acid) and Fura-2 were from Molecular Probes (Eugene, OR).
Ca \(^{2+}\). The currents also showed inward rectification and reversed direction at a potential positive of \(+30\) mV (not shown).

Under these conditions adenophostin A (1.08 \pm 0.12 \mu M), thapsigargin (1.05 \pm 0.14 \mu M), and ionomycin (1.29 \pm 0.13 \mu M) caused similar increases in \(I_{\text{crac}}\) (Fig. 2F), but all of the values were significantly less than those measured with strong Ca \(^{2+}\) buffering (Fig. 1C), being 45 \pm 5, 43 \pm 6, and 46 \pm 3\%, respectively.

Following break in with pipettes containing adenophostin A, or following application of ionomycin, activation of \(I_{\text{crac}}\) occurred after short delays of 49 \pm 11 and 88 \pm 9 s, respectively (Fig. 2, A and E). However, there was a considerably longer delay between the delivery of thapsigargin and subsequent activation of \(I_{\text{crac}}\) (181 \pm 19 s) (Fig. 2D). \(I_{\text{crac}}\) was activated by thapsigargin with a mean development time (time from the initial increase in current to development of current; see Ref. 13)) of 375 \pm 35 s, considerably slower than that for adenophostin A (132 \pm 25 s) or ionomycin (145 \pm 22 s) (Fig. 2F, open bars). The initial rise in Ca \(^{2+}\) induced by thapsigargin indicates blockade of endoplasmic reticulum Ca \(^{2+}\)-ATPases and the subsequent leak of Ca \(^{2+}\) from intracellular stores. The delay before detectable \(I_{\text{crac}}\) activation and the slow time course of development presumably reflect the time it takes to empty, to the necessary degree, the stores linked to \(I_{\text{crac}}\) activation. It is because of this delayed and slow activation of \(I_{\text{crac}}\) with thapsigargin that our earlier study failed to detect activation of \(I_{\text{crac}}\) by thapsigargin in RBL-1 cells (10). Also, in our earlier study (10) ionomycin failed to activate \(I_{\text{crac}}\). However, a substantially higher concentration of ionomycin was used (5 \mu M, as opposed to 500 nM in the current study), and at this concentration ionomycin raises [Ca \(^{2+}\)]\text{c}\) to extremely high levels, in excess of 1 \mu M, which likely caused a strong Ca \(^{2+}\)-dependent inactivation of \(I_{\text{crac}}\).

**Activation of \(I_{\text{crac}}\) at Elevated [Ca \(^{2+}\)]\text{c}\)**—We next considered how minimal Ca \(^{2+}\) buffering might prevent \(I_{\text{crac}}\) and GPIP2 from activating detectable \(I_{\text{crac}}\). Direct effects of global [Ca \(^{2+}\)]\text{c}\) on CRAC channels is unlikely to be responsible, because the agents that activated \(I_{\text{crac}}\) actually raised steady-state [Ca \(^{2+}\)]\text{c}\) to somewhat higher levels than did IP\(_{3}\) or GPIP2. Thus, either IP\(_{3}\) and GPIP2 are capable of raising Ca \(^{2+}\) to higher levels than other agents in small, discrete regions close to the CRAC channels or, alternatively, \(I_{\text{crac}}\) activated by IP\(_{3}\) and GPIP2 is for some reason more sensitive to Ca \(^{2+}\) inhibition. Thus, we studied the effect of increased cytosolic Ca \(^{2+}\) on \(I_{\text{crac}}\) under conditions whereby both sub-plasmalemmal and global [Ca \(^{2+}\)]\text{c}\) were strongly buffered with 10 mM BAPTA. We buffered [Ca \(^{2+}\)]\text{c}\) to either a basal value ([Ca \(^{2+}\)]\text{c} = 100 nM) or a value ([Ca \(^{2+}\)]\text{c} = 500 nM) similar to that recorded in weakly buffered cells after activation with store depletion agents (Fig. 2E).

Under these conditions (10 mM BAPTA, [Ca \(^{2+}\)]\text{c} = 500 nM), adenophostin A (Fig. 3A), thapsigargin (Fig. 3D), and ionomycin (Fig. 3E) activated \(I_{\text{crac}}\). IP\(_{3}\) (Fig. 3B) and GPIP2 (Fig. 3C) did not. Thus, IP\(_{3}\) and GPIP2 apparently fail to activate \(I_{\text{crac}}\) because with these agents the signaling mechanism is more sensitive to inhibition by elevated [Ca \(^{2+}\)]\text{c}\).

