

Adenophostin A and Inositol 1,4,5-Trisphosphate Differentially Activate Cl^- Currents in *Xenopus* Oocytes Because of Disparate Ca^{2+} Release Kinetics*

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Depletion of endoplasmic reticulum Ca^{2+} stores induces Ca^{2+} entry from the extracellular space by a process termed “store-operated Ca^{2+} entry” (SOCE). It has been suggested that the novel fungal metabolite adenophostin-A may be able to stimulate Ca^{2+} entry without stimulating Ca^{2+} release from stores. To test this idea further, we compared Ca^{2+} release, SOCE, and the stimulation of Ca^{2+} -activated Cl^- currents in *Xenopus* oocytes in response to inositol 1,4,5-trisphosphate (IP_3) and adenophostin-A injection. IP_3 stimulated an outward Cl^- current, $I_{\text{Cl1-S}}$, in response to Ca^{2+} release from stores followed by an inward current, I_{Cl2} , in response to SOCE. In contrast, low concentrations of adenophostins (AdAs) activated I_{Cl2} without activating $I_{\text{Cl1-S}}$, consistent with the suggestion that AdA can activate Ca^{2+} entry without stimulating Ca^{2+} release. However, when Ca^{2+} entry has been stimulated by AdA, Ca^{2+} stores are largely depleted of Ca^{2+} , as assessed by the inability of ionomycin to release additional Ca^{2+} . The Ca^{2+} release stimulated by AdA, however, was 7 times slower than the release stimulated by IP_3 , which could explain the minimal activation of $I_{\text{Cl1-S}}$; when Ca^{2+} is released slowly, the threshold level required for $I_{\text{Cl1-S}}$ activation is not attained.

Ca^{2+} signals regulate many cellular processes including cell growth, fertilization, gene transcription, and apoptosis (1). Increases in cytosolic Ca^{2+} levels are produced both by Ca^{2+} released from internal stores and Ca^{2+} influxed from the extracellular space. A major pathway for Ca^{2+} mobilization from internal stores is through inositol 1,4,5-trisphosphate receptors (IP_3R)¹ after stimulation of G-protein- or tyrosine kinase-coupled plasma membrane receptors linked to phospholipase C (2–4). The decrease in the Ca^{2+} content of the internal store then stimulates Ca^{2+} entry through plasma membrane store-operated Ca^{2+} channels (SOCs) by a process called store-operated Ca^{2+} entry (SOCE) (5, 6). The mechanism by which a reduction in the content of store Ca^{2+} results in opening of SOCs remains unknown, but there are two major hypotheses. The conformational coupling hypothesis suggests that there is direct physical contact between IP_3R s and SOCs such that conformational changes in the IP_3R occurring upon Ca^{2+} de-

pletion of the internal store can affect the opening of SOCs (7, 8). The diffusible messenger hypothesis suggests that the Ca^{2+} store (endoplasmic reticulum) produces a diffusible messenger that opens SOCs (9, 10).

Recently, a novel family of compounds called adenophostins (AdAs), which are structurally distinct from IP_3 , have been isolated from cultures of the fungus *Penicillium brevicompactum* (11, 12). The AdAs are 10–100-fold more potent than IP_3 in opening IP_3R s (13) and are capable of activating all three IP_3R subtypes (13–15). Recently, Hartzell *et al.* (16) and DeLisle *et al.* (17) have shown that in *Xenopus* oocytes, low concentrations of AdA stimulate Ca^{2+} -activated Cl^- currents that are activated by Ca^{2+} influx more than Cl^- currents that are activated by Ca^{2+} released from stores. Based on these observations, DeLisle *et al.* (17) suggested that AdA may be capable of activating store-operated Ca^{2+} entry without first stimulating Ca^{2+} release from stores. This is significant because it suggests that AdA may share structural features with the putative diffusible Ca^{2+} entry signal released by Ca^{2+} -depleted endoplasmic reticulum.

