Adenophostin A and ribophostin, but not inositol 1,4,5-trisphosphate or *manno*-adenophostin, activate the Ca^{2+} release-activated Ca^{2+} current, I_{CRAC}, in weak intracellular Ca^{2+} buffer

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Under physiological conditions of weak intracellular Ca^{2+} buffering (0.1 mM EGTA), the second messenger Ins(1,4,5)P_3 often fails to activate any detectable store-operated Ca^{2+} current. However, it has been reported that the fungal metabolite adenophostin A [which has a severalfold higher affinity than Ins(1,4,5)P_3 for Ins(1,4,5)P_3 receptors] consistently activates the current under similar conditions. Here, whole-cell patch clamp experiments have been performed to examine how adenophostin A can activate the store-operated Ca^{2+} current (I_{CRAC}) in RBL-1 (rat basophilic leukaemia) cells. In a strong intracellular Ca^{2+} buffer, saturating concentrations of adenophostin A activated I_{CRAC} maximally and the current amplitude and kinetics were indistinguishable from those obtained with high concentrations of Ins(1,4,5)P_3. In a weak Ca^{2+} buffer, adenophostin A consistently activated I_{CRAC}, but the current was submaximal. High concentrations of Ins(1,4,5)P_3 or the non-metabolizable analogue Ins(2,4,5)P_3 were largely ineffective under these conditions. The size of I_{CRAC} to adenophostin A in weak Ca^{2+} buffer could be significantly increased by either inhibiting sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase ('SERCA') pumps with thapsigargin or enhancing mitochondrial Ca^{2+} uptake, although blocking the mitochondrial Ca^{2+} uniporter with Ruthenium Red did not suppress the activation of the current. Changing the levels of free ATP in the recording pipette did not enhance the size of I_{CRAC} evoked by adenophostin A. We also examined two structurally distinct analogues of adenophostin A (*manno*-adenophostin and ribophostin), for which the affinities for the Ins(1,4,5)P_3 receptor are similar to that of Ins(1,4,5)P_3 in equilibrium binding experiments. Although these analogues were able to activate I_{CRAC} to its maximal extent in strong buffer, ribophostin, but not *manno*-adenophostin, consistently activated the current in weak buffer. We conclude that adenophostin A and ribophostin are able to activate I_{CRAC} in weak buffer through a mechanism that is quite distinct from that employed by Ins(1,4,5)P_3 and *manno*-adenophostin and is not related to equilibrium affinities.

Key words: calcium release, calcium stores, store-operated calcium entry.

INTRODUCTION

In non-excitable cells, one major source of Ca^{2+} influx is through the store-operated pathway by which the process of emptying the intracellular Ca^{2+} stores results in the activation of store-operated Ca^{2+} channels in the plasma membrane [1,2]. Although several types of store-operated channel have been described electrophysiologically, the best characterized and most widely distributed are the CRAC (Ca^{2+} release-activated Ca^{2+}) channels [3,4].

The Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) has generally been measured in the presence of strong intracellular Ca^{2+} buffer [several mM EGTA or bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid (BAPTA)] because this decreases both store refilling and Ca^{2+}-dependent inactivation of the channels [4]. Under these conditions, the second messenger Ins(1,4,5)P_3 activates I_{CRAC} to its maximal extent in RBL-1 (rat basophilic leukaemia) cells [5,6]. However, in the presence of weak Ca^{2+} buffer (0.1 mM EGTA or BAPTA), Ins(1,4,5)P_3 is largely ineffective in spite of releasing Ca^{2+} from the stores [6–11]. It seems that sufficient Ca^{2+} is taken back up into the stores by the very active sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) pumps so that the store Ca^{2+} content not to fall sufficiently for I_{CRAC} to activate. Only when the SERCA pumps are blocked or some of the released Ca^{2+} is taken up into mitochondria can Ins(1,4,5)P_3 activate I_{CRAC} in weak Ca^{2+} buffer [10,11].

Although Ins(1,4,5)P_3 is the most potent physiological agonist of Ins(1,4,5)P_3-gated Ca^{2+} channels, the fungal metabolite adenophostin A is much more potent in releasing Ca^{2+} from internal stores [12]. This is thought to reflect the approx. 10-fold higher affinity of adenophostin A for Ins(1,4,5)P_3 receptors compared with that of Ins(1,4,5)P_3. Apart from this difference, adenophostin A is believed to interact with Ins(1,4,5)P_3 receptors in a manner identical with that for Ins(1,4,5)P_3. Like Ins(1,4,5)P_3, the adenophostins show a quantal pattern of Ca^{2+} release [13], evoke intracellular Ca^{2+} oscillations [7] and induce the down-regulation of type I Ins(1,4,5)P_3 receptors after chronic exposure to the ligand [14]. Furthermore, both Ca^{2+}-dependent activation and inactivation of receptors are indistinguishable in the presence of saturating concentrations of either Ins(1,4,5)P_3 or adenophostin A [15], and calmodulin promotes the inactivation of Ca^{2+} release irrespective of whether either Ins(1,4,5)P_3 or adenophostin A is the activating ligand [16]. At the single-channel level, open and closed channel dwell times, conductance and Ca^{2+} dependence are identical for Ins(1,4,5)P_3 and adenophostin A [17].

However, experiments using adenophostin A to activate store-operated Ca^{2+} entry are not easily reconciled with the notion that adenophostin A interacts with the Ins(1,4,5)P_3 receptor in a manner indistinguishable from that of Ins(1,4,5)P_3. In RBL-1 cells, adenophostin A was found to activate I_{CRAC} in weak Ca^{2+} buffer whereas Ins(1,4,5)P_3 was ineffective under similar condi-

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid; RBL, rat basophilic leukaemia; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase.

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tions [7,8]. It was suggested that, because of their higher affinity for adenophostin A, Ins(1,4,5)P_3 receptors might be less sensitive to Ca^{2+}-dependent inactivation in the presence of adenophostin A than to Ins(1,4,5)P_3 [8], although more recent reports on Ins(1,4,5)P_3 receptors do not indicate such a difference (see above). Additionally, adenophostin A, but not Ins(1,4,5)P_3, might have decreased the Ca^{2+}-dependent inactivation of CRAC channels [7].