Adenophostin A (2.18 \pm 0.03 pA/pF), thapsigargin (2.57 \pm 0.65 pA/pF), and ionomycin (2.95 \pm 0.45 pA/pF) caused similar increases in \(I_{\text{crac}}\). These values were also similar to measurements made with free Ca \(^{2+}\) buffered to 100 nM (Fig. 1C), being 90 \pm 3, 104 \pm 26, and 105 \pm 20\%, respectively. It is worth noting, however, that although the peak magnitude of \(I_{\text{crac}}\) measured with free Ca \(^{2+}\) set to 100 or 500 nM was similar, a slow inactivation of the current was prominent at the higher cytosolic [Ca \(^{2+}\)]. \(I_{\text{crac}}\) activated by adenophostin A fell to 90 \pm 12 and 56 \pm 9\% of the peak after 300 s, at 100 and 500 nM free Ca \(^{2+}\), respectively. Over the same period, \(I_{\text{crac}}\) activated by ionomycin also declined more at 500 nM, falling to 62 \pm 9\% of the peak compared with 87 \pm 11\% with 100 nM free Ca \(^{2+}\).

The mean development time of \(I_{\text{crac}}\) for thapsigargin at 500 nM [Ca \(^{2+}\)]\text{c} = 415 \pm 27 s) was again much slower than for adenophostin A (94 \pm 8 s) and ionomycin (68 \pm 5 s). There was also a considerable delay before thapsigargin induced detectable activation of \(I_{\text{crac}}\) (295 \pm 34 s) compared to when free [Ca \(^{2+}\)]\text{c} was set to 100 nM (91 \pm 14 s) (Fig. 3D). For both adenophostin A and ionomycin, activation of \(I_{\text{crac}}\) was seen after only a short delay (42 \pm 12 and 88 \pm 9 s, respectively).

**Role of the IP\(_{3}\) Receptor and SERCA Pumps—Buffering sub-plasmalemmal [Ca \(^{2+}\)]**—Buffering sub-plasmalemmal [Ca \(^{2+}\)] gradients (10 mM BAPTA), and raising bulk cytosolic [Ca \(^{2+}\)] (500 nM), mimicked some of the effects of low Ca \(^{2+}\) buffering, namely failure of GPIP2 and IP\(_{3}\) to activate \(I_{\text{crac}}\) and slow activation of \(I_{\text{crac}}\) by thapsigargin. Given that adenophostin A, ionomycin, and thapsigargin all activate \(I_{\text{crac}}\) under conditions where IP\(_{3}\) and GPIP2 are ineffective, direct Ca \(^{2+}\)-dependent inactivation of the CRAC channel seems unlikely to be the cause. Rather, an involvement of the IP\(_{3}\) receptor itself would be indicated. Thus, \(I_{\text{crac}}\) could be inactivated if, when IP\(_{3}\) and GPIP2 are used, the IP\(_{3}\) receptors were desensitized, and as a result the stores signaling \(I_{\text{crac}}\) activation were refilled. Increases in [Ca \(^{2+}\)]\text{c}\) are known to promote dissociation of IP\(_{3}\) from the IP\(_{3}\) receptor leading to faster IP\(_{3}\) receptor inactivation (14). Adenophostin A, by virtue of its high affinity for the IP\(_{3}\) receptor, may be less susceptible to this increased rate of dissociation. Adenophostin A would therefore prolong IP\(_{3}\) receptor activation, relative to the lower affinity agonists (GPIP2 and IP\(_{3}\)), and maintain depleted Ca \(^{2+}\) stores.
If indeed IP₃ receptor inactivation does lead to rapid store refilling and turns off detectable $I_{\text{crac}}$, then prevention of Ca²⁺ re-uptake (with thapsigargin) should prevent the re-uptake of Ca²⁺ that occurs with IP₃ and GPIP₂, and thus these agents would act more like adenophostin A. To test this hypothesis, thapsigargin was applied to cells loaded with Fura-2 and patched-clamped in the cell-attached mode (Fig. 4A). Patch pipettes contained 100 μM GPIP₂ and 0.1 mM BAPTA. After 50–70 s, a slight increase in [Ca²⁺], was detected indicating SERCA inhibition (Fig. 4A, upper trace). The whole-cell mode was then established in order to deliver GPIP₂ and measure $I_{\text{crac}}$ (Fig. 4A, lower trace). GPIP₂ caused a further, more substantial increase in [Ca²⁺], which was sustained (compare with GPIP₂ alone, Fig. 2C). Importantly, the increase in [Ca²⁺], that occurred upon delivery of GPIP₂ was accompanied by rapid activation of $I_{\text{crac}}$; $I_{\text{crac}}$ was activated after only a short delay (33 ± 8 s) and with a time course of 86 ± 14 s. Had the current been activated by thapsigargin alone, the delay from break-in would have been in excess of 3 min and the development time in excess of 5 min (inferred from Fig. 2D). Development of $I_{\text{crac}}$ in the presence of GPIP₂ is therefore triggered by the rapid release of Ca²⁺ through activated IP₃ receptors and independent of the slow leak of Ca²⁺ induced by thapsigargin alone. The peak amplitude of $I_{\text{crac}}$ was not significantly altered by the presence (1.53 ± 0.23 pA/pF) or absence of GPIP₂ (1.05 ± 0.14 pA/pF, Fig. 2F). This is to be expected if GPIP₂ increases the rate of release, through opening IP₃ receptors, but not the overall extent.

