Effect of Adenophostin A on Ca$^{2+}$ Entry and Calcium Release-activated Calcium Current ($I_{\text{crac}}$) in Rat Basophilic Leukemia Cells*

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Yi Huang‡, Masaaki Takahashish, Kazuhiko Tansawaš, and James W. Putney, Jr.§

From the §Calcium Regulation Section, Laboratory of Signal Transduction, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709 and the ¶Biological Research Laboratories, Sankyo Co., Ltd., Tokyo 140, Japan

In most non-excitatory cells, calcium influx is signaled by depletion of intracellular calcium stores by a process known as capacitative calcium entry. Adenophostin A, a potent activator of the inositol 1,4,5-trisphosphate receptor, has been shown to activate Ca$^{2+}$ entry in Xenopus oocytes to a greater extent than expected on the basis of its ability to release calcium stores. In this study, we compared the abilities of adenophostin A and inositol 2,4,5-trisphosphate ([2,4,5]IP$_3$) to release Ca$^{2+}$ from intracellular stores, to activate Ca$^{2+}$ entry, and to activate calcium release-activated calcium current ($I_{\text{crac}}$) in rat basophilic leukemia cells. Under conditions of low intracellular Ca$^{2+}$ buffering (0.1 mM BAPTA), adenophostin A-induced Ca$^{2+}$ release and activation of $I_{\text{crac}}$ could be monitored simultaneously. However, other reagents that would be expected to deplete Ca$^{2+}$ stores ([2,4,5]IP$_3$, 3-fluoro-inositol 1,4,5-trisphosphate, thapsigargin, and ionomycin) were unable to activate $I_{\text{crac}}$ under this low Ca$^{2+}$ buffering condition. Adenophostin A activated $I_{\text{crac}}$ after a significant delay, longer than the delay for Ca$^{2+}$ release. Thus, adenophostin A activates $I_{\text{crac}}$ as a consequence of release of intracellular Ca$^{2+}$, rather than directly acting on store-operated channels. The unique ability of adenophostin A to activate $I_{\text{crac}}$ under conditions of low intracellular Ca$^{2+}$ buffering suggests an additional site of action, perhaps in preventing or reducing rapid Ca$^{2+}$-dependent inactivation of store-operated Ca$^{2+}$ channels.

Stimulation of G-protein-coupled receptors and tyrosine kinase receptors activates phospholipase C, which generates inositol 1,4,5-trisphosphate ([1,4,5]IP$_3$) and diacylglycerol. ([1,4,5]IP$_3$) binds to its receptor on the membrane of the endoplasmic reticulum and releases Ca$^{2+}$ stored therein. The depletion of intracellular Ca$^{2+}$ stores then signals the opening of plasma membrane Ca$^{2+}$ channels, a process known as capacitative calcium entry (1, 2). The signaling mechanism for the activation of capacitative calcium entry is not understood. A direct coupling model suggests that store depletion involves a functional coupling between ([1,4,5]IP$_3$) receptors on the endoplasmic reticulum membrane and Ca$^{2+}$ entry channels on the plasma membrane (3, 4). Other models suggest that store depletion generates one or more second messengers which in turn activate Ca$^{2+}$ entry channels (5).