It is important to understand why adenophostin A is more effective than Ins(1,4,5)P_3 in activating I_{CRAC} in weak Ca^{2+} buffer, because this will provide insight into the regulation of store-operated influx under physiological conditions. In the present study, we examined various potential mechanisms whereby adenophostin A might be more effective than Ins(1,4,5)P_3 in activating I_{CRAC}; we also investigated the effects of two structurally distinct analogues of adenophostin A (*manno*-adenophostin and ribophostin) that have similar affinities to Ins(1,4,5)P_3 for the Ins(1,4,5)P_3 receptor, and we compared these with Ins(1,4,5)P_3 and adenophostin A.

EXPERIMENTAL

RBL-1 cells were bought from the Cell Bank at the Sir William Dunn School of Pathology, Oxford University and were cultured as described previously [6,9].

Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (20–23 °C) as described previously [5,6]. Sylgard-coated, fire-polished pipettes had d.c. resistances of 3.5–5 MΩ when filled with standard internal solution that contained (in mM): caesium glutamate 145, NaCl 8, $MgCl_2$ 1, EGTA 0.1, $MgATP^{2-}$ 2 and Hepes 10, made to pH 7.2 with CsOH. A correction of +10 mV was applied to the subsequent liquid junction potential that arose from this glutamate-based internal solution. In some experiments, cells were dialysed with a pipette solution in which Ca^{2+} was strongly buffered at 140 nM (10 mM EGTA/4.6 mM $CaCl_2$). Mitochondrial cocktail contained (in mM): pyruvic acid 2, malic acid 2, NaH_2PO_4 1, cAMP 0.5 and GTP 0.5 and $MgCl_2$ 0.5. Extracellular solution contained (in mM): NaCl 145, KCl 2.8, $CaCl_2$ 10, $MgCl_2$ 2, CsCl 10, glucose 10, Hepes 10, made to pH 7.4 with NaOH. I_{CRAC} was measured as described previously [5] by applying voltage ramps (−100 to +100 mV in 50 ms) at 0.5 Hz from a holding potential of 0 mV. Currents were filtered with an eight-pole Bessel filter at 2.5 kHz and digitized at 100 μs. Currents were normalized by dividing the amplitudes (measured from the voltage ramps at −80 mV) by the cell capacitance. Compensation was made for capacitive currents before each ramp by using the automatic compensation of the EPC 9-2 amplifier. All leak currents were subtracted by averaging the first few ramp currents (usually two) and then subtracting this from all subsequent currents.

Adenophostin A and *manno*-adenophostin were synthesized as described [18] and were used as the sodium salts. Ribophostin was synthesized as described [19] and used as the hexapotassium salt. The structures of Ins(1,4,5)P_3 and of the analogues are shown in Figure 1. Thapsigargin was purchased from Alomone laboratories. Ins(1,4,5)P_3 was from Calbiochem. Ins(2,4,5)P_3 was a gift from Andrew Letcher and Robin Irvine (Cambridge, U.K). All other chemicals were purchased from Sigma.

RESULTS

Activation of I_{CRAC} by adenophostin A in strong Ca^{2+} buffer

Figure 2(A) (▲) shows the time course of activation of I_{CRAC} for an RBL-1 cell dialysed with a patch pipette solution containing 1 μM adenophostin A (see Figure 1 for structure). The pipette Ca^{2+} concentration was strongly buffered at 140 nM to prevent the spontaneous depletion of stores [9]. The current–voltage relationship (taken at 80 s) is depicted in Figure 2(B). Decreasing the adenophostin A concentration to 200 nM also activated I_{CRAC} but only after a sizeable delay (Figure 2A, ●). A lower concentration (5 nM) failed to activate any current (Figure 2A, ○; the current–voltage relation is shown in Figure 2B). Figure 2(C) plots the amplitude of I_{CRAC} against adenophostin A concentration (each point is the mean of 5–12 cells). Whereas adenophostin A or less failed to generate any current at 5 nM,

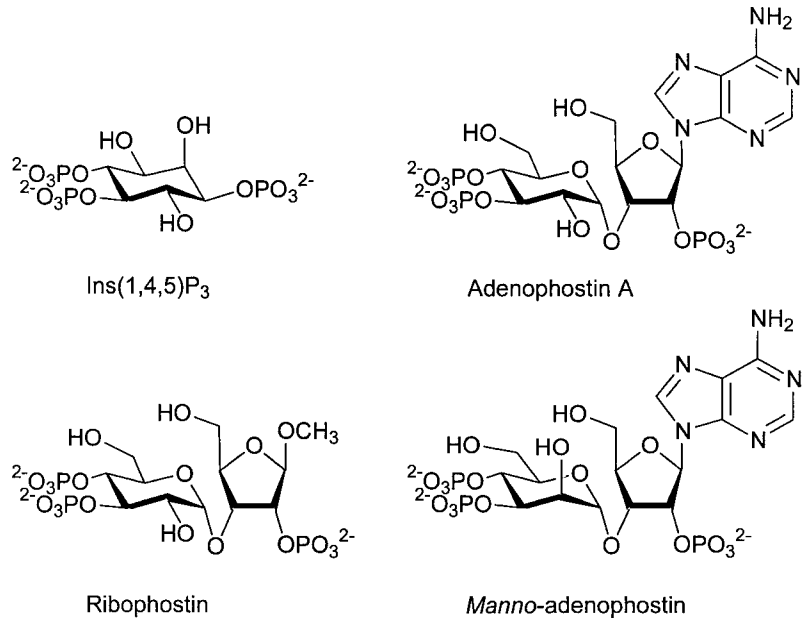


Figure 1 Structures of Ins(1,4,5)P_3, adenophostin A, ribophostin and *manno*-adenophostin

