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# Effects of Adenophostin-A and Inositol-1,4,5-trisphosphate on $Cl^-$ Currents in *Xenopus laevis* Oocytes

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## SUMMARY

Adenophostin-A, a novel compound isolated from cultures of *Penicillium brevicompactum*, has been shown to stimulate $Ca^{2+}$ release from inositol-1,4,5-trisphosphate ($IP_3$)-sensitive $Ca^{2+}$ stores in microsomal preparations, permeabilized cells, and lipid vesicles containing purified $IP_3$ receptor. The purpose of the current study was to compare the effects of adenophostin-A and $IP_3$ on $Ca^{2+}$ release from stores and $Ca^{2+}$ influx in intact *Xenopus laevis* oocytes. $Ca^{2+}$ influx though store-operated $Ca^{2+}$ channels and $Ca^{2+}$ release from stores were monitored by measuring two $Ca^{2+}$ -activated $Cl^-$ currents that can be used as real-time indicators of $Ca^{2+}$ release and $Ca^{2+}$ influx ($I_{Cl-1}$ and $I_{Cl-2}$, respectively). We find that high concentrations (final intraoocyte concentrations of 5–10 $\mu$M) of adenophostin-A and $IP_3$ stimulate a large $Ca^{2+}$ release from stores (as measured by $I_{Cl-1}$) followed by $Ca^{2+}$ influx (as measured by $I_{Cl-2}$). Low concentrations (~50 nM) of $IP_3$ stimulate oscillations in $Ca^{2+}$ release without stimulating $Ca^{2+}$ influx. In contrast, low concentrations of adenophostin-A can stimulate $Ca^{2+}$ influx without stimulating a large $Ca^{2+}$ release. However, $Ca^{2+}$ influx did not occur in the complete absence of $Ca^{2+}$ release. Therefore, it is unlikely that adenophostin-A directly stimulates store-operated $Ca^{2+}$ channels. We hypothesize that adenophostin-A releases $Ca^{2+}$ from a subpopulation of stores that is tightly coupled to store-operated $Ca^{2+}$ channels.

The concentration of cytosolic free $Ca^{2+}$ regulates many physiological processes as diverse as fertilization and programmed cell death. One of the key pathways that controls the level of cytosolic free $Ca^{2+}$ involves G protein-coupled and tyrosine kinase-coupled receptor stimulation of phospholipase C, production of $IP_3$, and the release of $Ca^{2+}$ from internal stores (1–4). Release of $Ca^{2+}$ from internal stores is often followed by a sustained influx of extracellular $Ca^{2+}$ (5–8). This influx [capacitative $Ca^{2+}$ entry (9)] is mediated by SOCCs in the plasmalemma that are apparently controlled by the level of $Ca^{2+}$ in the internal store.

Although $IP_3$ is a very potent stimulator of $Ca^{2+}$ release from internal stores ($ED_{50}$ ~ 200 nM), it has recently been reported that a structurally different compound, adenophostin-A, is ~100-fold more potent than $IP_3$ in releasing $Ca^{2+}$ from internal stores (10). Adenophostin-A, isolated from the broth of cultures of *Penicillium brevicompactum*, is 2′-AMP linked through its 3′-hydroxyl to glucose-3,4-diphosphate. It has been proposed that the 3- and 4-phosphates on the glucose ring of adenophostin-A assume the same role as the 4- and 5-phosphates in $IP_3$ (10). Consistent with this idea is the finding that 2-hydroxyethyl-α-D-glucopyranoside-2,3′,4′-trisphosphate is also capable of binding to the $IP_3$ receptor and releasing $Ca^{2+}$ from internal stores, although with ~1000-fold lower potency than adenophostin-A (11). Although the effects of adenophostin-A on $Ca^{2+}$ release have been demonstrated in microsomal preparations (10), permeabilized cells (10), and purified reconstituted $IP_3$ receptors (12), the effects of adenophostin-A in intact cells have not been investigated. In the current study, we examined the effects of adenophostin-A injected into *Xenopus laevis* oocytes and compared its effects with those of $IP_3$.

*X. laevis* oocytes are a very useful model system for studying $Ca^{2+}$ signaling, in part because they express $Ca^{2+}$-activated $Cl^-$ channels that can be used as real-time indicators of cytosolic $Ca^{2+}$ concentration (13) and in part because their large size facilitates the study of spatial and temporal changes in cytosolic $Ca^{2+}$ concentrations (14). We have recently described two distinct $Ca^{2+}$-activated $Cl^-$ currents in *X. laevis* oocytes whose activation depends on the source of $Ca^{2+}$: $I_{Cl-2}$ is activated only by $Ca^{2+}$ influx through SOCCs, and $I_{Cl-1}$ can be activated both by $Ca^{2+}$ influx and by $Ca^{2+}$ release from internal stores, depending on the voltage protocol used (15). The purpose of the current study was to use these two currents to compare the effects of intracellular injection of $IP_3$ and adenophostin-A on $Ca^{2+}$ release from

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**ABBREVIATIONS:** $IP_3$, inositol-1,4,5-trisphosphate; SOCC, store-operated $Ca^{2+}$ channel; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $[adenophostin\text{-}A]_{CALC}$; calculated adenophostin-A concentration; $I_{Cl-1}$ and $I_{Cl-2}$, $Ca^{2+}$-activated $Cl^-$ currents.

internal stores and the subsequent capacitative $Ca^{2+}$ influx through SOCCs.

## Materials and Methods

**Electrophysiological methods.** *X. laevis* oocytes (stage V–VI) were isolated according to the method of Dascal (13) and voltage-clamped with two microelectrodes filled with 3 M KCl (1–2 MΩ) as described previously (15). Typically, the membrane was held at −35 mV, and voltage steps were applied as described in the text. Stimulation and data acquisition were controlled by pCLAMP 6.01 (Axon Instruments, Burlingame, CA) via a Digidata 1200 A/D-D/A converter (Axon Instruments) and a Gateway P5–90 computer (Intel Pentium, 90 MHz). During recording, the oocyte was superfused with normal Ringer's solution at a rate of 2 ml/min (~300-μl chamber). Experiments were performed at room temperature (22–26°).

**Microinjection.** Oocytes were injected with $IP_3$ or adenophostin-A using a Drummond Nanoject Automatic Oocyte Injector (Broomall, PA). The injection pipette was pulled from glass capillary tubing in a manner similar to the recording electrodes and then broken so that it had a beveled tip with an inside diameter of 10–20 μm. The final concentrations ([X]calc) of injected solutions in the oocyte were calculated assuming an oocyte volume of 1 μl and uniform distribution of the solute in the oocyte. The figures show the pipette concentrations and volumes of solutions injected.

**Solutions.** Normal Ringer's solution consisted of 123 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1.8 mM $MgCl_2$, and 10 mM HEPES, pH 7.4. Zero-$Ca^{2+}$ Ringer's was the same except $CaCl_2$ was omitted, $MgCl_2$ was increased to 5 mM, and 0.1 mM EGTA was added. Stock solutions of $IP_3$ and adenophostin-A were made at 10 mM in $H_2O$, stored at −20°, and diluted in water to the final concentrations indicated for injection. In all cases, injection of the same volume of water had no effect on the $Cl^-$ currents. Adenophostin-A was the generous gift of Drs. M. Takahashi, S. Takahashi, and K. Tanzawa (Sankyo Co., Ltd., Tokyo, Japan).