Adenophostin A, a compound isolated from the culture broth of the Penicillium brevicompactum, is the most potent known agonist for the ([1,4,5]IP$_3$) receptor. Its affinity for the ([1,4,5]IP$_3$) receptor is about 100-fold greater than that of ([1,4,5]IP$_3$) (6). Recently, two reports (7, 8) have shown that injection of adenophostin A into Xenopus oocytes produces a greater activation of Ca$^{2+}$ entry (based on the activity of Ca$^{2+}$-dependent Cl$^{-}$ channels) than would be expected solely as a result of release of intracellular Ca$^{2+}$ stores, that is when compared with ([1,4,5]IP$_3$). With low concentrations of adenophostin A, DeLisle et al. (7) reported a stimulation of Ca$^{2+}$-dependent Cl$^{-}$ current without a detectable release of intracellular Ca$^{2+}$. This suggests that in addition to its action as an agonist for the ([1,4,5]IP$_3$) receptor, adenophostin A either acts downstream of ([1,4,5]IP$_3$) in the pathway signaling capacitative calcium entry (perhaps on the channels themselves) or preferentially releases Ca$^{2+}$ from stores coupled to the activation of Ca$^{2+}$ entry. In this study, we have examined the effects of adenophostin A on Ca$^{2+}$ entry in a mammalian cell line, rat basophilic leukemia cells (RBL-1). Adenophostin A and ([2,4,5]IP$_3$), both non-metabolizable analogs of ([1,4,5]IP$_3$), were compared with regard to their abilities to release Ca$^{2+}$ from intracellular stores, to activate Ca$^{2+}$ entry, and to activate the well characterized calcium release-activated calcium current ($I_{\text{crac}}$) (9). While we find no evidence for a direct activation of calcium entry or of $I_{\text{crac}}$ by adenophostin A, we have discovered an unusual and unique ability of adenophostin A to support $I_{\text{crac}}$ under conditions of limited intracellular calcium buffering. Adenophostin A will be a useful tool for further investigations into the signaling mechanisms for capacitative calcium entry.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Rat basophilic leukemia cells (RBL-1, ATCC 1378-CRL, batch number F-13352) were cultured as recommended by ATCC. Adenophostin A was isolated as described previously (10). Ionomycin and 3-deoxy-3-fluoro-1,4,5-trisphosphate (3-F-IP$_3$) were obtained from Calbiochem, and ([2,4,5]IP$_3$) was obtained from Calbiochem or Sigma. Thapsigargin was purchased from LC Laboratories.

**Fura-2 Loading**—The attached cells were mounted in a Teflon chamber and incubated with 1 μM fura-2/AM (Molecular Probes) for 25 min at room temperature. The cells were then washed and bathed in a HEPES-buffered physiological saline solution (in mM: 150 NaCl, 4.7 KCl, 1.8 CaCl$_2$, 1.13 MgCl$_2$, 10 glucose, and 10 HEPES, pH 7.2; HPSS) at room temperature for at least 10 min before Ca$^{2+}$ measurements were made.

**Fluorescence Measurements**—The fluorescence of the fura-2-loaded cells was monitored with a photomultiplier-based system, mounted on a Nikon Diaphot microscope equipped with a Nikon 40× (1.3 N.A.) Neofluor objective. The fluorescence light source was provided by a Deltascan D101 (Photon Technology International Ltd.), equipped with a light path chopper and dual excitation monochromators. The light
path chopper enabled rapid interchange between two excitation wavelengths (340 and 380 nm), and a photomultiplier tube monitored the emission fluorescence at 510 nm, selected by a barrier filter (Omegaf). All experiments were carried out at 24 °C with cells incubated in HPSS. Calibration and calculation of [Ca2+]i were carried out as described previously (11).

Electrophysiology—The normal external saline was HPSS described above. Nominally Ca2+-free saline was the same except no CaCl2 was added. The bath volume (0.5 ml) was rapidly exchanged with a gravity perfusion system. The labels in the figures indicate the exact times when new bath solution was introduced, without any correction for the dead time required for a new solution to reach the cell. For fluorescence experiments, the patch pipette (2–4 MS, Corning glass, 7052) contained (in mM) 140 NaCl, 2 MgCl2, 10 HEPES, 10 EGTA. The bath solution supplemented with 2 mM adenosphostin A (•••) or 100 μM (2,4,5)IP3 (-----). Bottom, pipette solution supplemented with 0.25 μM adenosphostin A (•••) or 25 μM (2,4,5)IP3 (-----).