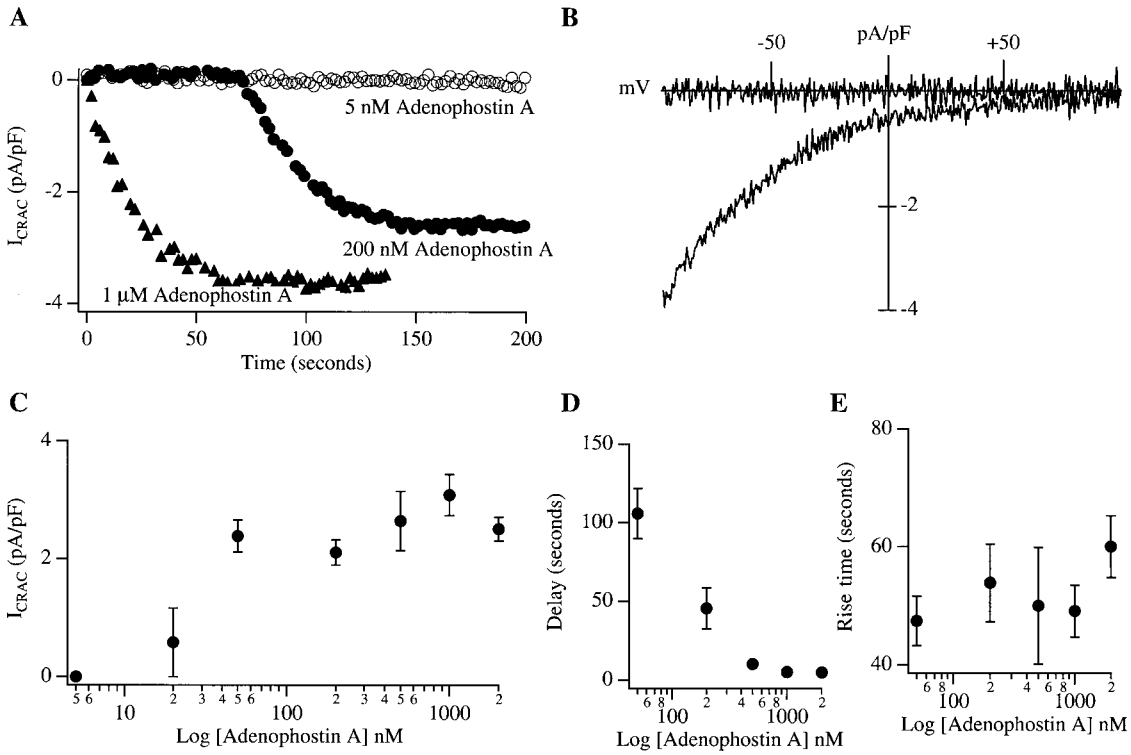


Figure 2 Ability of different concentrations of adenophostin A to activate I_{CRAC} in strong intracellular Ca^{2+} buffer

(**A**) Time course of I_{CRAC} activation after dialysis with different concentrations of adenophostin A as indicated. (**B**) The current–voltage relationships for 1 μM adenophostin A (taken at 80 s) and 5 nM adenophostin A (taken at 100 s, and which failed to respond) are shown. (**C**) Adenophostin A concentration plotted against I_{CRAC} amplitude for several cells. (**D**, **E**) The delay before I_{CRAC} activates (**D**) and the rise time (time over which the current increases from 10 to 90% of steady-state amplitude) (**E**) are plotted against adenophostin A concentration. Values are means ± S.E.M.

50 nM was maximally effective. A concentration of 20 nM was close to the threshold concentration in that only one of five cells responded; however, the current in this cell was maximal (−3 pA/pF). The delay before I_{CRAC} activated and the rate of development of the current (measured as the rise time from 10% to 90% of the steady-state current amplitude) as a function of adenophostin A concentration are shown in Figures 2(D) and 2(E). As the adenophostin concentration increased (range 50–500 nM), the delay decreased significantly ($P < 0.0.1$) but the rise time remained relatively constant. Activation of I_{CRAC} by a maximally effective dose of adenophostin A is virtually indistinguishable from that seen with a maximal concentration of Ins(1,4,5)P_3 in that the delay, activation time-constant, peak amplitude and time to peak are very similar [see [5,6,9] for the kinetics of I_{CRAC} response to Ins(1,4,5)P_3].

Activation of I_{CRAC} by adenophostin A in weak Ca^{2+} buffer

We repeated the above experiments but now in the presence of weak, and hence more physiological, levels of intracellular Ca^{2+} buffering. Cells were dialysed with different concentrations of adenophostin A in the presence of weak buffer (0.1 mM EGTA). The time courses of activation of I_{CRAC} for three different concentrations of adenophostin A are shown in Figure 3(A) and the corresponding dose–response curve is plotted in Figure 3(B). Although adenophostin A activated I_{CRAC} in weak buffer (consistent with [7,8]), it was striking that even a maximally effective concentration (1 μM) only activated the current partly, such that the size of the current was almost 4-fold smaller than in strong buffer (−0.81 ± 0.14 pA/pF compared with −3.18 ± 0.25 pA/pF respectively; means ± S.E.M.). The kinetics of I_{CRAC} development was also altered in the presence of weak buffer in that, with 1 μM adenophostin A, the delay before I_{CRAC} activated was significantly longer (approx. 14-fold) than in strong buffer (compare Figure 3C with Figure 2D). The rise time was also slower in weak buffer but to a smaller extent (compare Figure 3D with Figure 2E).

Comparison of maximally effective concentrations of adenophostin A with Ins(1,4,5)P_3 and Ins(2,4,5)P_3 in weak Ca^{2+} buffer

A maximally effective concentration of Ins(1,4,5)P_3 (30 μM) fails to activate any detectable I_{CRAC} in weak Ca^{2+} buffer in most RBL-1 cells. Only between 10% and 30% of the cells respond and the current is submaximal ([6–9]; see also Figure 8). In contrast, almost all cells (18 of 19) respond to adenophostin A under similar conditions (Figure 2). Hence in the presence of weak Ca^{2+} buffer, the probability that a cell will respond to adenophostin A is much higher than for Ins(1,4,5)P_3 (both at maximally effective concentrations). The amplitude of I_{CRAC} for those cells that did respond to 30 μM Ins(1,4,5)P_3 was around −1 pA/pF [20]. Strikingly, this was similar to the amplitude seen with adenophostin A (Figure 3B). This suggests that, if a cell responded to Ins(1,4,5)P_3, then it did so by evoking a current similar in size to that evoked by adenophostin A. Hence, in weak Ca^{2+} buffer, adenophostin A seemed only to increase the probability of activating I_{CRAC} but did not affect the subsequent size.