## Results

**Effects of $IP_3$ and adenophostin-A at a constant membrane potential of −100 mV.** Initially, we examined the effects of $IP_3$ and adenophostin-A on the membrane current recorded at a constant holding potential of −100 mV (Fig. 1). When 13 nl of a 1 mM solution of $IP_3$ was injected into an *X. laevis* oocyte, an inward current developed. The current was biphasic: the initial component peaked in ~30 sec and was followed by a slowly developing current that took ~10 min to develop fully (Fig. 1A). A similar result was obtained when the oocyte was injected with 10 nl of a 1 mM solution of adenophostin-A (Fig. 1B). These concentrations of drug produced calculated drug concentrations in the oocyte of 5–13 μM (assuming an oocyte volume of 1 μl), which would be expected to be supramaximal for stimulating $Ca^{2+}$ release from $IP_3$-sensitive stores (4, 10). In contrast, when the oocyte was injected with 200-fold less adenophostin-A (intraoocyte concentration ~50 nM), the transient phase was not detectable and only the slow phase was present (Fig. 1C). The slow phase of the currents evoked by $IP_3$ or adenophostin-A were abolished by removal of extracellular $Ca^{2+}$ (not shown). This confirms the results of other studies (15–20) that have shown that the transient phase of the inward current produced by $IP_3$ injection corresponds to a $Cl^-$ current that is activated by $Ca^{2+}$ released from internal stores and the slowly developing phase is a $Cl^-$ current that requires $Ca^{2+}$ influx from the extracellular space. The data of Fig. 1C, therefore, suggest that low concentrations of adenophostin-A might stimulate

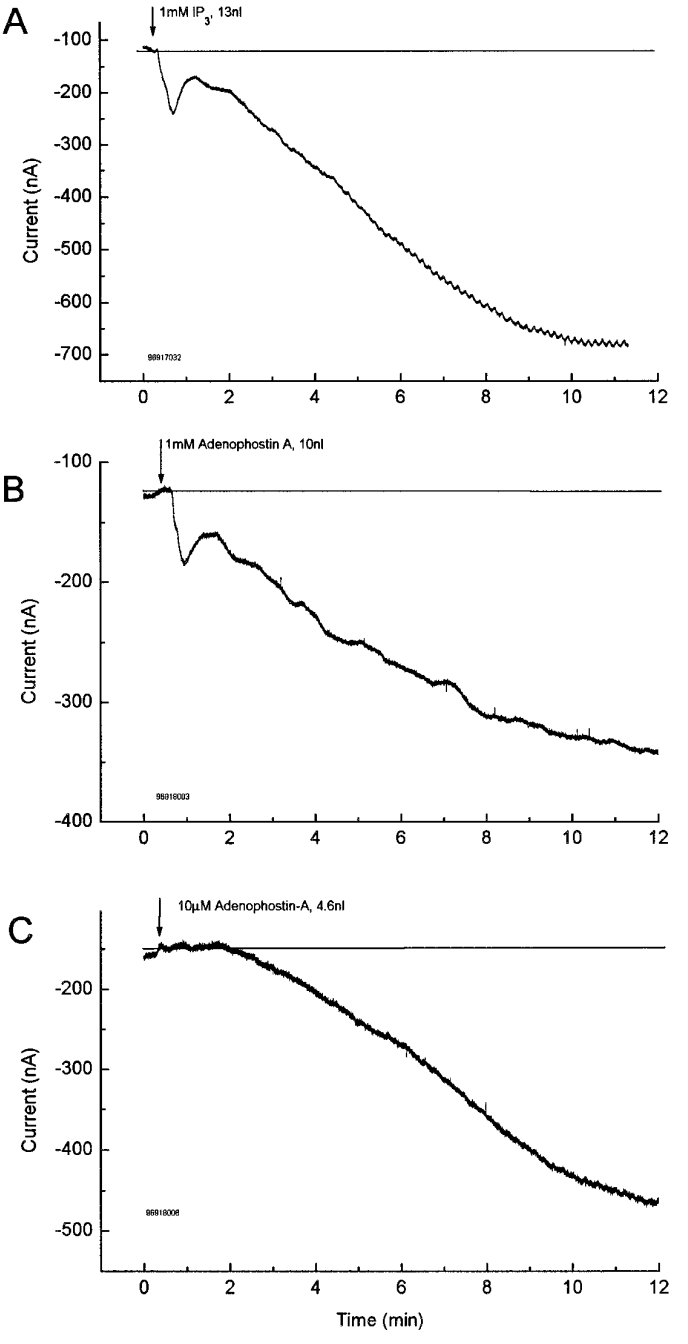


**Fig. 1.** Effects of injection of $IP_3$ and adenophostin in *X. laevis* oocytes at −100 mV. Oocytes were held at −100 mV and (A) 13 nl of 1 mM $IP_3$ (calculated $IP_3$ concentration = 13 μM), (B) 10 nl of 1 mM adenophostin-A ($[adenophostin\text{-}A]_{CALC}$ = 10 μM), or (C) 4.6 nl of 10 μM adenophostin-A ($[adenophostin\text{-}A]_{CALC}$ = 46 nM) was injected at the time indicated (*arrows*).

$Ca^{2+}$ influx without first causing a substantial release of $Ca^{2+}$ from internal stores.

Although the voltage protocol used in Fig. 1 has been a standard approach for investigating $Ca^{2+}$ release and influx in *X. laevis* oocytes, it is not very sensitive for detecting $Ca^{2+}$ release from stores because the transient current ($I_{Cl\text{-}1}$) that is stimulated by $Ca^{2+}$ released from stores is not significantly activated at negative potentials (15). At −100 mV, $I_{Cl\text{-}1}$ is a rather insensitive indicator of $Ca^{2+}$ release from stores (15).

**Effects of $IP_3$ on $I_{Cl-1}$ and $I_{Cl-2}$.** To examine this possibility more closely, we compared the effects of $IP_3$ and adenophostin-A on $I_{Cl-1}$ and $I_{Cl-2}$ as we have previously described (15). Fig. 2 shows the effect of injection of large concentrations of $IP_3$ into an oocyte. The potential of the oocyte was repetitively stepped from a holding potential of −35 mV to +40 mV for 1 sec and then to −120 mV for 1 sec. The current at the end of the +40 mV pulse was taken as a measure of $I_{Cl-1}$, and the current at the end of the −120 mV pulse was taken as a measure of $I_{Cl-2}$. We have previously shown that under these conditions, $I_{Cl-1}$ is an indicator of $Ca^{2+}$ released from stores and $I_{Cl-2}$ is an indicator of $Ca^{2+}$ influx (15). Injection of 4.6 nl of 10 mM $IP_3$ into an oocyte caused an immediate but transient increase in $I_{Cl-1}$ (Fig. 2, A and B). This increase peaked in ~1 min and declined back to base-line in ~2 min. As $I_{Cl-1}$ at +40 mV declined, $I_{Cl-2}$ at −120 mV began to increase and reached a maximum in ~10 min. The time courses of $I_{Cl-1}$ and $I_{Cl-2}$ paralleled the time courses of the transient and slow phases, respectively, of the current recorded in Fig. 1. A qualitatively similar result was obtained when a 100-fold lower amount of $IP_3$ was injected (Fig. 2, C and D).

In contrast, when very low concentrations of $IP_3$ were injected into the oocyte, a different result was obtained (Fig. 3). In the experiment illustrated, the oocyte was injected three times with 10 μM $IP_3$. The first injection of 10 nl of 10 μM $IP_3$ produced a small $I_{Cl-1}$ and no $I_{Cl-2}$. The second injection of the same amount produced a somewhat larger $I_{Cl-1}$ that oscillated in amplitude for several minutes before it declined to base-line. No $I_{Cl-2}$ was detected. The third injection of 23 nl produced an even larger $I_{Cl-1}$. $I_{Cl-1}$ reached a maximum after ~4 min but then began to oscillate and declined to base-line after ~8 min. In response to this injection, $I_{Cl-2}$ was stimulated. $I_{Cl-2}$ began to develop ~1 min after $I_{Cl-1}$ began to increase and reached a peak and declined to zero with approximately the same time course as $I_{Cl-1}$. The decline of $I_{Cl-2}$ coincided with the onset of $I_{Cl-1}$ oscillation. The observation that the amplitudes of $I_{Cl-1}$ and $I_{Cl-2}$ were not linearly related suggested that release of $Ca^{2+}$ from internal stores needed to reach a threshold level before $Ca^{2+}$