The preceding data suggest that when Ca²⁺ is free to increase, re-uptake of Ca²⁺ prevents detectable activation of $I_{\text{crac}}$ by GPIP₂. If this assumption is correct, then delivery of thapsigargin before GPIP₂, with Ca²⁺ clamped to 500 nM with 10 mM BAPTA, should also allow rapid activation of $I_{\text{crac}}$ (Fig. 4B). As predicted, in cells pretreated with thapsigargin for 50–70 s, delivery of GPIP₂ led to a rapid activation of $I_{\text{crac}}$. Activation was detected after only a short delay (34 ± 11 s) and had a development time of 81 ± 13 s, compared with 415 ± 27 s for thapsigargin alone. The amplitude of the current induced by GPIP₂ and thapsigargin combined (3.16 ± 0.28 pA/pF) was once again not significantly greater than that seen with thapsigargin alone (2.57 ± 0.65 pA/pF).

These results reveal that SERCA pumps need to be blocked if GPIP₂ is to activate detectable $I_{\text{crac}}$ when [Ca²⁺], is weakly buffered. Refilling of stores by SERCA pumps must occur very quickly, and therefore, a decrease in IP₃ receptor activity must also occur quickly to explain the complete lack of detectable...
$I_{\text{crac}}$ activation by GPIP$_2$ or IP$_3$. GPIP$_2$ is successful in activating $I_{\text{crac}}$ after thapsigargin addition because despite rapid IP$_3$ receptor desensitization, Ca$^{2+}$ cannot be re-accumulated into the critical stores. This predicts that if the addition of the SERCA inhibitor is delayed for even a short interval following addition of GPIP$_2$, $I_{\text{crac}}$ activation will still fail. As shown in Fig. 4C, this is indeed the case. When thapsigargin was added 50–70 s after break-in with GPIP$_2$, no rapid activation of $I_{\text{crac}}$ was observed; rather $I_{\text{crac}}$ activation occurred slowly (311 ± 7 s) and after a latency of 222 ± 21 s, similar to activation of $I_{\text{crac}}$ by thapsigargin alone (see Fig. 2D).

Collectively these results indicate that IP$_3$ and GPIP$_2$ activate IP$_3$ receptors and allow a rapid release of Ca$^{2+}$ from intracellular stores, but this receptor activity and enhanced Ca$^{2+}$ release are transient. Hence, when re-uptake of Ca$^{2+}$ is blocked prior to GPIP$_2$ exposure, the release induced by GPIP$_2$ is sufficiently sustained to activate $I_{\text{crac}}$ rapidly (Fig. 4A). However, if Ca$^{2+}$ re-uptake is allowed to proceed for even a minute after GPIP$_2$ delivery, blockade of SERCA pumps at this stage does not rapidly activate $I_{\text{crac}}$ because the IP$_3$ receptors have already desensitized and Ca$^{2+}$ has been re-accumulated (Fig. 4C).