Unlike adenophostin A, Ins(1,4,5)P_3 is rapidly metabolized both by Ins(1,4,5)P_3 3-kinases and 5-phosphatases [21]. To determine whether Ins(1,4,5)P_3 metabolism was a major factor

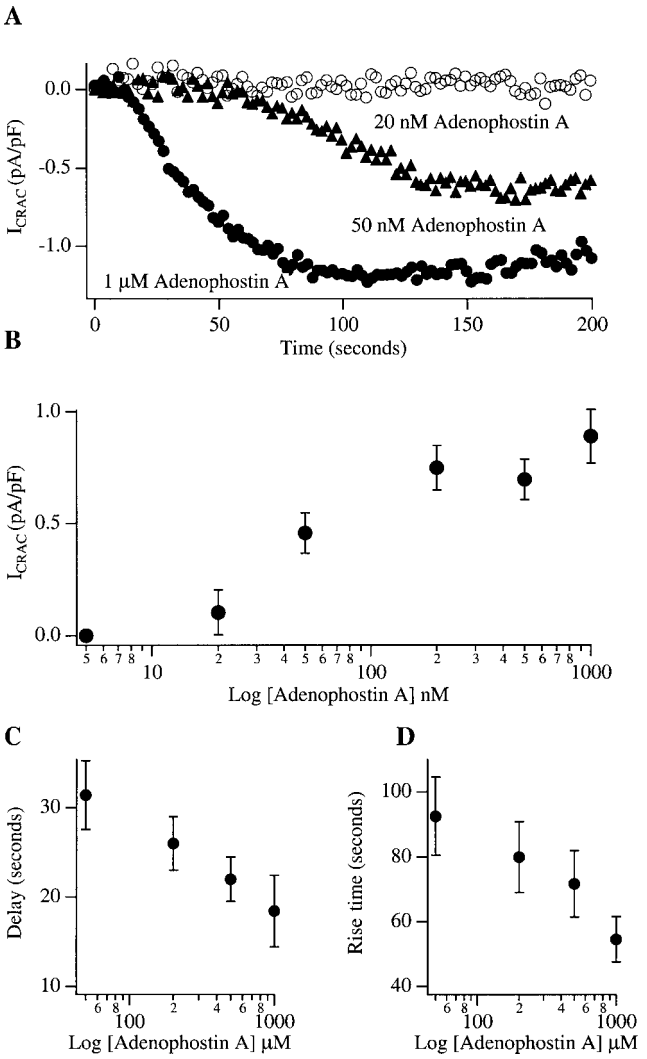


Figure 3 Ability of different concentrations of adenophostin A to activate I_{CRAC} in weak intracellular Ca^{2+} buffer

(**A**) Time course of I_{CRAC} after dialysis with different concentrations of adenophostin A as indicated. (**B**) Relationship between adenophostin A concentration and size of I_{CRAC}. (**C**, **D**) The delay before I_{CRAC} activates (**C**) and the rise time (**D**) are plotted against adenophostin A concentration. Values are means ± S.E.M.

underlying its relative inability to activate I_{CRAC} in weak Ca^{2+} buffer, we used the non-metabolizable analogue Ins(2,4,5)P_3 instead. Cells were dialysed with 50 μM Ins(2,4,5)P_3, a maximally effective concentration. However, I_{CRAC} was still very small (the mean was -0.43 ± 0.15 pA/pF; nine cells) and only 44 % of cells responded. These results were consistent with a previous report, in which Ins(2,4,5)P_3 was also ineffective [7].

Adenophostin A and Ca^{2+}-dependent fast inactivation

To account for the greater ability of adenophostin A than Ins(1,4,5)P_3 to activate I_{CRAC} in weak buffer, it has been suggested that the former might decrease the amount of fast Ca^{2+}-dependent inactivation of CRAC channels [7]. To test this, we evoked different extents of fast inactivation and compared this between control cells [where I_{CRAC} was evoked by Ins(1,4,5)P_3] and those in which the current was activated by adenophostin A instead. Fast inactivation was induced by applying hyperpolarizing steps from 0 mV to more negative voltages (-100 to -40) for 250 ms. We used strong Ca^{2+} buffer in these experiments (buffered at 140 nM Ca^{2+} to prevent the passive depletion of stores) because the current was maximal under these conditions, hence increasing our resolution. The upper panel in Figure 4(A) shows a typical voltage protocol to -80 mV; the lower panel shows recordings taken in 30 μM Ins(1,4,5)P_3 and 1 μM adenophostin A. In both cases the current declines at a similar rate to reach a similar extent at the end of the hyperpolarizing pulse. Pooled data from several cells are summarized in Figure 4(B). The more negative the potential, the greater is the electrical driving force for Ca^{2+} influx, and hence the greater the extent of fast inactivation [22,23]. However, the extent of inactivation of I_{CRAC} when adenophostin A was used to evoke the current was very similar to that seen in control cells. Hence adenophostin A did not decrease the ability of Ca^{2+} to induce fast inactivation in strong buffer.

In three cells in which adenophostin A evoked a current greater than -1 pA/pF in weak buffer, we applied hyperpolarizing steps to -80 mV and measured the extent of inactivation. This was 48 ± 5 % and was not significantly different from that seen in strong buffer. Hence adenophostin A did not seem to alter the extent of fast inactivation in either strong or weak buffer.

Adenophostin A only partly depletes Ca^{2+} stores in weak Ca^{2+} buffer

The submaximal I_{CRAC} evoked by adenophostin A in weak Ca^{2+} buffer could reflect a partial depletion of stores to below the level required for I_{CRAC} to activate fully. If this is true, then the size of I_{CRAC} evoked by adenophostin A ought to be increased by promoting further store emptying. Figure 5(A) shows that the size of I_{CRAC} response to 1 μM adenophostin A was increased by the inclusion of 2 μM thapsigargin, a SERCA pump blocker, in the recording pipette ($P < 0.01$). However, neither the delay before the current started to activate nor the rate of development was affected (Figures 5B and 5C). Hence adenophostin A emptied Ca^{2+} stores sufficiently for I_{CRAC} to activate in weak buffer but not to the extent that the current activated fully. Store refilling could occur in spite of the continuous presence of a saturating concentration of adenophostin A.

Effects of mitochondrial Ca^{2+} uptake on I_{CRAC} evoked by adenophostin A

Although Ins(1,4,5)P_3 is largely ineffective in activating I_{CRAC} in weak Ca^{2+} buffer, it can consistently evoke the current provided mitochondria are maintained in an energized state in the whole-cell configuration of the patch-clamp technique [11]. We considered that adenophostin A might modulate mitochondrial Ca^{2+} uptake directly, which might explain its greater potency than that of Ins(1,4,5)P_3 in activating I_{CRAC} in weak buffer. However, dialysis with 100 μM Ruthenium Red (an inhibitor of the mitochondrial uniporter) failed to alter the size of I_{CRAC} evoked by 1 μM adenophostin A (-0.82 ± 0.2 compared with 0.7 ± 0.1 pA/pF in the presence and the absence of Ruthenium Red; seven cells each). Dialysis with the mitochondrial cocktail solution together with adenophostin A resulted in a small but significant increase in the size of the current (-1.1 ± 0.12 compared with -0.7 ± 0.1 pA/pF in the presence and the absence of cocktail; $P < 0.05$; seven cells each). Mitochondrial Ca^{2+} uptake was therefore not required for adenophostin A to activate I_{CRAC}, but it enhanced the size of the current.