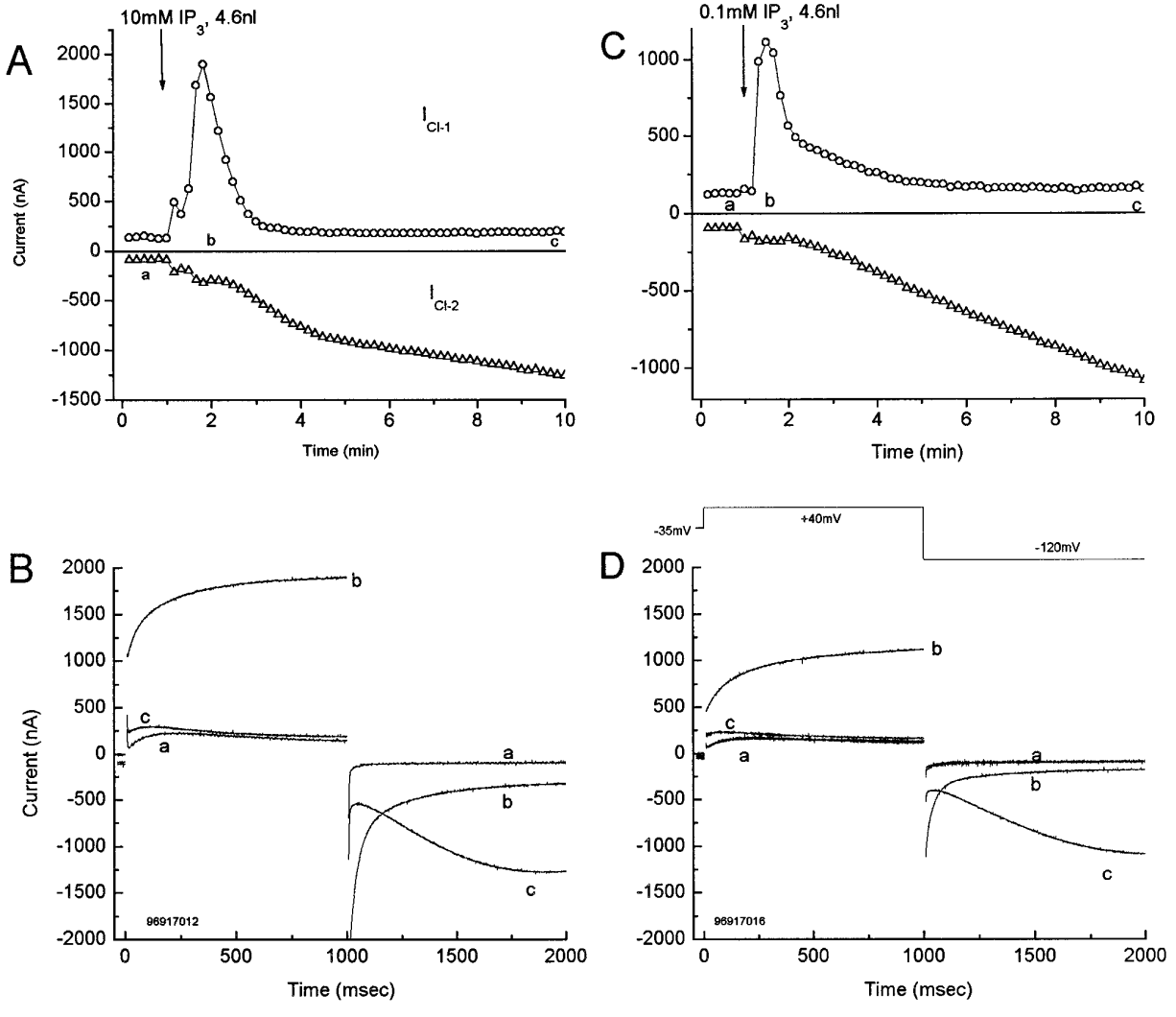


**Fig. 2.** Effects of high concentrations of $IP_3$ on two $Cl^-$ currents. Oocytes were voltage-clamped and repeatedly stepped from −35 mV to +40 mV for 1 sec and then to −120 mV for 1 sec (D, *top trace*). The current at the end of the +40-mV pulse was taken as $I_{Cl-1}$, and the current at the end of the −120-mV pulse was taken as $I_{Cl-2}$. A and C, Plot of the change in (○) $I_{Cl-1}$ and (△) $I_{Cl-2}$ in response to (A) 4.6 nl of 1 mM $IP_3$ (calculated $IP_3$ concentration = 4.6 μM) or (C) 4.6 nl of 0.1 mM $IP_3$ (calculated $IP_3$ concentration = 460 nM). B and D, Selected traces corresponding to the plots in A and C, respectively. *a–c,* Times in A–C at which the traces were selected.

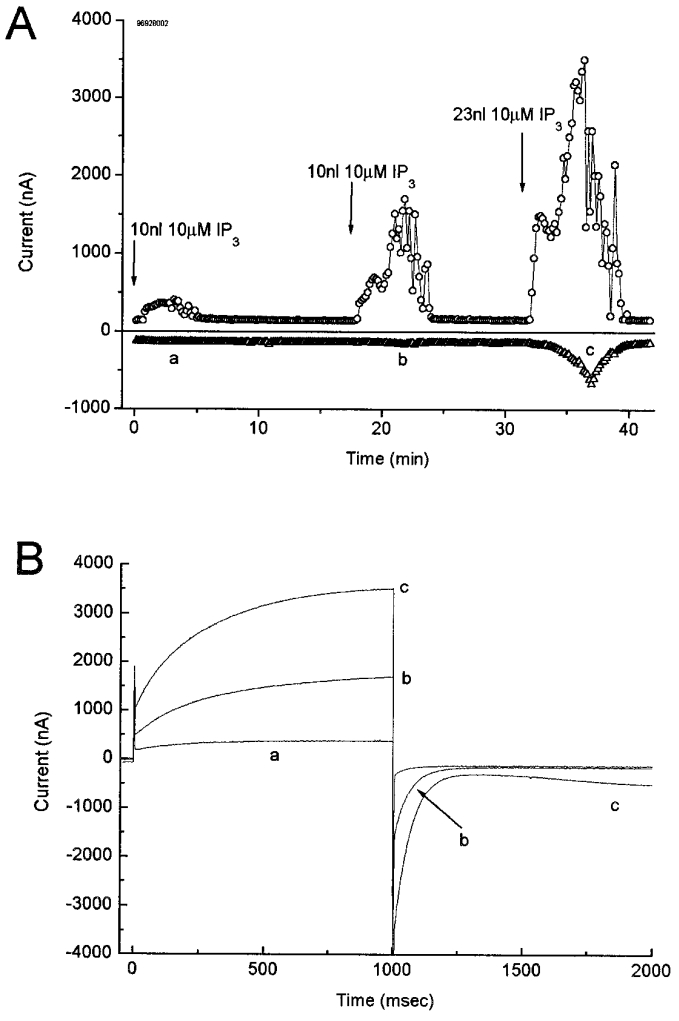


**Fig. 3.** Effects of low concentrations of $IP_3$ on $Cl^-$ currents measured as described in legend to Fig. 2. A, Plot of (○) $I_{Cl\text{-}1}$ and (△) $I_{Cl\text{-}2}$ as a function of time. $IP_3$ (10 μM in the pipette) was injected at the three times indicated (*arrows*). B, *Traces* (*a–c*) corresponding to the times indicated in A.