**The Size of the Critical Stores Regulating $I_{\text{crac}}$**—Finally, we attempted experiments designed to demonstrate more directly the re-uptake of Ca$^{2+}$ into pools regulating $I_{\text{crac}}$ and to determine their size relative to the presumably larger IP$_3$ and thapsigargin-sensitive stores. To this end, we activated signaling in cells with either GPIP$_2$ or adenophostin A, and we examined the size of the [Ca$^{2+}$]$_i$ signal on application of thapsigargin in Ca$^{2+}$-free media (Fig. 5, lower panels). On delivery of adenophostin A or GPIP$_2$, cells were exposed to either Ca$^{2+}$-free or Ca$^{2+}$-containing media before assessing the thapsigargin-sensitive store content in order to determine the contribution of Ca$^{2+}$ influx to the content of the stores. A residual, thapsigargin-sensitive, Ca$^{2+}$ store was detected in cells after exposure to adenophostin A (Fig. 5A) or GPIP$_2$ (Fig. 5B) in the absence of Ca$^{2+}$ influx (dotted lines). When Ca$^{2+}$ influx was allowed before store assessment (dashed lines), some refilling of stores occurred after both agents, although slightly more refilling appeared to occur in the presence of GPIP$_2$. In each case, however, Ca$^{2+}$ stores were not completely refilled, because control cells exposed only to thapsigargin showed the largest release of Ca$^{2+}$ (solid lines).

Assessment of the Ca$^{2+}$ stores by inhibition of SERCA pumps revealed Ca$^{2+}$ release kinetics for adenophostin A (Fig. 5A, dashed lines) which were distinct from that in control cells (Fig. 5, solid line) or GPIP$_2$-treated cells (Fig. 5B, dashed lines). For adenophostin A-treated cells, the rate of Ca$^{2+}$ release was faster than in control cells, with [Ca$^{2+}$]$_i$ rising, only adenosinophostin A or GPIP$_2$ release (Fig. 5A, upper panels). In that study, thapsigargin (TG) was applied to the outside of the cell as indicated. Thick black traces represent control cells exposed only to thapsigargin. The lower panels show mean changes in [Ca$^{2+}$]$_i$, collated from single cells, and the upper panels show the simultaneous current densities measured from those same cells. Traces are mean ± S.E. ($n$ ≥ 4).

![Fig. 5. Extent of intracellular Ca$^{2+}$ pool depletion by adenophostin A and GPIP$_2$. Cells were loaded with Fura-2 AM and patched with pipettes containing 0.1 mM BAPTA, 50 μM Fura-2 free acid, and either 2 μM adenophostin A (AdA) (A) or 100 μM GPIP$_2$ (B). Establishment of the whole-cell configuration is indicated by the vertical arrows, at which time the voltage protocol described in Fig. 1 was initiated. Extracellular Ca$^{2+}$ was either completely omitted (black dotted lines) or removed as indicated (black solid lines). In each trace 1 μM thapsigargin (TG) was applied to the outside of the cell as indicated. Thick black traces represent control cells exposed only to thapsigargin. The lower panels show mean changes in [Ca$^{2+}$]$_i$, collated from single cells, and the upper panels show the simultaneous current densities measured from those same cells. Traces are mean ± S.E. ($n$ ≥ 4).](image)

**DISCUSSION**

Our laboratory previously established a method for the simultaneous measurement of $I_{\text{crac}}$ and [Ca$^{2+}$]$_i$ in RBL-1 cells (10). Prevention of Ca$^{2+}$ entry between current measurements (by holding the cell membrane at a positive voltage) was the key to minimizing Ca$^{2+}$-dependent inactivation of $I_{\text{crac}}$. In that study we showed that adenophostin A, but not IP$_3$ congeners,
IP$_3$ Receptors and I$_{crac}$

Fig. 6. Adenophostin A activates I$_{crac}$ to a similar extent as ionomycin and thapsigargin without depleting all thapsigargin-sensitive Ca$^{2+}$ stores. Cells were loaded with Fura-2 AM and patched with pipettes containing 0.1 mM BAPTA, 50 µM Fura-2 free acid, with (A) or without (B) 2 mM AdA. Establishment of the whole-cell configuration is indicated by the vertical arrows, at which time the voltage protocol described in Fig. 1 was initiated. Extracellular Ca$^{2+}$ was either completely omitted (dotted line in B) or removed where indicated (solid lines). Thapsigargin (TG, 1 µM) and ionomycin (IONO, 500 nM) were applied to the outside of the cell where indicated. The upper part of each panel shows the mean current densities measured simultaneously with [Ca$^{2+}$], in the same cells (lower traces). Traces are mean ± S.E. (n ≥ 3).