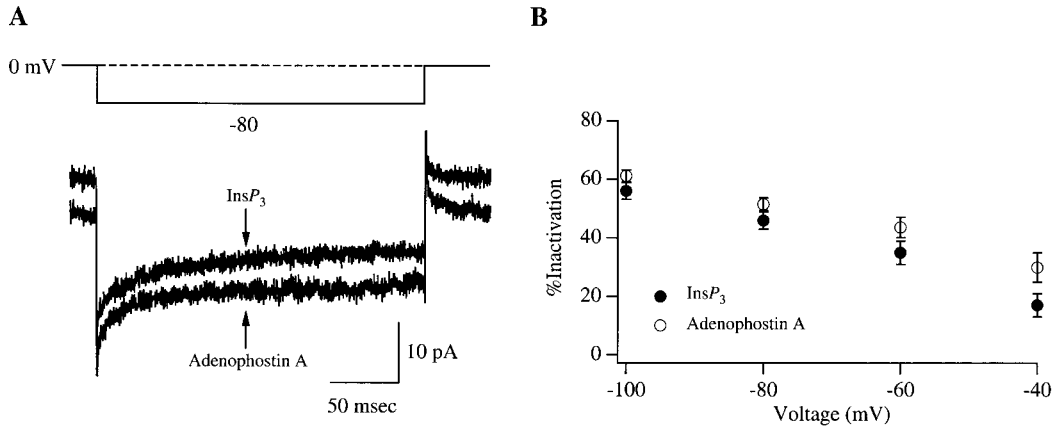


Figure 4 Adenophostin A does not alter the extent of Ca^{2+}-dependent fast inactivation

(**A**) Upper panel: the voltage protocol used to induce fast inactivation. Lower panel: raw traces. (**B**) The extent of inactivation (measured as the steady-state current at the end of the pulse divided by the current amplitude 2 ms after the onset of the pulse; see [23]) is plotted against the size of the hyperpolarizing pulse. The extent of inactivation was similar for control cells (●) [in which I_{CRAC} was evoked by Ins(1,4,5)P_3] compared with those dialysed with 1 μM adenophostin A (○). Values are means ± S.E.M.

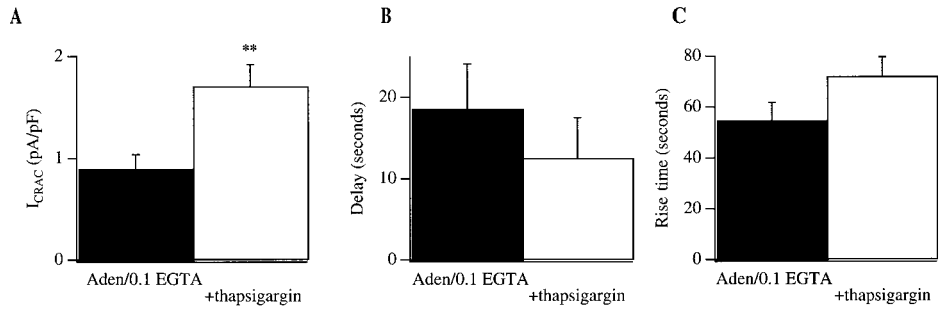


Figure 5 Thapsigargin increases the size of I_{CRAC} evoked by adenophostin A in weak Ca^{2+} buffer

(**A**) The mean amplitude of I_{CRAC} to 1 μM adenophostin A in weak (0.1 mM EGTA) buffer is compared with that obtained when the SERCA pump blocker thapsigargin (2 μM) was also included. The difference in amplitudes was significant ($P < 0.01$). (**B**, **C**) The delay before I_{CRAC} activated (**B**) was not significantly altered by the additional presence of thapsigargin, nor was the rise time of the current (**C**). I_{CRAC} was measured as in Figure 2 by using voltage ramps (−100 to +100 mV in 50 ms) applied every 2 s. Values are means ± S.E.M.

Effects of varying the concentration of free ATP on I_{CRAC} response to adenophostin A in weak Ca^{2+} buffer

Single-channel studies on type I Ins(1,4,5)P_3 receptors in the outer membrane of isolated *Xenopus laevis* oocyte nuclei have suggested that the potency of adenophostin A apparently depends on the ambient concentration of free ATP [17]. In the presence of free ATP (0.5 mM), adenophostin A was 60 times more potent than Ins(1,4,5)P_3, whereas in the absence of ATP adenophostin was only twice as potent. Free ATP in our internal solution was estimated to be 52 μM. We therefore increased free ATP levels in weak Ca^{2+} buffer by using two different methods to see whether this increased the size of I_{CRAC} response to adenophostin A. First, we used 10 mM $MgATP^{2-}$ (no added free Mg^{2+}; calculated free ATP 0.506 mM; calculated free Mg^{2+} 0.504 mM); secondly, we used a mixture of 2 mM Mg^{2+} and 2.5 mM K_2ATP^{2-} (calculated free ATP 0.587 mM; calculated free Mg^{2+} 0.088 mM). We first investigated the effects of free ATP on the amplitude and rate of activation of the current after dialysis with Ins(1,4,5)P_3 in strong buffer. The results are summarized in Figure 6. In comparison with our standard conditions (1 mM Mg^{2+}/2 mM $MgATP^{2-}$), the amplitude of I_{CRAC} was decreased only slightly in the presence of 10 mM $MgATP^{2-}$ but not when K_2ATP^{2-} was used instead (Figure 6A). Neither the delay before the current developed (Figure 6B) nor the rise time (Figure 6C) was significantly affected. In weak buffer, increasing the free ATP concentration failed to increase the size of I_{CRAC} in comparison with our standard conditions (Figure 6A, right-hand histogram). Similarly, neither the delay before the current started to develop (Figure 6B) nor the rise time (Figure 6C) was significantly affected by increasing the free ATP levels.