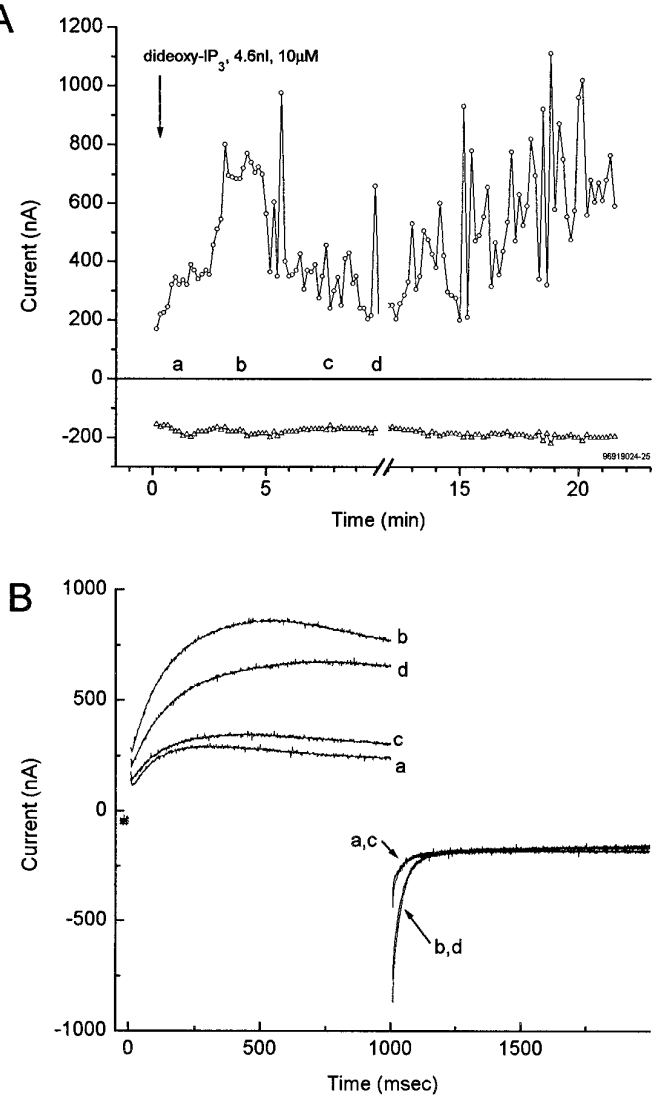


**Fig. 4.** Effects of low concentration of 2,3-dideoxy-$IP_3$ on $Cl^-$ currents. A, Plot of (○) $I_{Cl\text{-}1}$ and (△) $I_{Cl\text{-}2}$ as a function of time. Dideoxy-$IP_3$ (4.6 nl, 10 μM) was injected at the time indicated (*arrow*) (calculated dideoxy-$IP_3$ concentration = 46 nM). B, *Traces* (*a–c*) corresponding to the times indicated in A.

influx was stimulated (21). The first two injections of $IP_3$ failed to release sufficient $Ca^{2+}$ from the store to activate influx, whereas the last injection produced sufficient release to stimulate influx. We believe that the influx was transient because release was terminated as the injected $IP_3$ was metabolically inactivated and the stores were refilled by $Ca^{2+}$ influx. The length of time $I_{Cl\text{-}2}$ remained elevated correlated with the dose of $IP_3$ injected. With large injections of $IP_3$ (50 μM intraoocyte concentration), as in Fig. 2A, $Ca^{2+}$ influx usually remained elevated for >1 hr, but with lower concentrations (0.5 μM) as in Fig. 2B, $I_{Cl\text{-}2}$ declined to base-line in ~30 min.

In an attempt to simplify the interpretation of these experiments, we repeated them using the metabolically stable derivatives of $IP_3$, 2,3-dideoxy $IP_3$ and 2-deoxy-3-fluoro $IP_3$ (21). Injection of high concentrations of these analogs (4.6 nl of a 1 mM solution) had the same effect as injections of similarly high concentrations of native $IP_3$ (as in Fig. 2, but not shown). However, injection of 4.6 nl of 10 μM 2,3-dideoxy-$IP_3$ invariably produced an increase in $I_{Cl\text{-}1}$ that then oscillated in amplitude for >20 min (Fig. 4). Under these conditions, when $I_{Cl\text{-}1}$ was oscillating, $I_{Cl\text{-}2}$ was usually not activated or was activated only transiently. Presumably, when $I_{Cl\text{-}1}$ was oscillating, the level of $Ca^{2+}$ in the stores did not reach a sufficiently low level for a sufficiently long time to initiate the signal required to activate $Ca^{2+}$ influx.

**Effects of adenophostin-A on $I_{Cl\text{-}1}$ and $I_{Cl\text{-}2}$.** Injection of high concentrations of adenophostin-A produced a similar response to that seen with injection of high concentrations of $IP_3$ (Fig. 5). It seemed that $I_{Cl\text{-}1}$ declined more slowly in response to adenophostin-A than in response to $IP_3$ (compare Figs. 2 and 5), but this was not analyzed quantitatively. In contrast, injection of a low concentration of adenophostin-A produced a different response than injection of a low concentration of $IP_3$ (Fig. 6). Fig. 6 shows an example typical of >50 oocytes, in which the injection of 4.6 nl of a 1 μM solution of adenophostin-A stimulated $I_{Cl\text{-}1}$ only a little, whereas $I_{Cl\text{-}2}$ was strongly stimulated. Compare this result (Fig. 6) with that shown in Fig. 3, in which $IP_3$ stimulated a larger $I_{Cl\text{-}1}$ but no $I_{Cl\text{-}2}$.

To verify that the currents stimulated by adenophostin-A

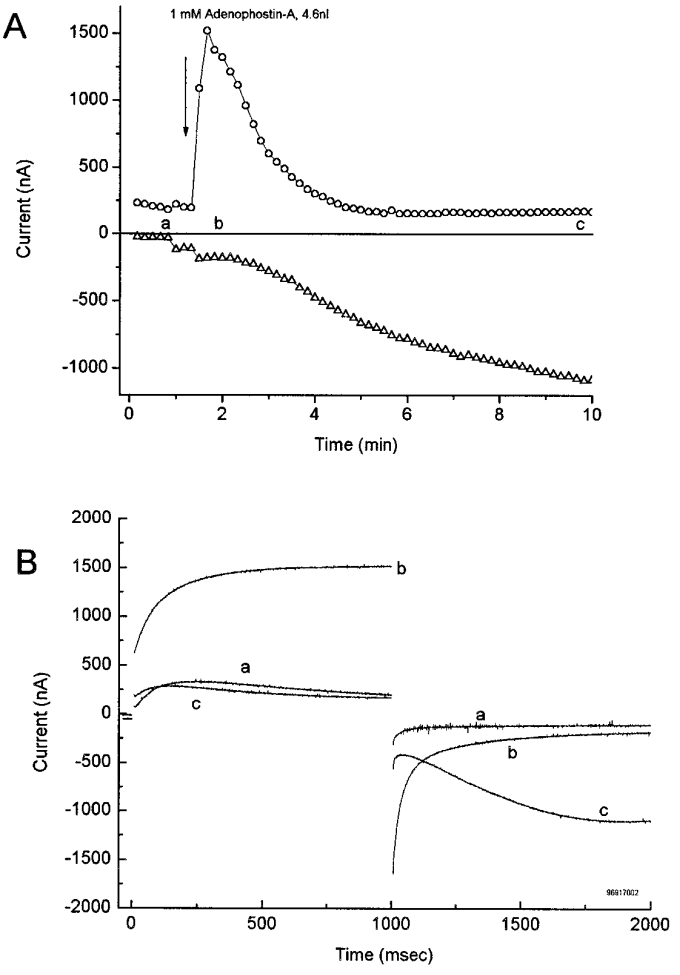


**Fig. 5.** Effect of a high concentration of adenophostin-A on $Cl^-$ currents. A, Plot of (○) $I_{Cl-1}$ and (△) $I_{Cl-2}$ as a function of time. Adenophostin-A (4.6 nl of 1 mM) was injected at the time indicated (*arrow*) ([adenophostin-A]$_{CALC}$ = 4.6 $\mu$M). B, *Traces* (*a–c*) corresponding to the times indicated in A.