Ca^{2+} -activated Cl^- currents have been used for many years as real time indicators of sub-plasmalemmal Ca^{2+} in *Xenopus* oocytes (18–24), but clearly Cl^- currents are only indirect indicators of Ca^{2+} concentration. Consequently, conclusions about cytosolic Ca^{2+} concentration derived from these measurements are subject to different interpretations. We have recently found that there are two Ca^{2+} -activated Cl^- currents in the oocyte that are selectively activated by Ca^{2+} released from stores and by Ca^{2+} influx (24). The Ca^{2+} release-activated Cl^- current ($I_{\text{Cl1-S}}$) has an outwardly rectifying steady-state current-voltage relationship, whereas the Ca^{2+} influx-activated Cl^- current (I_{Cl2}) has an inwardly rectifying steady-state current-voltage relationship (24). This means that Ca^{2+} -activated Cl^- currents measured at constant negative membrane potentials, as was done in the experiments of DeLisle *et al.* (17), are relatively insensitive indicators of Ca^{2+} released from stores. In our experiments (16), we measured $I_{\text{Cl1-S}}$ as an outward current at positive membrane potentials and I_{Cl2} as an inward current at negative membrane potentials to more clearly differentiate between Ca^{2+} influx and Ca^{2+} release and to increase the sensitivity of detection of Ca^{2+} release. Using this protocol, IP_3 activated both $I_{\text{Cl1-S}}$ (“ Ca^{2+} release”) and I_{Cl2} (“ Ca^{2+} influx”), but low concentrations of AdA often activated only a tiny amount of $I_{\text{Cl1-S}}$, even though I_{Cl2} was robustly activated. But, because we could not find a concentration of AdA which could activate I_{Cl2} without activating some small amount of $I_{\text{Cl1-S}}$, we concluded that AdA did not activate SOCE independently of Ca^{2+} release from stores. We hypothesized that AdA activated relatively little $I_{\text{Cl1-S}}$ either because AdA released Ca^{2+} from stores very slowly or that AdA released Ca^{2+} from a subpopulation of stores which was tightly coupled to SOCs.

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¹ The abbreviations used are: IP_3R , inositol 1,4,5-trisphosphate receptors; SOC, store-operated Ca^{2+} channels; SOCE, store-operated Ca^{2+} entry; AdA, adenophostins.