Effects of adenophostin A analogues on I_{CRAC}

Several analogues of adenophostin A have been developed that exhibit different potencies towards the Ins(1,4,5)P_3 receptor. *Manno*-adenophostin and ribophostin (see Figure 1 for structures) are two such compounds that were particularly effective for our purposes because they both have similar affinities to that of Ins(1,4,5)P_3 for the Ins(1,4,5)P_3 receptor, as well as similar EC_{50} values to Ins(1,4,5)P_3 for Ca^{2+} release in permeabilized hepatocytes [24]. If the higher affinity of adenophostin A than that of Ins(1,4,5)P_3 for Ins(1,4,5)P_3 receptors was the main factor

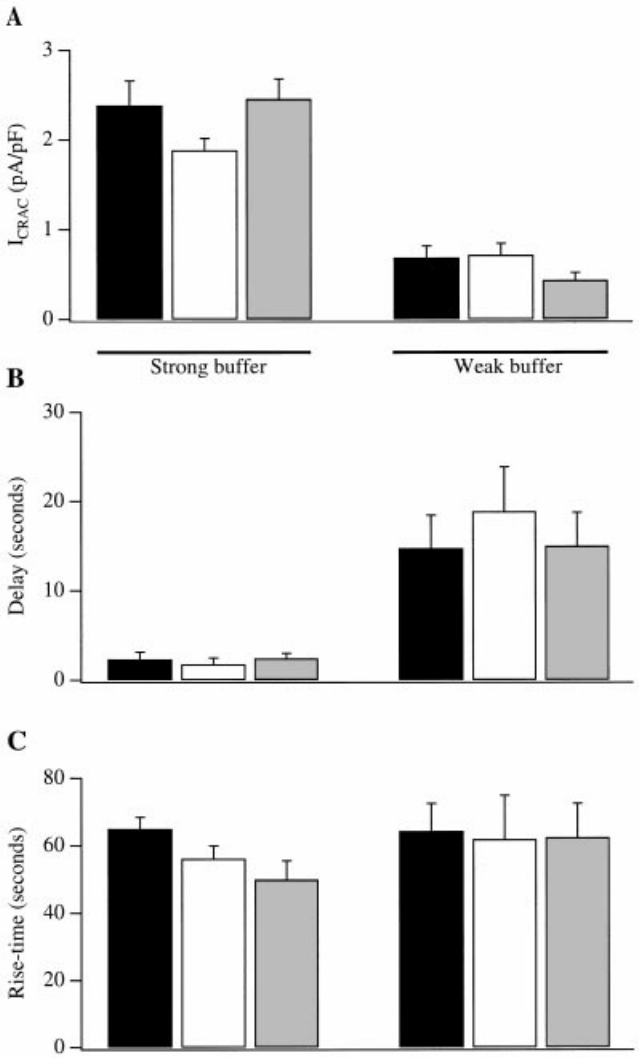


Figure 6 Effect of increasing the concentration of free ATP on I_{CRAC}

(**A**) The left-hand histogram plots the size of I_{CRAC} after dialysis with Ins(1,4,5)P_3 (30 μM) and strong buffer (10 mM EGTA) for control conditions (filled bar, 1 mM $MgCl_2$ and 2 mM $MgATP^{2-}$ in the recording pipette) compared with recordings in 10 mM $MgATP^{2-}$ (open bar, no added $MgCl_2$ and 10 mM $MgATP^{2-}$) and K_2ATP^{2-} (grey bars, 2 mM $MgCl_2$ and 2.5 mM K_2ATP^{2-}). The right-hand histogram summarizes aggregate data when cells were dialysed with 1 μM adenophostin A in weak Ca^{2+} buffer in the presence of different free ATP levels. (**B**, **C**) A comparison of the delay (**B**) and rise time (**C**) of the currents under the different conditions. There were no significant differences between the size of I_{CRAC}, the delay and the rise time for the various conditions in strong buffer or weak buffer. Note that the size of I_{CRAC} and also the delay were still significantly smaller in weak buffer than in strong buffer for each given condition (i.e. compare 10 mM $MgATP^{2-}$ in weak and strong buffers). Values are means $\pm$ S.E.M.

underlying its ability to activate I_{CRAC} in weak Ca^{2+} buffer, then one would predict that neither *manno*-adenophostin nor ribophostin should activate the current in weak buffer because these analogues have similar affinities to that of Ins(1,4,5)P_3 for the Ins(1,4,5)P_3 receptor. We therefore examined whether *manno*-adenophostin and ribophostin activated I_{CRAC} in weak buffer.

Activation of I_{CRAC} by *manno*-adenophostin and ribophostin in strong Ca^{2+} buffer

Figure 7(A) plots the concentration of each analogue against the amplitude of I_{CRAC} in strong buffer. For both analogues, concentrations approx. 0.1 μM were at the threshold for eliciting a response, and maximally effective concentrations were greater than 1 μM. In contrast, 50 nM adenophostin A was maximally effective (Figure 2), indicating that both *manno*-adenophostin and ribophostin were approx. 20 times less potent. This is consistent with Ca^{2+} release data from permeabilized hepatocytes [24]. Both the delay before I_{CRAC} activated (Figure 7B) and the rise time (Figure 7C) were similar when the current was evoked by either analogue. Superimposed in Figure 7 are data obtained under identical conditions with 10 μM Ins(1,4,5)P_3, a maximally effective concentration. There were no significant differences between any of the parameters measured for Ins(1,4,5)P_3, *manno*-adenophostin and ribophostin (all at 10 μM).

Activation of I_{CRAC} by *manno*-adenophostin and ribophostin in weak Ca^{2+} buffer

Figure 8 compares the ability of *manno*-adenophostin, ribophostin and Ins(1,4,5)P_3 (all at saturating concentrations of 30 μM) to activate I_{CRAC} in weak Ca^{2+} buffer. *Manno*-adenophostin, like Ins(1,4,5)P_3, was relatively ineffective (Figure 8A). Only 7 of 15 cells responded and the mean amplitude of the current was small (Figure 8B). However, results with ribophostin were markedly different: all cells (14 of 14) responded and the current was significantly larger than that with *manno*-adenophostin or Ins(1,4,5)P_3 (Figure 8B; $P < 0.01$). The size of I_{CRAC} evoked by ribophostin was not significantly different from that with adenophostin A. The kinetics of development of the currents, for cells that responded, was not significantly different between Ins(1,4,5)P_3, *manno*-adenophostin and ribophostin (results not shown).

DISCUSSION

Our main finding is that adenophostin A and ribophostin both activate I_{CRAC} in weak intracellular Ca^{2+} buffer, whereas Ins(1,4,5)P_3, Ins(2,4,5)P_3 and *manno*-adenophostin are largely ineffective. All agonists are equally effective in strong buffer. Resolution of this is important not only for the interpretation of results using adenophostin A but also because it will provide new insight into the regulation of store-operated Ca^{2+} influx under physiological conditions of weak intracellular Ca^{2+} buffering.