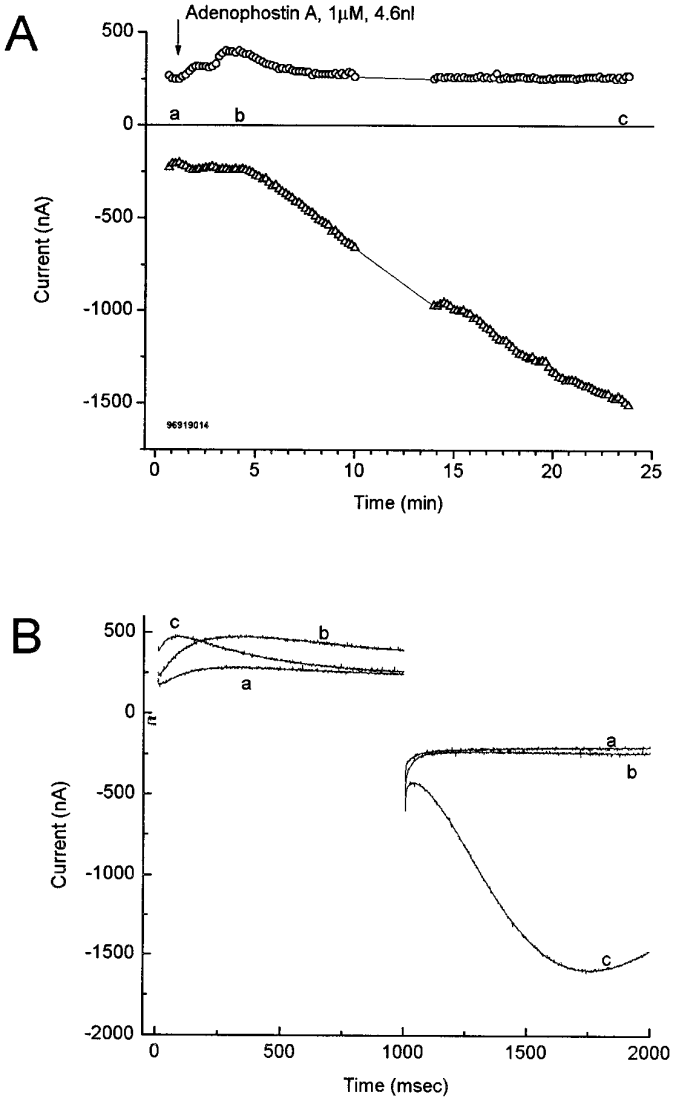


**Fig. 6.** Effect of a low concentration of adenophostin-A on $Cl^-$ currents. A, Plot of (○) $I_{Cl-1}$ and (△) $I_{Cl-2}$ as a function of time. Adenophostin-A (4.6 nl of 1 $\mu$M) was injected at the time indicated (*arrow*) ([adenophostin-A]$_{CALC}$ = 4.6 nM). Recording was interrupted between 10 and 14 min while other voltage protocols were being run. B, *Traces* (*a–c*) corresponding to the times indicated in A.

had the same properties as those stimulated by $IP_3$, we characterized the adenophostin-A evoked currents in more detail. In Fig. 7, we tested the dependence of $I_{Cl-1}$ and $I_{Cl-2}$ on extracellular $Ca^{2+}$ . In Fig. 7A,10 nl of 100 $\mu$M adenophostin was injected at 40 sec. At ~30 sec after $I_{Cl-1}$ became maximally stimulated, we switched to zero-$Ca^{2+}$ Ringer's solution. Removal of extracellular $Ca^{2+}$ had little effect on $I_{Cl-1}$, but $I_{Cl-2}$ did not develop until $Ca^{2+}$ was added back to the extracellular solution at ~9 min. This shows that $I_{Cl-1}$ is not dependent on $Ca^{2+}$ influx, whereas $I_{Cl-2}$ is dependent on $Ca^{2+}$ influx. This confirms that the currents stimulated by adenophostin-A have the same dependence on store-released $Ca^{2+}$ and influxed $Ca^{2+}$ as the currents stimulated by $IP_3$ (15). Furthermore, the adenophostin-A-stimulated currents have the same voltage-dependent activation and current-voltage relationships as the $IP_3$-stimulated currents (data not shown).

To compare the responses to adenophostin-A and $IP_3$ quantitatively, we measured the maximal amplitude of $I_{Cl-1}$ and the amplitude of $I_{Cl-2}$ 10 min after injection of adenophostin-A or 2,3-dideoxy $IP_3$. For these experiments, we wanted to compare concentrations of adenophostin-A and 2,3-dideoxy $IP_3$ that produced the minimal possible stimulation of $I_{Cl-1}$. We determined that the minimal [adenophostin-A]$_{CALC}$ required to produce an effect on $I_{Cl-2}$ varied from oocyte to oocyte but was ~5 nM. The minimal calculated dideoxy-$IP_3$ concentration required to produce a response was ~25 nM. To compare the effects of low concentrations of these drugs, we began by injecting 5–10 nl of a 0.5–2 $\mu$M solution of adenophostin-A or 5–10 nl of a 1–10 $\mu$M solution of 2,3-dideoxy $IP_3$. If the first injection did not produce a response in 2 min, a second injection was given and the final concentration of drug was calculated as the sum of the two injections. For the data shown in Fig. 8, the average calculated intraoocyte concentration of 2,3-dideoxy $IP_3$ was 60 ± 20 nM, and the average adenophostin-A concentration was 18 ± 4 nM. The principal difference between the responses to adenophostin-A and $IP_3$ was that adenophostin-A was much less effective than $IP_3$ in stimulating $I_{Cl-1}$ but was more effective than $IP_3$ in stimulating $I_{Cl-2}$. Adenophostin-A produced a ~4-fold smaller $I_{Cl-1}$ (237 ± 39 nA, 25 cells, for adenophostin-A;

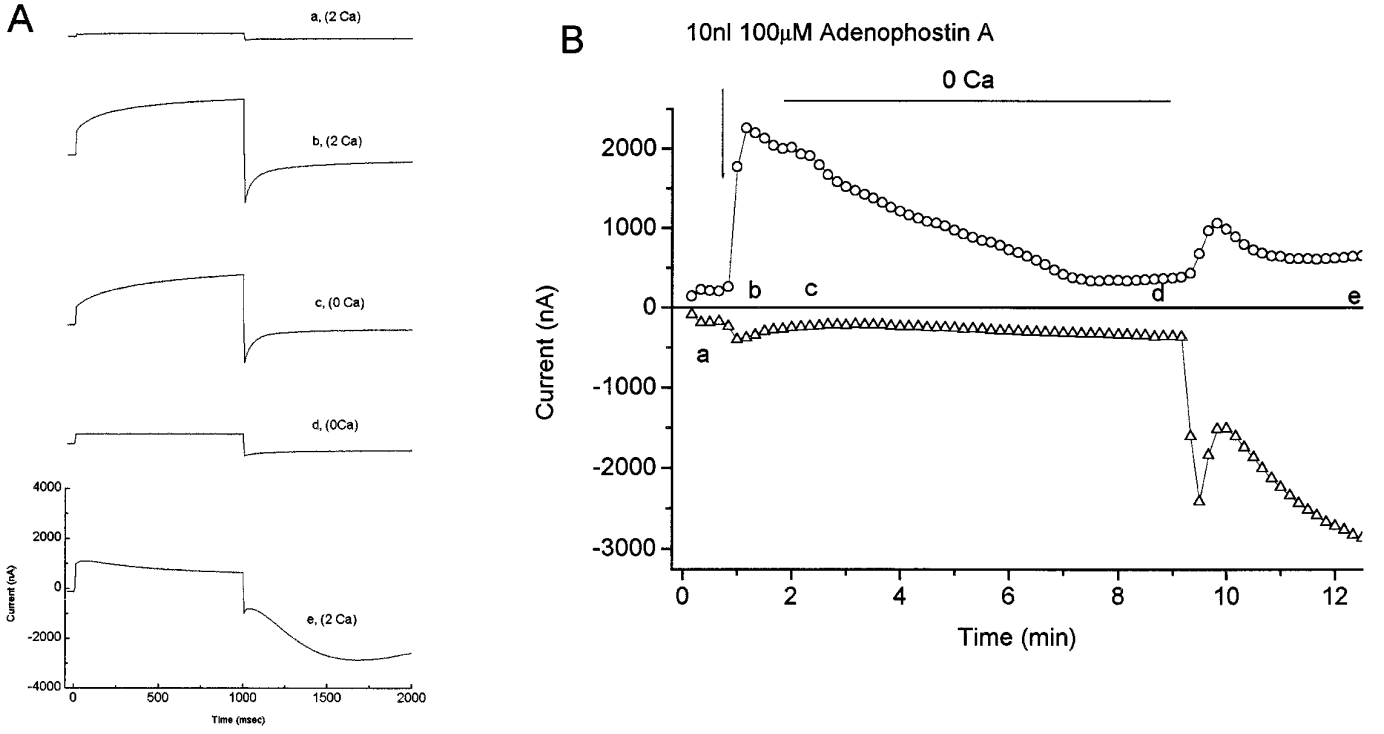


**Fig. 7.** Effect of zero external $Ca^{2+}$ on currents evoked by adenophostin-A. A, *Traces* (*a–e*) corresponding to the times indicated in B. B, Plot of (○) $I_{Cl\text{-}1}$ and (△) $I_{Cl\text{-}2}$ as a function of time. Adenophostin-A (10 nl of 100 μM) was injected at the time indicated (*arrow*) ([adenophostin-A]$_{CALC}$ = 1 μM). The solution was normal Ringer's except for the period during which the solution was switched to zero-$Ca^{2+}$ Ringer's (*0 Ca*) (see Methods).