RESULTS

Fig. 1 illustrates the protocol used to assess Ca2+ entry with a series of concentrations of (2,4,5)IP3 and adenosphostin A. Cells patch-clamped in the whole cell configuration were held at 30 mV to prevent Ca2+ entry. The pipettes contained differing concentrations of (2,4,5)IP3 or adenosphostin A which induced release of intracellular Ca2+. For (2,4,5)IP3, this release was generally accompanied by the appearance of [Ca2+]i oscillations. This was true for adenosphostin A as well, but only with the lower concentrations. Where indicated, the holding potential was changed to −60 mV to provide a controlled driving force for Ca2+ entry, and a subsequent rise in [Ca2+]i occurred. The [Ca2+]i rise at −60 mV with the protocol in Fig. 1 may give a reasonable estimate of Ca2+ entry at different concentrations of (2,4,5)IP3 or adenosphostin, but the initial [Ca2+]i spike is not likely a reliable indicator of the magnitude of release. As in previous studies (14, 15), we utilized the ionomycin residual technique, illustrated in Fig. 2. In this experiment, a cell incubated in nominally Ca2+-free medium (+ 1 mM EGTA to prevent Ca2+ entry due to ionomycin) was activated by 250 nM adenosphostin A in the pipette. Subsequent addition of a high concentration (10 μM) of ionomycin induced a small release of Ca2+ compared with a control cell, indicative of significant depletion of intracellular Ca2+ stores by adenosphostin A. Fig. 3 (top) summarizes the data for Ca2+ release by adenosphostin A and (2,4,5)IP3. It was not possible to obtain graded release of Ca2+ with adenosphostin A. 0.25 μM adenosphostin A depleted Ca2+ to the same extent as the highest concentration tested, 10 μM, and lower concentrations (0.1 and 0.05 μM) failed to consistently activate release and entry. However, as reported previously (15), one concentration of (2,4,5)IP3, 10 μM, induced a consistent and partial depletion of Ca2+ stores.

Fig. 3 (bottom) shows data for Ca2+ entry in response to adenosphostin A and (2,4,5)IP3 within the concentration range of reproducible Ca2+ release. At the highest concentrations tested, both adenosphostin A (10 μM) and (2,4,5)IP3 (100 μM) released Ca2+ and activated Ca2+ entry to a similar extent. At 10 μM (2,4,5)IP3, release of Ca2+ was incomplete, and likewise, entry was lower than at other concentrations. Interestingly, at the intermediate concentrations of (2,4,5)IP3, even though (2,4,5)IP3 appeared to release Ca2+ maximally, the Ca2+ entry response was significantly less that that seen with adenosphostin A.

The level of Ca2+ entry in Figs. 1 and 3 is determined not only by Ca2+ entry but also the extrusion across the plasma membrane and uptake into the endoplasmic reticulum. Therefore, we decided to examine the effect of adenosphostin A and (2,4,5)IP3 on Ca2+ entry by using techniques that avoid these complicating factors. In the experiments shown in Fig. 4, prior to changing the holding potential to −60 mV, the external medium was changed from one containing 10 mM Ba2+ to one containing 10 mM Ba2+. Ba2+ is able to permeate the channels opened by depleting intracellular stores but is not a substrate

FIG. 1. Ca2+ release and entry with adenosphostin A and (2,4,5)IP3. Single RBL-1 cells, loaded with fura-2, were held at 30 mV under conventional whole cell configuration. With the establishment of whole cell configuration, adenosphostin A or (2,4,5)IP3 present in the patch clamp pipettes caused a transient Ca2+ release from intracellular Ca2+ stores without detectable Ca2+ entry. (2,4,5)IP3 caused [Ca2+]i oscillations at both high and low concentrations, whereas adenosphostin A only caused [Ca] oscillations only at lower concentrations. At the indicated time, the holding potential was changed to −60 mV to allow Ca2+ entry to occur. Top, pipette solution supplemented with 2 μM adenosphostin A (•••) or 100 μM (2,4,5)IP3 (-----). Bottom, pipette solution supplemented with 0.25 μM adenosphostin A (•••) or 25 μM (2,4,5)IP3 (-----).