Equilibrium binding studies have revealed that adenophostin A has a much higher affinity than Ins(1,4,5)P_3 for Ins(1,4,5)P_3 receptors [24]. Because open channel duration is inversely related to the rate of agonist dissociation, one simple explanation is that Ins(1,4,5)P_3-sensitive channels stay open longer with adenophostin A bound than with Ins(1,4,5)P_3; this would translate into greater store depletion and hence a larger I_{CRAC}. However, the rate of dissociation of adenophostin A is only 1.9-fold slower than that of Ins(1,4,5)P_3, at least in hepatocytes [15]. Moreover, if this model is true, it predicts that analogues of adenophostin A with similar affinities to that of Ins(1,4,5)P_3 for the Ins(1,4,5)P_3 receptor should be equally ineffective in activating I_{CRAC} in weak buffer. Although this was certainly true for *manno*-adenophostin, the opposite was true for ribophostin. This latter analogue, which has a similar affinity to that of *manno*-adenophostin for Ins(1,4,5)P_3 receptors [24], was nevertheless as effective as adenophostin A in evoking I_{CRAC} in weak Ca^{2+} buffer. Therefore differences in equilibrium affinities alone cannot explain why adenophostin A and ribophostin activate I_{CRAC}, but Ins(1,4,5)P_3 and *manno*-adenophostin do not, in weak Ca^{2+} buffer.

Two possible mechanisms can be envisaged to account for the preferential activation of I_{CRAC} by adenophostin A and ribophostin in weak buffer in comparison with Ins(1,4,5)P_3 and *manno*-adenophostin: (1) different extents of store depletion

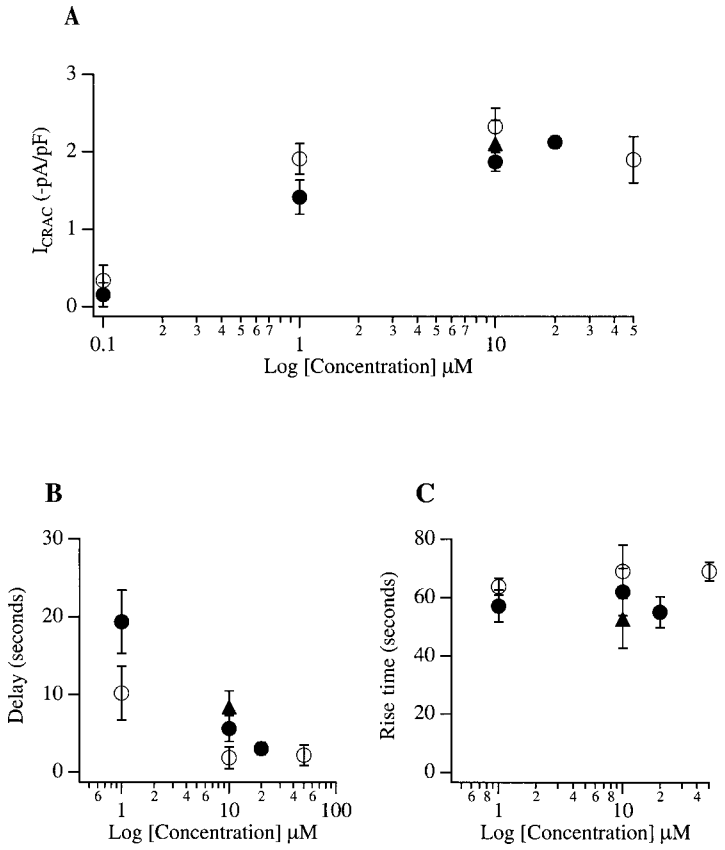


Figure 7 Effects of *manno*-adenophostin and ribophostin on I_{CRAC} in strong Ca^{2+} buffer

(**A**) The mean amplitude of I_{CRAC} is plotted against concentration of *manno*-adenophostin (○) and ribophostin (●). The response to 10 μM Ins(1,4,5)P_3 is also included (▲). (**B**, **C**) The delay (**B**) and rise time (**C**) of the current for different concentrations of each agonist. Cells were dialysed with a pipette solution in which Ca^{2+} was strongly buffered at 140 nM. Values are means ± S.E.M.

brought about by different gating properties of the occupied Ins(1,4,5)P_3 receptor or (2) a novel action of adenophostin A and ribophostin in addition to their actions on the Ins(1,4,5)P_3 receptor.

Gating kinetics of Ins(1,4,5)P_3 receptor

Scheme 1 is a simplified gating scheme of Ins(1,4,5)P_3 receptor activity in the presence of a ligand (A) and is used for narration purposes only. R refers to the resting state (non-liganded form of the Ins(1,4,5)P_3 receptor), A-R the state with ligand bound, A-R* the open channel and A-R*-I the inactivated state.

Although binding studies have established that the equilibrium dissociation constants (k_{-1}/k_{+1}) for Ins(1,4,5)P_3, *manno*-adenophostin and ribophostin are similar [24], they do not address the key issue of whether the kinetics of channel opening, once ligand has bound, are different (k_{-2}/k_{+2}). Differences in binding to form A-R do not translate automatically into different kinetics of channel gating. It is possible that either k_{-2} or k_{+2} (or both) depend on the nature of the agonist so that adenophostin A and ribophostin promote more rapid and/or sustained opening relative to Ins(1,4,5)P_3 or *manno*-adenophostin. In this scheme, ribophostin (and adenophostin A) would be more efficacious than Ins(1,4,5)P_3 and *manno*-adenophostin in spite of their having similar affinities. This would be analogous to the activation of nicotinic acetylcholine receptors in the neuromuscular junction, where acetylcholine, which has a lower affinity than that of suberyldicholine, opens the channels with greater efficacy [25]. Alternatively, the rate and extent of inactivation might be different in the presence of the different agonists (i.e. k_{-3} might depend on the nature of the agonist–receptor complex).