904 ± 184 nA, 19 cells, for 2,3-dideoxy $IP_3$),but stimulated $I_{Cl\text{-}2}$ to a greater extent (−483 ± 71 nA for adenophostin-A; −298 ± 101 nA for 2,3-dideoxy $IP_3$). Thus, the ratio of $I_{Cl\text{-}2}$ to $I_{Cl\text{-}1}$ was 5-fold greater for adenophostin-A than for $IP_3$ (Fig. 8B).

These results, and those of Fig. 1, suggest that adenophostin-A is capable of stimulating $Ca^{2+}$ influx without depleting the $Ca^{2+}$ stores to the same extent as $IP_3$. Indeed, Fig. 1 might suggest that $Ca^{2+}$ release from stores is not at all necessary to stimulate $Ca^{2+}$ influx, but as we pointed out, the voltage protocol used in Fig. 1 was very insensitive for measuring $I_{Cl\text{-}1}$. Using the more sensitive protocol of Fig. 8, we have never seen development of $I_{Cl\text{-}2}$ without some stimulation of $I_{Cl\text{-}1}$. Injection of 5–10 nl of 0.5–1 μM adenophostin-A either had no effect on $I_{Cl\text{-}1}$ or $I_{Cl\text{-}2}$ or produced an increase in $I_{Cl\text{-}2}$ that was preceded by an increase in $I_{Cl\text{-}1}$, albeit sometimes the increase was very small (for example, Fig. 9B). These concentrations of adenophostin-A are in the range of concentrations that are effective in releasing $Ca^{2+}$ via $IP_3$ receptors (10, 12). In the range below 1 nM, adenophostin-A had no discernible effect on the $Ca^{2+}$-activated $Cl^-$ currents. We wondered whether the stimulation of $I_{Cl\text{-}1}$ by low concentrations of adenophostin might be explained by the higher concentration of adenophostin near the tip of the injection pipette on injection. To test this possibility, we injected larger volumes (≤46 nl) of lower pipette concentration adenophostin solutions (≤50 nM). Although in a few of these injections increases in $I_{Cl\text{-}1}$ were not detectable at +40 mV, they were detectable at +80 mV. Furthermore, under these conditions, the development of $I_{Cl\text{-}2}$ was extremely slow: it often took >2 hr for $I_{Cl\text{-}2}$ to develop fully. This observation suggested that at low [adenophostin-A]$_{CALC}$, release of $Ca^{2+}$ from stores was very slow but was eventually sufficient to trigger $Ca^{2+}$ influx. This conclusion has recently been supported by confocal imaging of $Ca^{2+}$-green-1 fluorescence.[1] On injection of 9.2 nl of 0.2 μM adenophostin-A, we observed release of $Ca^{2+}$ from stores. Obviously, this does not prove that $Ca^{2+}$ must be released from stores for $I_{Cl\text{-}2}$ to be stimulated, but we have so far been unable to dissociate the two processes.

**Effect of $IP_3$ after adenophostin-A.** To determine whether adenophostin-A could stimulate influx without depleting $Ca^{2+}$ stores, we investigated whether $Ca^{2+}$ could be released from stores by $IP_3$ after $I_{Cl\text{-}2}$ had developed fully in response to a previous adenophostin-A injection. In Fig. 9A, injection of 10 nl of 1 μM adenophostin produced a ~400 nA $I_{Cl\text{-}1}$ and a ~4500 nA $I_{Cl\text{-}2}$, which developed over ~35 min. After $I_{Cl\text{-}2}$ had plateaued, 4.6 nl of 1 mM $IP_3$ was injected. This produced only a very small increase in $I_{Cl\text{-}1}$ and $I_{Cl\text{-}2}$. These results suggested that adenophostin-A under these conditions had either emptied the $IP_3$-sensitive stores or had somehow inactivated the $IP_3$ receptor. In other cells, however, a different result was obtained, as shown in Fig. 9B. In this experiment, a slightly smaller amount of adenophostin (4.6 nl of 0.5 μM adenophostin) produced a ~10-fold smaller increase in $I_{Cl\text{-}1}$ (~70 nA, *inset*) and a smaller $I_{Cl\text{-}2}$ (~1000 nA). After $I_{Cl\text{-}2}$ had plateaued, 10 nl of 1 mM $IP_3$ was injected. In this cell, the $IP_3$ injection produced a large $I_{Cl\text{-}1}$ and rapidly stimulated $I_{Cl\text{-}2}$. Thus, in this cell, the $IP_3$-sensitive stores were clearly not completely depleted of Ca, even though a large $I_{Cl\text{-}2}$ current was present in response to adenophostin-A injection. Similar results were obtained in seven other cells injected with adenophostin to give ~5–25 nM calculated final concentration. In contrast, after a first

[1] K. Machaca and H. C. Hartzell. Manuscript in preparation.

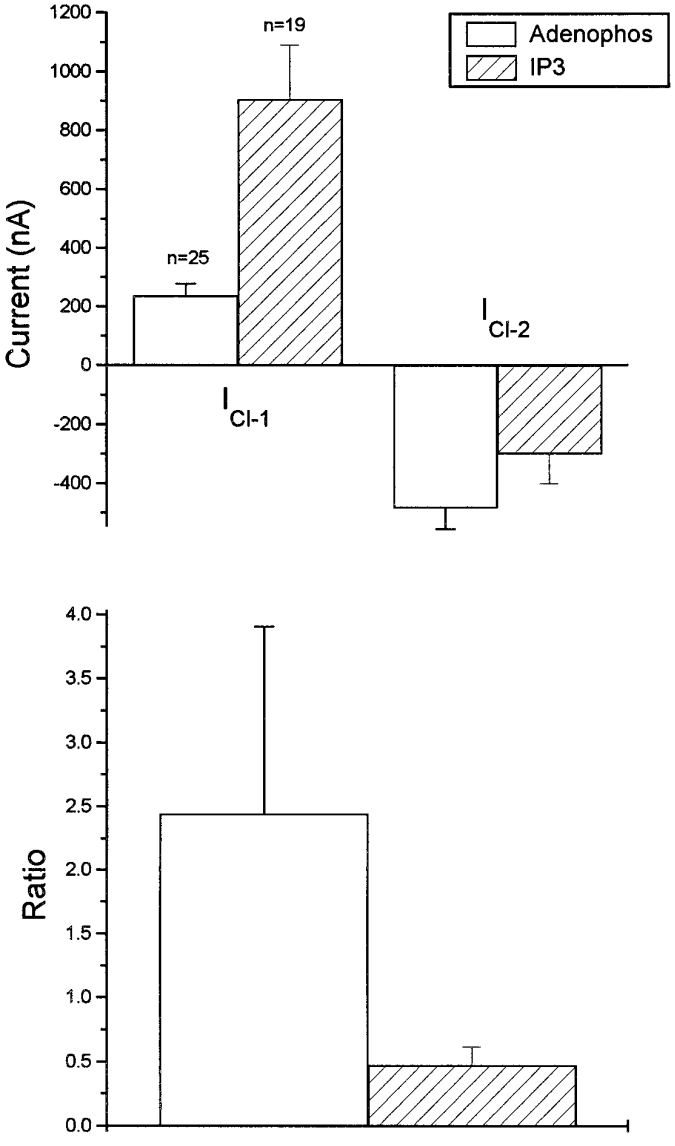


**Fig. 8.** Summary of the effect of low concentrations of 2,3-dideoxy $IP_3$ and adenophostin on $Cl^-$ currents. A, The maximal amplitude of $I_{Cl-1}$ and the amplitude of $I_{Cl-2}$ at 10 min after injection of drug were measured. *Error bars,* standard errors. B, The ratio of $I_{Cl-2}$ at 10 min to the maximal $I_{Cl-1}$ observed in that oocyte was calculated for each individual oocyte in A.

injection of $IP_3$ activated $I_{Cl-2}$, a second injection of $IP_3$ never evoked $I_{Cl-1}$ as long as $I_{Cl-2}$ was present.