FIG. 2. Protocol for determining ionomycin residual Ca2+ stores. In this example, on break-in the patch pipette contained 250 nM adenosphostin A (AdA) (----). Subsequent addition of 10 μM ionomycin (Iono.) 5 min later reveals a substantial depletion of the ionomycin-releasable Ca2+ store, when compared with a control cell not stimulated with adenosphostin A (---).
for plasma membrane or endoplasmic reticulum Ca²⁺ pumps (16–18). The summarized data in Fig. 4 shows that Ba²⁺ entry is not significantly different when activated by 25 μM (2,4,5)IP₃ or 0.25 μM adenophostin A.

Another technique for assessing Ca²⁺ entry that avoids effects of Ca²⁺ pumps is to measure the current associated with entry directly, the calcium release-activated calcium current, or I_{crac} (19). Fig. 5 shows that adenophostin A activates an inward current in RBL-1 cells and that this current has the following properties indicating that it is I_{crac}: (i) the current is strongly inwardly rectifying with a magnitude and current-voltage relationship similar to that previously described for I_{crac} by Hoth and Penner (19); (ii) the current is observed with strong intracellular calcium buffering (10 mM BAPTA) but is lost when external calcium is removed (Fig. 5). Consistent with the result from the Ba²⁺ experiments, the amount of I_{crac} activated with adenophostin A was the same as that with (2,4,5)IP₃ at all concentrations except for 10 μM (2,4,5)IP₃, which only partially depleted the intracellular Ca²⁺ stores (Fig. 6). Therefore, the results suggest that adenophostin A and (2,4,5)IP₃ activate Ca²⁺ entry similarly. Because of the different results from those obtained in the Ca²⁺ entry experiments, in some experiments we followed the exact same protocol as used in Fig. 1, that is holding the cell at 30 mV and measuring I_{crac} with 1.8 mM external Ca²⁺ at 300 s after the establishment of whole cell configuration. Again, there was no difference in the amount of I_{crac} activated (1.84 ± 0.28 pA/pF, n = 7, for 0.25 μM adenophostin A versus 1.82 ± 0.11 pA/pF, n = 6, for 25 μM (2,4,5)IP₃). The potential meaning of the disparate findings with measurement of net [Ca²⁺], changes compared with Ba²⁺ entry or I_{crac} will be addressed under “Discussion.”

The results to this point reveal no clear distinctions between the actions of (2,4,5)IP₃ and adenophostin A. Thus, the ability of adenophostin A to activate Ca²⁺ entry and I_{crac} may result from its ability to release intracellular Ca²⁺ stores, rather than from a direct effect on calcium entry channels. To investigate further this issue, we examined the temporal relationship between the activation of I_{crac} and Ca²⁺ release, as we did in an earlier study for (2,4,5)IP₃ (15). We measured the latency for I_{crac} activation with adenophostin A and compared that with the latency for Ca²⁺ release from intracellular stores. We found that adenophostin A activated I_{crac} with a latency correlating to, but longer than, the latency for Ca²⁺ release (Fig. 7). A similar pattern for Ca²⁺ release and I_{crac} activation was found for (2,4,5)IP₃ (15). Thus, adenophostin A appears to activate I_{crac} only after Ca²⁺ release from intracellular Ca²⁺ stores is well under way, suggesting that adenophostin A activates I_{crac} as a result of the release of intracellular Ca²⁺, rather than through a direct action on store-operated Ca²⁺ channels or some other site downstream of Ca²⁺ release.