Can we distinguish between an action of adenophostin A and ribophostin on k_2/k_{-2} or on k_{-3}? Unfortunately, no study so far has addressed the kinetics of Ins(1,4,5)P_3 receptor gating in RBL cells with adenophostin A and related compounds. We are therefore forced to draw on findings from other systems, which might not be wholly applicable. In both permeabilized hepatocytes [15] and nuclei of *Xenopus* oocytes [17], Ins(1,4,5)P_3 receptors inactivate in a manner independent of whether the activating ligand is Ins(1,4,5)P_3 or adenophostin A. However, in RBL cells Ins(1,4,5)P_3 receptors do not seem to inactivate in the presence of high levels of Ins(1,4,5)P_3 [10,26], so differences in inactivation rates are unlikely to account fully for the different abilities of the agonists to activate I_{CRAC}.

Is there any evidence that adenophostin A and ribophostin have a higher efficacy than Ins(1,4,5)P_3 and *manno*-adenophostin in opening the channels? Adenophostin A and Ins(1,4,5)P_3 are thought to have similar efficacies because the corresponding EC_{50}/K_d ratios (EC_{50} for Ca^{2+} release and K_d for affinity constant) are similar. However, these determinations are performed under drastically different experimental conditions, rendering it difficult to compare the two parameters [16,24]. An increased efficacy of adenophostin A might be revealed as an increase in channel mean open time or burst frequency. Single-

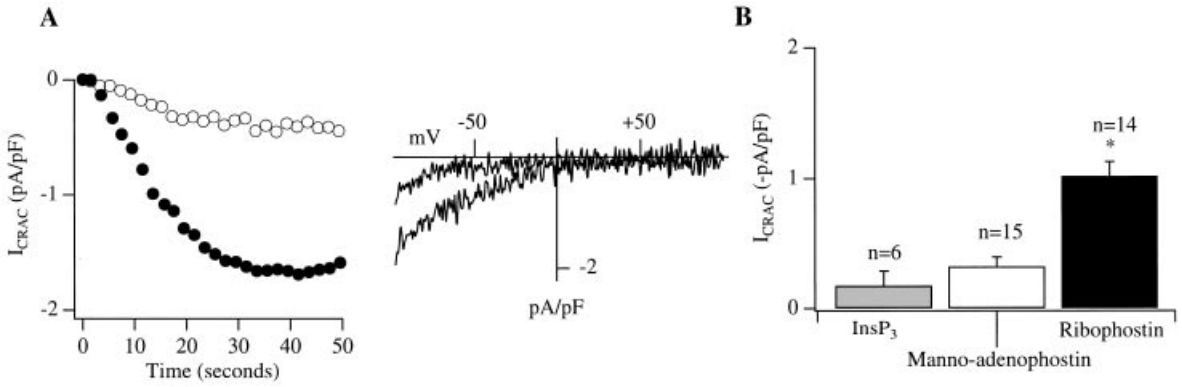
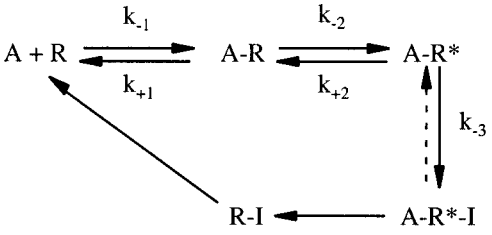


Figure 8 Ribophostin, but not *manno*-adenophostin, activates I_{CRAC} in weak Ca^{2+} buffer

(**A**) Left panel: the time course of development of I_{CRAC} for a cell dialysed with 30 μM ribophostin (●) and for one dialysed with 30 μM *manno*-adenophostin (○). Right panel: corresponding current–voltage relationships, taken at 40 s for each. (**B**) The mean amplitude of I_{CRAC} for the different conditions depicted (all in weak Ca^{2+} buffer). The difference in current size between ribophostin and those for Ins(1,4,5)P_3 and *manno*-adenophostin was significant ($P < 0.01$). Values are means ± S.E.M.



Scheme 1 Simplified gating scheme for Ins(1,4,5)P_3 receptor activation

channel recordings on type I Ins(1,4,5)P_3 receptors from *Xenopus* oocyte nuclei failed to reveal any difference in channel properties in the presence of Ins(1,4,5)P_3, adenophostin A or ribophostin [17]. However, in RBL cells the type II Ins(1,4,5)P_3 receptor isoform dominates, although the type I and III receptors are also expressed [27]. It is likely that heteromultimers form with distinct properties, as in other systems [28]. It is conceivable that different combinations of subunits produce channels with gating features dictated by the agonist. Furthermore, probably only small changes in any of the rate constants would be sufficient to account for the greater efficacy of adenophostin A and ribophostin, because the latter increase the probability of a cell responding but the size of I_{CRAC} for all responders is similar irrespective of the agonist. Our previous work suggests that there is a threshold below which intraluminal Ca^{2+} needs to decrease for macroscopic I_{CRAC} to activate [6,10]. Ins(1,4,5)P_3 empties stores close to the threshold but often does not exceed it. Hence a small change in k_2/k_{-2} might well be sufficient to ensure that the threshold is consistently overcome and that I_{CRAC} subsequently develops, albeit at a submaximal level. Such small changes are likely to be masked in strong Ca^{2+} buffer, in which SERCA activity is lower and Ca^{2+} feedback effects are decreased, rendering it easier to deplete the stores for I_{CRAC} to activate. In addition, k_2/k_{-2} might be somewhat dependent on Ca^{2+}.

Novel action of adenophostin A and ribophostin?

It is possible that adenophostin A and ribophostin activate I_{CRAC} in weak buffer through a mechanism additional to activating Ins(1,4,5)P_3 receptors, and that this step cannot be triggered by *manno*-adenophostin or Ins(1,4,5)P_3. Huang et al. [7] suggested that adenophostin A might alter the extent of Ca^{2+}-dependent fast inactivation; however, our results (Figure 4) indicate that this is not a prominent effect. Adenophostin A is also not likely to facilitate mitochondrial Ca^{2+} uptake through stimulation of the uniporter because Ruthenium Red failed to suppress the current. Finally, we do not consider that adenophostin A and ribophostin somehow preferentially stabilize coupling between Ins(1,4,5)P_3 receptors on the stores and the store-operated channels in the plasma membrane [29,30] because we have recently reported that Ins(1,4,5)P_3 receptors do not seem to be involved in the activation of I_{CRAC} in RBL-1 cells and that manoeuvres that interfere with such coupling in other cell types consistently fail to affect I_{CRAC} at all [31].

Understanding how adenophostin A and ribophostin are able to activate I_{CRAC} in weak Ca^{2+} buffer, as well as the molecular structures that underlie this, will provide insight not only into mechanisms that control store-operated Ca^{2+} influx but also into the kinetic profile of Ca^{2+} release mechanisms.

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