## Discussion

These experiments demonstrate a difference in the ability of adenophostin-A and $IP_3$ to activate two $Cl^-$ currents in *X. laevis* oocytes. We have previously shown that one of these $Cl^-$ currents ($I_{Cl-1}$) is activated by $Ca^{2+}$ released from internal stores because it is absent unless the oocyte is injected with $IP_3$ and that its activation on stepping from −35 mV to positive potentials is independent of extracellular $Ca^{2+}$ but is blocked by intracellular 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (15). The other current ($I_{Cl-2}$) is activated by $Ca^{2+}$ influx and is abolished by removal of extracellular $Ca^{2+}$ (15). These currents are most likely due to different channels because $I_{Cl-1}$ has a linear instantaneous current-voltage relationship with an activation range at positive potentials and $I_{Cl-2}$ has a strongly outwardly rectifying current-voltage relationship with activation at negative potentials.

**Does adenophostin activate SOCCs without depleting stores?.** If we assume that $I_{Cl-1}$ is a reliable indicator of $Ca^{2+}$ release from stores (see below) and that $I_{Cl-2}$ is a reliable indicator of $Ca^{2+}$ influx, these data suggest the possibility that adenophostin-A may be capable of activating $Ca^{2+}$ influx through SOCCs without depleting $Ca^{2+}$ stores. If this is true, this is very exciting because it suggests that the signal transmitted from $Ca^{2+}$ stores to SOCCs may be an adenophostin-A-like compound. At the present time, the nature of the signaling pathway between stores and SOCCs remains completely unknown (8). One hypothesis for the coupling mechanism states that when store $Ca^{2+}$ falls, the store releases a diffusible messenger. However, experiments supporting the existence of a diffusible messenger remain controversial (22–25). The alternative hypothesis, proposed by Berridge *et al.* (8, 26) hypothesizes that a "$Ca^{2+}$-sensor" in the membrane of the $Ca^{2+}$ store directly couples to the SOCC and opens it. This conformational coupling hypothesis is attractive because it is analogous to the dihydropyridine receptor-ryanodine receptor coupling that occurs in skeletal muscle excitation-contraction coupling, but there remains little direct evidence either in support of or against this hypothesis (8).

The idea that adenophostin-A may activate SOCCs without depleting $Ca^{2+}$ stores is supported by the finding that adenophostin-A stimulates a larger $I_{Cl-2}$, whereas stimulation of $I_{Cl-1}$ is >20-fold less compared with $IP_3$ (compare Figs. 9B and 2C). If $I_{Cl-1}$ is an accurate indicator of $Ca^{2+}$ released from stores, then the data suggest that adenophostin-A can activate SOCCs without depleting $Ca^{2+}$ stores to the extent that is necessary for $IP_3$ to activate SOCCs. Furthermore, in cells in which $I_{Cl-2}$ is activated but $I_{Cl-1}$ is stimulated only marginally by adenophostin-A, $IP_3$ is capable of stimulating a large $I_{Cl-1}$ by releasing $Ca^{2+}$ from stores. Thus, it is clear that $Ca^{2+}$ stores are not completely depleted of $Ca^{2+}$ even though $I_{Cl-2}$ has developed significantly. In contrast, a second injection of $IP_3$ at any time after $I_{Cl-2}$ has begun to develop in response to an initial $IP_3$ injection is ineffective in stimulating additional $Ca^{2+}$ release (as measured by $I_{Cl-1}$). Thus, it seems that $IP_3$-sensitive stores must be more fully emptied of $Ca^{2+}$ in response to $IP_3$ than in response to adenophostin-A to initiate $Ca^{2+}$ influx.

**$I_{Cl-1}$ may be an imperfect indicator of store-released $Ca^{2+}$.** There are two possible explanations of these results. The first explantation, as suggested above, is that adenophostin-A has a direct effect on SOCCs. This interpretation should be accepted with caution, however, because we have never observed the development of $I_{Cl-2}$ in the complete absence of stimulation of $I_{Cl-1}$. This observation could simply be explained if adenophostin-A has two sites of action, the $IP_3$ receptor and the SOCC, and the dose-response curves for the two sites overlap partially. However, another possibility is that $I_{Cl-1}$ is an imperfect indicator of $Ca^{2+}$ release from stores. For example, the response of the $I_{Cl-1}$ channel may depend on the rapidity with which $Ca^{2+}$ is released from the store (different rates of release resulting in different local concentrations of cytosolic $Ca^{2+}$) or possibly the temporal pattern of $Ca^{2+}$ release from the store. Thus, if low concen-

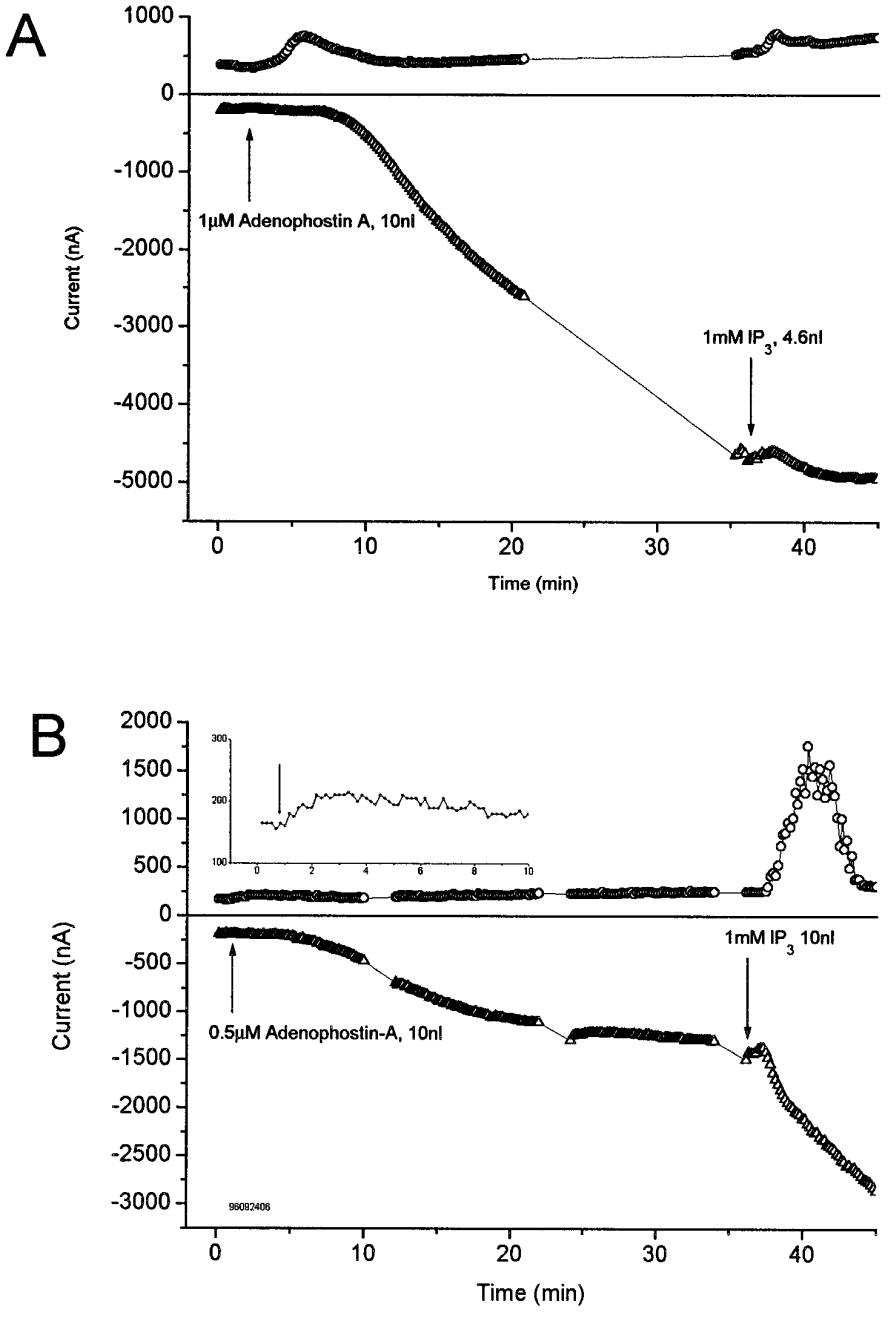


**Fig. 9.** The effect of $IP_3$ on $Cl^-$ currents after injection of adenophostin-A. A, Plot of (○) $I_{Cl-1}$ and (△) $I_{Cl-2}$ as a function of time. Adenophostin-A (10 nl of 1 $\mu$M) was injected at the time indicated (*first arrow*) ([adenophostin-A]$_{CALC}$ = 10 nM). At ~30 min later (*second arrow*), 4.6 nl of 1 mM $IP_3$ was injected (calculated $IP_3$ concentration = 4.6 $\mu$M). B, Another oocyte showing a different result. *Inset,* On an expanded scale, the increase in $I_{Cl-1}$ after adenophostin-A injection.

trations of adenophostin-A release $Ca^{2+}$ from stores slowly, it might not be revealed as an increase in $I_{Cl-1}$. Alternatively, because adenophostin-A binds to the $IP_3$ receptor with very high cooperativity (10, 12), it is possible that adenophostin-A stimulates a very rapid release of $Ca^{2+}$ from stores that is too fast to be detected by the $I_{Cl-1}$ channel. In support of the idea that $I_{Cl-1}$ may be an imperfect indicator of steady state cytosolic $Ca^{2+}$ concentration is the finding by Parker and Yao (27) that $Cl^-$ current amplitude correlated better with the rate of rise in the $Ca^{2+}$ transient measured by Fluo-3 fluorescence than with the steady state cytosolic $Ca^{2+}$ level.