One complication in the interpretation of the kinetic data in Fig. 7 is that the measurement of Ca²⁺ release and I_{crac} are carried out under different experimental conditions. In the fura-2 experiments to measure Ca²⁺ release, the pipette solution contains a modest amount of Ca²⁺ buffer (0.1 mM EGTA) so that net changes in cytoplasmic Ca²⁺ can be observed. On the other hand, measurements of I_{crac} in this study (as well as in all other published studies) were carried out with a much higher concentration of Ca²⁺ buffer in the pipette (10 mM BAPTA or EGTA) to minimize Ca²⁺-dependent inactivation of I_{crac}. Thus, we attempted to design a protocol that would permit measurement of I_{crac} and Ca²⁺ release (with fura-2 simulta-
neously on a single RBL-1 cell. The cells were loaded with fura-2 (see “Experimental Procedures”), and cells were patch-clamped with pipettes containing the Cs\(^+\)-containing \(I_{\text{crac}}\) in intracellular solution with 50 \(\mu M\) fura-2 but with BAPTA reduced to 0.1 mM. To minimize Ca\(^{2+}\) entry and Ca\(^{2+}\)-induced inactivation, we held the cell at \(+50\) mV. \(I_{\text{crac}}\) was assayed by a short ramp (from \(-100\) to 60 mV for 160 ms) every 1–5 s. With this protocol, we expect Ca\(^{2+}\) entry to be greatly reduced during the inter-ramp interval and to be mainly limited to the short duration of the ramp. As shown in Figs. 8 and 9, this procedure resulted in the development of a significant inward current when 0.5 \(\mu M\) adenophostin A was included in the pipette. This current had an I-V relationship expected of \(I_{\text{crac}}\) (Fig. 8); with these ionic conditions, no other known current could produce such an I-V relationship. The current was dependent on extracellular Ca\(^{2+}\) but did not depend on the level of [Ca\(^{2+}\)]\(_{i}\), consistent with Ca\(^{2+}\) acting as a charge carrier rather than as an activator of the current. Thus, the current has all of the properties expected of the depletion-activated current, \(I_{\text{crac}}\), seen with stronger intracellular Ca\(^{2+}\) buffers. Surprisingly, (2,4,5)IP\(_3\) (two batches from two different sources) completely failed to activate any detectable \(I_{\text{crac}}\) (Table I) with this protocol even though Ca\(^{2+}\) is almost depleted to the same extent as with adenophostin A (Fig. 9). Furthermore, three other store-depletion reagents (3-F-IP\(_3\), thapsigargin, and ionomycin) similarly failed to activate \(I_{\text{crac}}\) with the low Ca\(^{2+}\) buffer protocol (Table I). 3-F-IP\(_3\) is another nonhydrolyzable (1,4,5)IP\(_3\) analog. The \(K_d\) for 3-F-IP\(_3\) for the (1,4,5)IP\(_3\) receptor is comparable to that of (1,4,5)IP\(_3\) (13 nM compared with 6 nM for (1,4,5)IP\(_3\) by the same assay) but still higher than that for adenophostin A. The average peak Ca\(^{2+}\) release induced by 3-F-IP\(_3\) was 256.8 \pm 23.5 nM (n = 9 at 50 \(\mu M\)) and 330.0 \pm 1.0 nM (n = 2 at 100 \(\mu M\)), respectively, similar to that observed for (2,4,5)IP\(_3\) and adenophostin A (Fig. 1 and not shown). Finally, with the high Ca\(^{2+}\) buffer protocol, all of the above store-depletion reagents activated \(I_{\text{crac}}\) to a similar extent with almost 100% success rate (not shown), suggesting that the difference between adenophostin A and other store-depletion reagents is only seen with the more physiological low Ca\(^{2+}\) buffering protocol.