was an effective activator of I$_{crac}$ when [Ca$^{2+}$]$_i$ was weakly buffered and free to rise. We now show that adenophostin A is effective because it keeps IP$_3$ receptors active, maintaining specific, critical stores sufficiently depleted of Ca$^{2+}$ to signal I$_{crac}$ activation. In contrast, other IP$_3$ receptor agonists, including IP$_3$ itself and GPIP$_2$, fail to maintain active IP$_3$ receptors, allowing refilling of the critical stores. Depletion of Ca$^{2+}$ stores represents the sine qua non for capacitative calcium entry, and so the signal for I$_{crac}$ is substantially diminished and the current is not activated to a detectable level. This is a novel mode of Ca$^{2+}$-dependent inactivation of I$_{crac}$ in that the Ca$^{2+}$-feedback occurs at the level of the IP$_3$ receptor, rather than the CRAC channel. The possible involvement of direct Ca$^{2+}$-dependent inactivation of the CRAC channel being responsible for the failure of GPIP$_2$ or IP$_3$ to activate I$_{crac}$ is ruled out by the ability of other store-depleting agents, including thapsigargin and ionomycin, to activate I$_{crac}$ under similar conditions.

Our data reveal how IP$_3$ receptor activity can strongly influence the activity of CRAC channels. The IP$_3$ receptor participates “indirectly” in regulating CRAC channels in RBL-1 cells, because its activity determines the state of filling of a subset of Ca$^{2+}$ stores intrinsically linked to I$_{crac}$ activation. When the IP$_3$ receptor activates, these stores rapidly refill, through the activity of SERCA pumps, minimizing the signal for CRAC channel activation. Recently, the IP$_3$ receptor was also suggested to have a “direct” role in the regulation of Htrp3 channels stably expressed in HEK293 cells (15). The Htrp3 protein has previously been shown to form a channel activated by phospholipase C-linked agonists (16-18). Kiseliov and colleagues (20) now report that overexpressed Htrp3 channels can also be activated by store depletion but that the IP$_3$ receptor directly interacts with the Htrp3 channel and that occupation of the IP$_3$ receptor is a requirement for Htrp3 activation. Whether the IP$_3$ receptor works to modulate directly endogenous Ca$^{2+}$ channels is unclear, although single channels that could be activated by either store depletion (in cell attached patches) or IP$_3$ (in excised patches) have been reported (19, 20).

In RBL-1 cells, when Ca$^{2+}$ is free to fluctuate, adenophostin A, GPIP$_2$, and IP$_3$ each readily release Ca$^{2+}$ from the specialized domains linked to I$_{crac}$ activation. However, only adenophostin A keeps these stores sufficiently empty to signal detectable I$_{crac}$ activation, because only it maintains IP$_3$ receptor activity. The distinct abilities of these agonists to maintain IP$_3$ receptor activity may reflect the very different affinities they display for the IP$_3$ receptor. Adenophostin A is ≈ 100-fold more potent than 1,4,5-IP$_3$ (11), whereas GPIP$_2$ is ≈ 10-fold weaker (21). Increases in Ca$^{2+}$ are reported to increase the rate of dissociation of IP$_3$ from the IP$_3$ receptor (14). The lower affinity agonists would dissociate from the IP$_3$ receptor more quickly than adenophostin A, leading to a more rapid receptor inactivation. In the continued presence of adenophostin A some slow inactivation of I$_{crac}$ is seen with higher cytosolic [Ca$^{2+}$]$_i$ (11), (Fig. 3A). This may reflect either the slow dissociation of adenophostin A from the IP$_3$ receptor, reducing the signal for I$_{crac}$ activation, or a slow Ca$^{2+}$-dependent inactivation of the CRAC channel (8, 22).