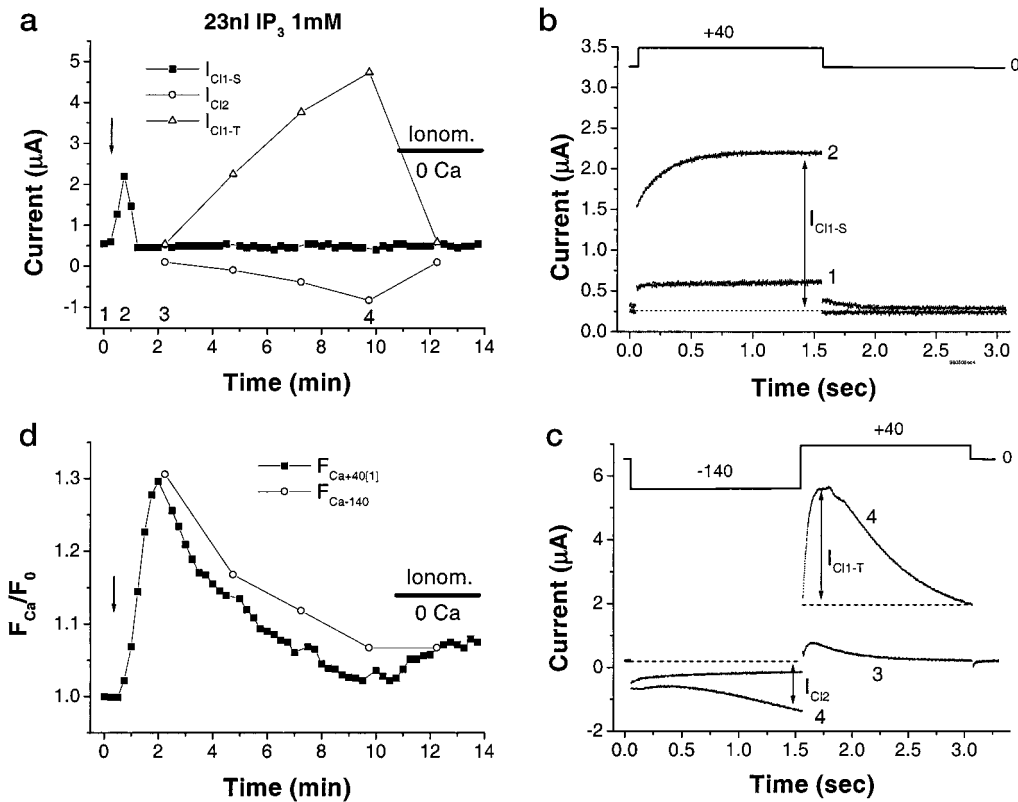


FIG. 1. Effect of large amounts of IP₃ on Cl⁻ currents and Ca²⁺ fluorescence in *Xenopus* oocyte. The voltage protocol was designed to minimize the amount of Ca²⁺ influx while still allowing the visualization of the time-dependent activation of the Cl⁻ channels after Ca²⁺ influx. The cell was stepped to +40 mV for 1.5 s from a holding potential of 0 mV every 15 s for 9 consecutive episodes. In the 10th episode, the cell was stepped to -140 mV then to +40 mV for 1.5 s each. Therefore, every 10th pulse elicited I_{Cl2} and I_{Cl1-T} , whereas the intervening pulses elicited I_{Cl1-S} . Cells were bathed in normal Ringer. The oocyte was injected with Ca-green-1 coupled to 70-kDa dextran 30 min before the experiment. The oocyte was voltage-clamped with two microelectrodes, and 23 nl of 1 mM IP₃ was injected at the arrow. At the end of the experiment, the oocyte was exposed to Ca²⁺-free Ringer containing 14 μ M ionomycin (Ionom.) to assess the Ca²⁺ content remaining in the stores. *a*, summary of Cl⁻ current amplitudes before and after IP₃ injection. *Filled squares*, I_{Cl1-S} ; *open circles*, I_{Cl2} ; and *open triangles*, I_{Cl1-T} . *b*, current traces corresponding to the +40 mV pulses labeled 1 and 2 in *a*. The voltage protocol used is shown at the top. I_{Cl1-S} was measured as the outward current at the end of the +40 mV pulse. *c*, current traces corresponding to the -140 mV/+40 mV pulse combination labeled 3 and 4 in *a*. The voltage protocol used is shown at the top. I_{Cl2} was measured as the inward current at the end of the -140 mV pulse. I_{Cl1-T} was measured as the peak time-dependent outward current during the +40 mV pulse after the -140 mV pulse. *d*, Ca²⁺ fluorescence measured simultaneously with the Cl⁻ currents. Ca²⁺ levels were measured by Ca-green-1 fluorescence at +40 mV during the +40 mV pulses from the 0-mV holding potential (F_{Ca+40} (*filled squares*)) and at -140 mV (F_{Ca-140} (*open circles*)) every 10th pulse. Ca²⁺ fluorescence levels were measured from the entire focal section and normalized to Ca²⁺-dependent fluorescence before IP₃ injection. At the end of the experiment, the oocyte was exposed to 14 μ M ionomycin in calcium-free Ringer (Ionom./0 Ca²⁺) to release any residual Ca²⁺ from stores (see Fig. 6). This cell is representative of 13 cells.

The purpose of this paper was to examine further the mechanisms of AdA regulation of Ca²⁺-activated Cl⁻ currents using confocal scanning microscopy of oocytes loaded with fluorescent Ca²⁺ indicators and two-microelectrode voltage clamp. Here we show that activation of SOCE following injection of low concentrations of AdA depends upon depletion of intracellular Ca²⁺ stores. However, at low AdA concentrations the kinetics of Ca²⁺ release from stores was >7-fold slower than that observed with IP₃. This slower mode of Ca²⁺ release is apparently not effective in activating I_{Cl1-S} . Therefore, different kinetics of Ca²⁺ release can differentially affect Cl⁻ current activation.

EXPERIMENTAL PROCEDURES

Isolation of *Xenopus* Oocytes—Stage V-VI oocytes were harvested from adult albino or normal *Xenopus laevis* females (*Xenopus* I) as described by Dascal (18). *Xenopus* were anesthetized by immersion in Tricaine (1.5 g/liter). Ovarian follicles were removed and digested in normal Ringer with no added calcium, containing 2 mg/ml collagenase type IA (Sigma Chemical Co., St. Louis, MO), for 2 h at room temperature. The oocytes were extensively rinsed with normal Ringer, placed in L-15 medium (Life Technologies, Inc., Gaithersburg, MD) and stored at 18 °C. Oocytes were usually used within 1–5 days after isolation.

Imaging and Electrophysiological Methods—*Xenopus* oocytes were injected with 9 nl Ca-green-1 coupled to 70kd dextran (333 μ M) for a final calculated oocyte concentration of \sim 3 μ M, and voltage-clamped with two-microelectrodes using a GeneClamp 500 (Axon Instruments,

Foster City, CA). Electrodes were filled with 3 M KCl and had resistances of 1–4 M Ω . Oocyte resting potentials were between -20 mV and -50 mV. Typically, the membrane was held at 0 mV and stepped to +40 mV for 1.5 s every 15 s to monitor I_{Cl1-S} . Every 2.25 min, a 1.5-s duration pulse to -140 mV followed by a 1.5-s duration pulse to +40 mV was given to monitor I_{Cl2} and I_{Cl1-T} , respectively. Images (256 \times 256 pixels) were acquired 500 ms after the onset of each voltage pulse using a Zeiss LSM 410 confocal box fitted to a Zeiss Axiovert 100TV inverted microscope using a Zeiss 10 \times objective (0.5 numerical aperture). The confocal aperture was set at the maximal opening, resulting in a focal section 1267 \times 1267 \times 35 μ m. Image data was analyzed using the LSM 410 software or NIH image 1.60 on a Mac IIx. Current data was analyzed on a Pentium PC using Origin 5.0 (Microcal Software, Northampton, MA). For plots of Ca²⁺ fluorescence, the fluorescence intensity of the entire confocal section was averaged and expressed as a ratio of the background fluorescence taken before IP₃ injection. Experiments were performed at room temperature (22–26 °C). Normal Ringer solution contained 123 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 1.8 mM MgCl₂, 10 mM HEPES, pH 7.4; Ca²⁺-free Ringer solution was the same except that CaCl₂ was omitted and, MgCl₂ was increased to 5 mM.