From the experiment depicted in Fig. 9, some interesting observations about the nature of the Ca\(^{2+}\)-mediated inactivation of \(I_{\text{crac}}\) can be made. In this particular experiment, the initial sampling of \(I_{\text{crac}}\) occurred every second. This resulted in an elevated [Ca\(^{2+}\)]\(_{i}\) which did not return to baseline, suggesting that with this schedule, sufficient Ca\(^{2+}\) enters during the ramp (presumably at a very high rate due to diminished inactivation) to maintain the average [Ca\(^{2+}\)]\(_{i}\) at a level similar to that seen with sustained activation at a physiological membrane potential (as in Fig. 1). Yet, this sustained level of around 250 nM [Ca\(^{2+}\)]\(_{i}\), is obviously insufficient to induce inactivation because a significant inward current develops. This is consistent with the prior suggestion (20, 21) that Ca\(^{2+}\)-dependent inactivation of \(I_{\text{crac}}\) results from the action of Ca\(^{2+}\) on a site very close to the mouth of the channel and is insensitive to the [Ca\(^{2+}\)]\(_{i}\) in the bulk of the cytoplasm (22). Note that when the inter-ramp interval was increased to 5 s, the average [Ca\(^{2+}\)]\(_{i}\), declined substantially, whereas \(I_{\text{crac}}\) changed little. Removal and restoration of external Ca\(^{2+}\) reversed and reinstated \(I_{\text{crac}}\) with minimal effects on [Ca\(^{2+}\)]\(_{i}\). Finally, changing the holding potential to \(-60\) mV caused \(I_{\text{crac}}\) to disappear completely, because sustained entry of Ca\(^{2+}\) through the channel causes inactivation to a level below that which can be detected. How-
however, the steady-state \([\text{Ca}^{2+}]_i\) level at \(-60\) mV was not substantially different from that in the initial phase of the experiment, when the inter-ramp interval was 1 s but \(I_{\text{crac}}\) was well developed. Again, this is consistent with the conclusion that it is the \(\text{Ca}^{2+}\) concentration in the microenvironment of the \(\text{Ca}^{2+}\) channel rather than the global, cytoplasmic \([\text{Ca}^{2+}]_i\), which is responsible for inactivation of the \(\text{Ca}^{2+}\) entry channels. It is presumed that when \(\text{Ca}^{2+}\) entry occurs physiologically, i.e. at \(-60\) mV, the observed sustained entry of \(\text{Ca}^{2+}\) is due to \(I_{\text{crac}}\) but the level of the current is below that which can be detected with current technology. However, this has not been unequivocally established by either the current studies or any previous work on the electrophysiology of store-operated \(\text{Ca}^{2+}\) channels.

The low \(\text{Ca}^{2+}\)-buffering protocol with adenophostin A permits simultaneous measurement of \(I_{\text{crac}}\) activation and \(\text{Ca}^{2+}\) release, and thus the latencies for these two events can be directly compared. Fig. 10 illustrates a typical finding with adenophostin A; there was a clear delay for the activation of \(I_{\text{crac}}\) after \(\text{Ca}^{2+}\) release was initiated. Fig. 11 summarizes a number of experiments using this technique to simultaneously examine \(\text{Ca}^{2+}\) release and \(I_{\text{crac}}\) activation. Two concentrations of adenophostin A, 0.5 and 2.0 \(\mu\text{M}\), were employed, and as was seen in the earlier experiments, at both concentrations there was a significant delay between the initiation of release and the initiation of \(I_{\text{crac}}\).

**DISCUSSION**

Previous reports suggest that adenophostin A can activate \(\text{Ca}^{2+}\) influx either in the absence of \(\text{Ca}^{2+}\) release (7) or with minimal \(\text{Ca}^{2+}\) release (8). These investigations based their conclusions on the activities of \(\text{Ca}^{2+}\)-dependent \(\text{Cl}^-\) channels, an indirect reporter of \([\text{Ca}^{2+}]_i\) changes. In this study, we ex-
Adenophostin A and Ca$^{2+}$ Entry

Analyzed the effects of adenophostin A and (2,4,5)IP$_3$ on Ca$^{2+}$ entry assessed indirectly by measurement of net [Ca$^{2+}$]$_i$ changes and directly by measuring the store-operated Ca$^{2+}$ current ($I_{\text{crac}}$). For the most part, our results suggest that adenophostin A acts in a manner similar to that of (2,4,5)IP$_3$ in activating Ca$^{2+}$ entry, through release of intracellular Ca$^{2+}$, rather than as a direct activator of store-operated Ca$^{2+}$ channels or through some other mechanism independent of intracellular Ca$^{2+}$ store depletion. We conclude this because with adenophostin A (i) we never observed activation of entry in the absence of Ca$^{2+}$ release, and (ii) there was a substantial delay between the activation of release and entry, similar to that observed previously for (2,4,5)IP$_3$.