RBL cells have been shown to express all three forms of the IP$_3$ receptor (23), with the predominating species being type 2. Because the number of IP$_3$ receptors involved in this subcompartment of the endoplasmic reticulum is likely small compared with the total number of receptors, any of the three types or possibly all three could be located there. There is evidence for specific involvement of the type 3 IP$_3$ receptor in regulating capacitative calcium entry (24). However, it is only the type-1 and type-2 receptors that appear to be regulated negatively by Ca$^{2+}$ (25, 26), and thus type 1 and/or type 2 receptors would more likely be involved in the negative regulation seen here.

In addition to the findings discussed above, our results also suggest that the stores linked to I$_{crac}$ activation in RBL-1 cells reside within a specialized subcompartment of the endoplasmic reticulum. Adenophostin A activates I$_{crac}$ by releasing only a fraction of the thapsigargin- and ionomycin-releasable Ca$^{2+}$ stores, yet further release of residual Ca$^{2+}$ stores does not increase I$_{crac}$ (Figs. 2F and 6, A and C). Hofer et al. (27) have also shown that in RBL-1 cells, full activation of I$_{crac}$ occurs with only partial depletion of intracellular stores by ionomycin. We cannot determine if the failure of ionomycin and thapsigargin to induce a larger activation of I$_{crac}$ than adenophostin A reflects subcompartments of the endoplasmic reticulum, some of which are and some of which are not linked to I$_{crac}$ activation, or more simply a requirement for only partial depletion of all (or of critical) stores for maximal I$_{crac}$ activation. However, we also observed that with minimal [Ca$^{2+}$]$_i$, buffering, GPIP$_2$ depletes nearly as much of the thapsigargin-sensitive store as does adenophostin A, yet GPIP$_2$ fails to activate detectable I$_{crac}$ and adenophostin A activates it maximally. This striking distinction in the action of GPIP$_2$ and adenophostin A therefore must reflect the filling state of a pool that is very small in comparison to the total IP$_3$-sensitive Ca$^{2+}$ stores.

There is already some evidence to suggest that specialized domains of the endoplasmic reticulum are coupled to activation of I$_{crac}$ in RBL-1 cells (13, 28). Parekh et al. (28) observed differences in the concentration-effect relationships for Ca$^{2+}$ release and I$_{crac}$ activation which they interpreted as reflecting heterologous Ca$^{2+}$ pools, only some of which were involved in I$_{crac}$ regulation. Huang and Putney (13) observed different latencies for (2,4,5)IP$_3$, thapsigargin, and ionomycin activation of
Ca\(^{2+}\) release and \(I_{\text{inac}}\). A significant delay was observed between the initiation of Ca\(^{2+}\) stores depletion and the activation of \(I_{\text{inac}}\) for (2,4,5)IP\(_3\) and thapsigargin. However, with ionomycin, little or no delay was observed. A small compartment of the endoplasmic reticulum was suggested to regulate Ca\(^{2+}\) entry, which was relatively resistant to store depletion by IP\(_3\) and thapsigargin but not by ionomycin. This could result from a non-homogenous distribution of IP\(_3\) receptors and leak channels. The more direct Ca\(^{2+}\)-transporting action of ionomycin would not be affected by such distributions. There is evidence that sub-compartments of the endoplasmic reticulum regulate Ca\(^{2+}\) influx in other cell types. Treatment of NIH-3T3 cells with the phorbol ester phorbol 12-myristate 13-acetate caused a loss of Ca\(^{2+}\) (~70\%) from thapsigargin- and IP\(_3\)-sensitive pools, but this depletion did not lead to activation of capacitative Ca\(^{2+}\) entry (29).