**Functionally different $IP_3$-sensitive stores.** An alternative explanation for the differential ability of adenophostin-A and $IP_3$ to stimulate $I_{Cl-1}$ and $I_{Cl-2}$ is that there are functionally different $IP_3$-sensitive $Ca^{2+}$ stores and that only a subset are tightly coupled to SOCCs. There is evidence in the literature that not all of the stores must be completely depleted of $Ca^{2+}$ to stimulate capacitative $Ca^{2+}$ entry through SOCCs. Montero *et al.* (28) suggested that there is a linear relationship between $Ca^{2+}$ influx and the amount of $Ca^{2+}$ in the store. However, there are suggestions that the relationship between "store $Ca^{2+}$" and $Ca^{2+}$ influx may be more complex. Mathes and Thompson (29) have shown that a ~60% reduction in store $Ca^{2+}$ is sufficient to maximally activate $Ca^{2+}$ influx in neuroblastoma cells. Furthermore, in *X. laevis* oocytes, it seems that there is no direct relationship between the level of store $Ca^{2+}$ depletion and $Ca^{2+}$ influx. Lupu-Meiri *et al.* (20) have shown that although acetylcholine or incubation of oocytes in zero-$Ca^{2+}$ solution produce comparable reductions in cell $Ca^{2+}$, only acetylcholine produces significant $Ca^{2+}$ influx. Likewise, different inositol phosphates have differential ability to activate $Ca^{2+}$ release

from stores and $Ca^{2+}$ influx (30). These and other data (31) suggest the possibility that there are discrete $Ca^{2+}$ stores in the cell and that only one subset of the stores is coupled to $Ca^{2+}$ influx. If this interpretation is true, it might suggest that low concentrations of adenophostin-A are capable of stimulating $Ca^{2+}$ release from a discrete $Ca^{2+}$ store that is more closely associated with SOCCs, whereas $IP_3$ may indiscriminately release $Ca^{2+}$ from all $IP_3$-sensitive stores in *X. laevis* oocytes.

One attractive hypothesis is that the type-3 $IP_3$ receptor is more tightly coupled to stores than the type-1 $IP_3$ receptor and that adenophostin-A has a higher affinity for the type-3 receptor than for the type-1 receptor. In support of this suggestion is the observation that overexpression of the type-3 $IP_3$ receptor in *X. laevis* oocytes increases capacitative $Ca^{2+}$ entry and that this receptor is preferentially localized near the plasma membrane (32).

Another important difference that we have observed between adenophostin-A and $IP_3$ is that low concentrations of $IP_3$ and $IP_3$ analogs produce oscillations in $I_{Cl\text{-}1}$, whereas adenophostin does not. Oscillations in $Cl^-$ currents produced by $IP_3$ injection have been described by other investigators (33–36). The oscillations of $I_{Cl\text{-}1}$ parallel the $Ca^{2+}$ waves produced by injection of oocytes with low concentrations of $IP_3$ analogs (37, 38) and are probably related to the bell-shaped $Ca^{2+}$ dependence of the $IP_3$ receptor (39–41). Thus, as $IP_3$ releases $Ca^{2+}$ from the store, the high concentration of cytosolic $Ca^{2+}$ inhibits the action of $IP_3$. As the cytosolic $Ca^{2+}$ is lowered by uptake into the endoplasmic reticulum by $Ca^{2+}$-ATPases, the inhibition is relieved and $IP_3$ can act again to stimulate release (42). The lack of oscillations with low concentrations of adenophostin suggests that $Ca^{2+}$ may not modulate the action of adenophostin-A as it modulates the effect of $IP_3$. An attractive hypothesis is that adenophostin-A may act on a subtype of $IP_3$ receptor that is modulated in a different way by $Ca^{2+}$ than is the type-1 receptor. In this regard, Mikoshiba *et al*. (43) recently showed that the type-1 and type-3 $IP_3$ receptors are regulated differently by $Ca^{2+}$ . Alternatively, the inactivation of the type-1 $IP_3$ receptor may depend on the agonist that activates it. If this is true, it suggests that inactivation may not be solely due to the increase by $IP_3$ of the access of $Ca^{2+}$ to an inhibitory binding site on the cytoplasmic surface of the $IP_3$ receptor (44).

**Other interpretations.** Another difference between adenophostin and $IP_3$ that should be considered is that adenophostin may be much more metabolically stable than $IP_3$. Although the metabolic pathways that use adenophostin as substrate and the products of adenophostin metabolism have not been characterized, it is known that $IP_3$ is relatively rapidly metabolized into other inositol phosphates that may have their own biological actions. Thus, the products of $IP_3$ metabolism could have complex effects that could explain the differences in responsiveness of $I_{Cl\text{-}1}$ and $I_{Cl\text{-}2}$ to adenophostin and $IP_3$. For example, it has been shown that inositol-3,4,5,6-tetrakisphosphate is capable of inhibiting $Ca^{2+}$-activated $Cl^-$ channels in colonic and intestinal epithelial cells (45, 46). If inositol-3,4,5,6-tetrakisphosphate or other inositol phosphates were to have effects not shared by adenophostin-A on $Cl^-$ channels, this could confound the interpretation of the results described here. One possible scenario would be that a metabolite of $IP_3$ selectively inhibits the $I_{Cl\text{-}2}$ channel because of the time required for metabolic conversion of the injected $IP_3$. The strongest argument against such a mechanism is shown in Fig. 9. This hypothesis would predict that $IP_3$ injection after $I_{Cl\text{-}2}$ had developed in response to adenophostin injection should produce an inhibition of the current. However, although the $IP_3$ injection does sometimes produce a very small and transient inhibition of $I_{Cl\text{-}2}$, this negative effect is too small and too short-lived to explain the differences we have described in the responses to $IP_3$ and adenophostin-A. Furthermore, we see a similar difference between adenophostin-A and two slowly metabolized analogs of $IP_3$, 2,3-dideoxy $IP_3$ and 2-deoxy-3-fluoro $IP_3$.

Another consequence of the difference in the metabolic stability of adenophostin-A and $IP_3$ might be spatial differences in the spread of the drugs through the cell after injection. For example, $IP_3$ may not diffuse throughout the cell before it is metabolized, whereas adenophostin-A might be able to diffuse a longer distance before it is inactivated. If so, compared with $IP_3$, adenophostin-A might deplete a larger fraction of the stores, which would activate more SOCCs, and activate more $I_{Cl\text{-}2}$. Although this could theoretically explain why adenophostin-A activates less $I_{Cl\text{-}1}$ and more $I_{Cl\text{-}2}$ than does $IP_3$, it is does not explain why $IP_3$ releases $Ca^{2+}$ from stores that activate $I_{Cl\text{-}1}$ and adenophostin-A does not (unless one assumes that adenophostin-A-sensitive stores are located farther from the membrane than $IP_3$-sensitive stores).

**Summary.** The results provide interesting suggestions regarding the possible mechanisms of action of adenophostin-A. However, distinguishing between the possibilities discussed above will require imaging changes in store $Ca^{2+}$ and cytosolic $Ca^{2+}$ using fluorescent or luminescent $Ca^{2+}$ probes and correlating these changes with the currents we have recorded here. These studies are currently in progress.

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