Had we only utilized [Ca$^{2+}$]$_i$ changes as an indicator of entry, we might have been misled into concluding that adenophostin was more efficient at inducing entry than (2,4,5)IP$_3$. With intermediate concentrations of (2,4,5)IP$_3$, net Ca$^{2+}$ entry was not maximal, despite apparent complete depletion of intracellular Ca$^{2+}$ stores (Fig. 3). There are two possible explanations for this phenomenon: either (i) (2,4,5)IP$_3$ (and not adenophostin A) activates a pathway for Ca$^{2+}$ removal, or (ii) despite the ability of (2,4,5)IP$_3$ to deplete stores completely in the absence of Ca$^{2+}$ entry (i.e. with the protocol shown in Fig. 2), in the presence of Ca$^{2+}$ entry some refilling of the stores occurs. We consider the latter alternative the more likely because previous studies have shown that (2,4,5)IP$_3$ acts as a partial agonist compared with (1,4,5)IP$_3$ when rate of release rather than extent of release is measured (23). Thus, in the absence of Ca$^{2+}$ entry, (2,4,5)IP$_3$ would be able to deplete completely the stores through submaximal activation of the (1,4,5)IP$_3$ receptor, but when entry elevates [Ca$^{2+}$]$_i$, some refilling would occur. Presumably adenophostin A is a full agonist for the receptor producing a maximal permeability increase, and little or no refilling would occur.

Despite similarities in the actions of adenophostin A and (2,4,5)IP$_3$, one particularly interesting and potentially significant difference was noted. Only adenophostin A was capable of consistently activating $I_{\text{crac}}$ in cells patch-clamped with low Ca$^{2+}$ buffer solutions, i.e. when [Ca$^{2+}$]$_i$ was permitted to fluctuate. (2,4,5)IP$_3$ failed to support $I_{\text{crac}}$ under this condition, even when employed at a concentration of 100 μM; at this concentration its Ca$^{2+}$ release and Ca$^{2+}$ entry activation appear maximal and similar to adenophostin A (Table I and Fig. 3). It has not previously been possible to observe $I_{\text{crac}}$ in RBL cells without the presence of high concentrations of intracellular buffers. In the present study, even with adenophostin A $I_{\text{crac}}$ could only be observed when entry was largely prevented by the use of a positive holding potential. We assume that during calcium entry under physiological conditions, i.e. when an increase in the fura-2 signal is observed, this entry is mediated at least in part by $I_{\text{crac}}$ at a level below that which can be detected.

| Store-depletion reagent | Concentration (μM) | Number of cells showing detectable $I_{\text{crac}}$/total cells tested |
|-------------------------|-------------------|---------------------------------------------------------------|
| AdA                     | 0.5               | 17/18                                                         |
|                         | 2                 | 6/6                                                           |
| (2,4,5)IP$_3$ (Sigma)   | 50                | 0/3                                                           |
|                         | 100               | 0/4                                                           |
| (2,4,5)IP$_3$ (Calbiochem) | 50         | 1/6                                                           |
|                         | 100               | 0/3                                                           |
| TG$^*$                  | 50                | 2/8                                                           |
|                         | 100               | 0/2                                                           |
| Ionomycin               | 2                 | 0/3                                                           |
|                         | 5                 | 0/3                                                           |

$^*$ TG is thapsigargin.

![Fig. 10](image1.png)

**Fig. 10.** Latency for activation of $I_{\text{crac}}$ and release of Ca$^{2+}$ assessed simultaneously in an RBL-1 cell patched with a pipette containing 2 μM adenophostin A. The figure to the right shows an expansion of the initial 50 s of the experiments revealing a clearly longer delay for $I_{\text{crac}}$ initiation compared with the delay required for the initiation of Ca$^{2+}$ release.