When Ca\(^{2+}\) stores are depleted by the SERCA inhibitor, thapsigargin, the release of Ca\(^{2+}\) depends on poorly understood “leak” channels in the endoplasmic reticulum. Interestingly, and consistent with our earlier conclusions (13), our data suggest that the specialized stores linked to \(I_{\text{inac}}\) activation are resistant to rapid emptying by the endogenous leak pathway. First, we observe a long delay between the initiation of Ca\(^{2+}\) store depletion and activation of \(I_{\text{inac}}\) by thapsigargin. Second, the current activated by thapsigargin in weakly buffered RBL-1 cells or in cells with [Ca\(^{2+}\)]\(_i\) set to 500 nM has very slow development kinetics. These sub-compartments therefore either possess a low number of leak channels or lack them entirely or possess thapsigargin-insensitive pools which act to slow the net Ca\(^{2+}\) leak (30, 31). The cause of the large increase in both the delay to onset and development kinetics of \(I_{\text{inac}}\) activation by thapsigargin after reduction of Ca\(^{2+}\) buffering from 10 to 0.1 mM BAPTA or on clamping [Ca\(^{2+}\)]\(_i\), to 500 nM is unknown but presumably results from elevated [Ca\(^{2+}\)]\(_i\). There are at least three explanations. 1) The endogenous leak of Ca\(^{2+}\) from intracellular stores is slower at higher cytoplasmic [Ca\(^{2+}\)]. For example if basally active IP\(_3\) receptors account for some of the endogenous leak. 2) In the presence of elevated [Ca\(^{2+}\)], thapsigargin-resistant Ca\(^{2+}\) entry still occurred as evidenced by a sustained increase in [Ca\(^{2+}\)], that reversed upon removal of extracellular Ca\(^{2+}\) (Fig. 2, B and C). Hence, the partially empty stores must signal some Ca\(^{2+}\) entry, but this is not detectable as a current. \(I_{\text{inac}}\) may be active under all conditions in which capacitative calcium entry occurs, but it may be below the threshold for detection. In the absence of specific pharmacological probes for these channels, it cannot be disproved that an as yet unidentified but distinct pathway contributes to entry under these conditions. Interestingly, Zhang and McCloskey (33) using the nystatin perforated patch technique and with no added intracellular calcium buffer were able to detect an inwardly rectifying Ca\(^{2+}\) current in RBL-2H3 cells in response to either immunoglobulin E (presumably acting through IP\(_3\)) or thapsigargin. This current was seen at 37 °C but not at room temperature. Hoth et al. (34) have demonstrated that in T-lymphocytes the ability to detect \(I_{\text{inac}}\) under conditions of low physiological calcium buffering in the patch pipette depends on endogenous calcium buffering by mitochondria. Thus, the ability to measure a calcium current associated with capacitative calcium entry may depend on a number of technical factors, including both experimentally applied as well as physiological calcium buffering. But it is clear from the data in this report and others in the literature that significant entry of calcium can occur through store-operated channels, whatever their nature, when the magnitude of the associated current is below the level of detection with presently available methodologies.

Because of these slow kinetics, in our experiments with IP\(_3\) and GPP\(_i\) as activators, the stores linked to \(I_{\text{inac}}\) activation may never be depleted sufficiently or for a long enough period to activate \(I_{\text{inac}}\) to a detectable level. Importantly, in our experiments, despite some refilling of Ca\(^{2+}\) stores in the presence of GPP\(_i\), stores were not completely refilled (Fig. 5B), and although the currents underlying Ca\(^{2+}\) entry for GPP\(_i\) and IP\(_3\) were beneath the level of detection, Ca\(^{2+}\) entry still occurred as evidenced by a sustained increase in [Ca\(^{2+}\)], that reversed upon removal of extracellular Ca\(^{2+}\) (Fig. 2, B and C). Hence, the partially empty stores must signal some Ca\(^{2+}\) entry, but this is not detectable as a current. \(I_{\text{inac}}\) may be active under all conditions in which capacitative calcium entry occurs, but it may be below the threshold for detection. In the absence of specific pharmacological probes for these channels, it cannot be disproved that an as yet unidentified but distinct pathway contributes to entry under these conditions. Interestingly, Zhang and McCloskey (33) using the nystatin perforated patch technique and with no added intracellular calcium buffer were able to detect an inwardly rectifying Ca\(^{2+}\) current in RBL-2H3 cells in response to either immunoglobulin E (presumably acting through IP\(_3\)) or thapsigargin. This current was seen at 37 °C but not at room temperature. Hoth et al. (34) have demonstrated that in T-lymphocytes the ability to detect \(I_{\text{inac}}\) under conditions of low physiological calcium buffering in the patch pipette depends on endogenous calcium buffering by mitochondria. Thus, the ability to measure a calcium current associated with capacitative calcium entry may depend on a number of technical factors, including both experimentally applied as well as physiological calcium buffering. But it is clear from the data in this report and others in the literature that significant entry of calcium can occur through store-operated channels, whatever their nature, when the magnitude of the associated current is below the level of detection with presently available methodologies.

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