![Fig. 11](image2.png)

**Fig. 11.** Relationship between Ca$^{2+}$ release and $I_{\text{crac}}$ activation utilizing the method for simultaneous assessment of Ca$^{2+}$ release and $I_{\text{crac}}$. The protocol was as depicted in Fig. 9 and the kinetic parameters as defined in Fig. 7. At both 0.5 and 2.0 μM adenophostin A, the latency for the development of $I_{\text{crac}}$ was significantly greater than the latency for initiation of Ca$^{2+}$ release as well as the time to peak Ca$^{2+}$ release. The data summarize data from 14 experiments at 0.5 μM and 5 experiments at 2.0 μM adenophostin A.
with present patch clamp technology. Adenophostin A provides for the first time a means to monitor the activity of the channels underlying $I_{\text{crac}}$ while observing changes in $[Ca^{2+}]_i$ due to intracellular $Ca^{2+}$ release.

Despite the appearance that adenophostin A and (2,4,5)IP$_3$ release $Ca^{2+}$ and activate $I_{\text{crac}}$ by similar mechanisms, we conclude that adenophostin A has some additional, unique action which (2,4,5)IP$_3$ does not share. Adenophostin A does not appear to prevent $Ca^{2+}$-dependent inactivation totally, as shown by the results in Fig. 9, that is when $Ca^{2+}$ entry proceeds continuously through the channels, neither agent produces detectable $I_{\text{crac}}$ because $Ca^{2+}$-dependent inactivation makes the current too small to detect. Note that $Ca^{2+}$-dependent inactivation is assumed to underlie the failure of (2,4,5)IP$_3$, thapsigargin, and ionomycin to activate detectable $I_{\text{crac}}$ with low $Ca^{2+}$ buffers, but this is not proven. The $Ca^{2+}$-dependent inactivation that occurs close to the mouth of the channel (21) should be considerably reduced by holding at +30 mV. However, there are other slower mechanisms of inactivation (24) that may be more sensitive to the global $[Ca^{2+}]$ in the cytoplasm. One possibility is that adenophostin A prevents or attenuates only the rapid inactivation that occurs in or near the cytoplasmic opening of the store-operated calcium channel. Regardless of the explanation, the ability to measure physiologically relevant changes in $[Ca^{2+}]_i$ resulting from $Ca^{2+}$ release simultaneously with measurement of $I_{\text{crac}}$ will be a useful tool for future research into the quantitative and temporal relationships between $Ca^{2+}$ discharge and $I_{\text{crac}}$ activation. One example in the present study is the clear demonstration of a delay between the release of $Ca^{2+}$ and the initiation of $I_{\text{crac}}$. This delay supports the idea of a diffusible signal responsible for the activation of the capacitative calcium entry channels, rather than a direct physical interaction between the endoplasmic reticulum and the plasma membrane (4). Such an observation has been made previously with (2,4,5)IP$_3$, but it was necessary to compare cells activated under very different conditions (release with low buffering; $I_{\text{crac}}$ with high buffering) (15) weakening the interpretation of these experiments considerably.

Unlike the previous work with Xenopus oocytes (7, 8), we found no evidence for a dissociation between the ability of adenophostin A to release $Ca^{2+}$ and its ability to activate entry. The substantial delay between release of $Ca^{2+}$ and activation of $I_{\text{crac}}$ argues against a direct activation of the channels by adenophostin A. The findings in oocytes may be related to the same property of adenophostin A which in the current study results in the consistent activation of $I_{\text{crac}}$ under low $Ca^{2+}$-buffering conditions. Alternatively, the studies in oocytes utilized $Ca^{2+}$-dependent chloride currents for assessment of $[Ca^{2+}]_i$ changes, and it is possible that they may sometimes underestimate the extent of $Ca^{2+}$ release, particularly if it occurs at sites which are not in the proximity of the $Ca^{2+}$-sensitive chloride channels. Regardless of the explanation, it is clear from the earlier work in oocytes and from the present work in RBL-1 cells that adenophostin A may have interesting actions in addition to its ability to activate (1,4,5)IP$_3$ receptors with high affinity. It may thus prove a useful tool for unraveling the molecular pathways that regulate capacitative calcium entry.

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