Oocytes were injected with IP₃ using a Nanoject automatic oocyte injector (Drummond Scientific Co., 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 <20 μ m. Solutions of IP₃ or AdA were prepared in Chelex resin-treated H₂O. The Ca²⁺ concentration in this solution was not buffered, but injection of H₂O produced no change in Ca-green

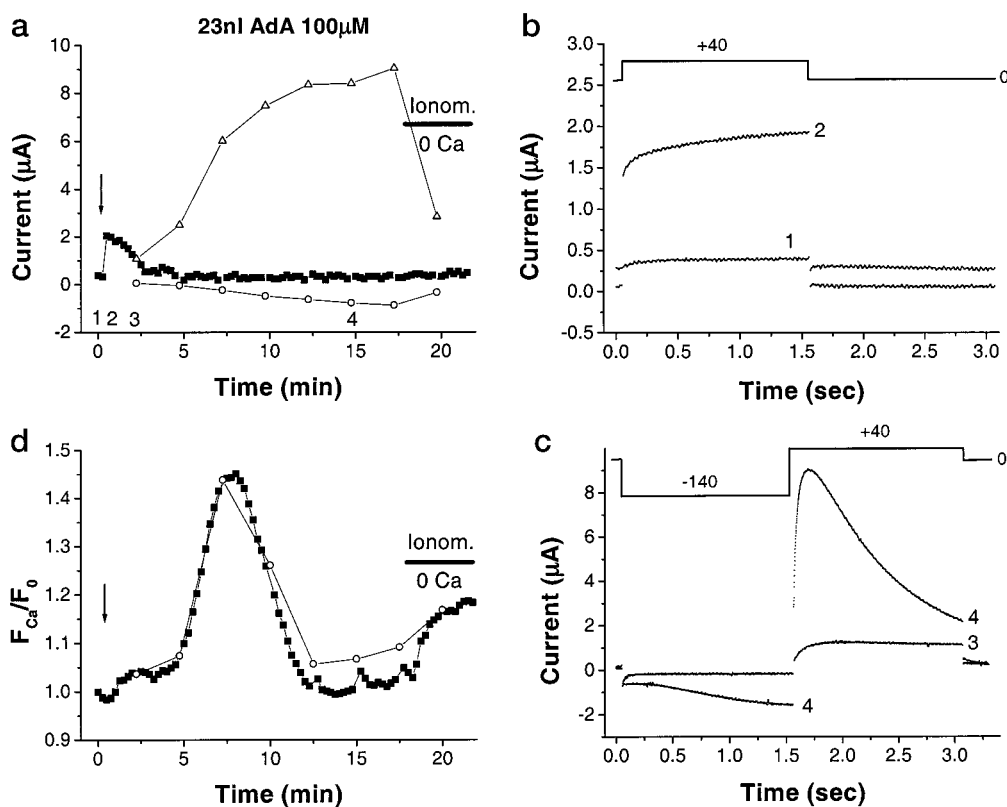


FIG. 2. Effect of large amounts of AdA on Cl⁻ currents and Ca²⁺ fluorescence in *Xenopus* oocyte. The conditions were identical to Fig. 1 except that 23 nl of 100 μM AdA was injected at the arrow. *a*, summary of Cl⁻ current amplitudes before and after AdA injection. Filled squares, $I_{\text{Cl1-S}}$; open circles, I_{Cl2} ; and open triangles, $I_{\text{Cl1-T}}$. *b*, current traces corresponding to the +40 mV pulses labeled 1 and 2 in *a*. *c*, current traces corresponding to the -140 mV/+40 mV pulse combination labeled 3 and 4 in *a*. *d*, Ca²⁺ fluorescence during the +40 mV pulses from the 0 mV holding potential ($F_{\text{Ca}^{+40}}$ (filled squares)) and at -140 mV ($F_{\text{Ca}^{-140}}$ (open circles)) every 10th pulse. At the end of the experiment the oocyte was exposed to 14 μM ionomycin in calcium-free Ringer (Ionom./0 Ca²⁺) to release any residual Ca²⁺ from stores (see Fig. 6). Note that $I_{\text{Cl1-T}}$ in this cell does not completely inactivate when the cell is switched to Ca²⁺-free Ringer, because not all of the Ca²⁺ in the bath had been removed by the time the pulse that stimulated $I_{\text{Cl1-T}}$ occurred. Typically, when the cell is switched to Ca²⁺-free Ringer after AdA injection, $I_{\text{Cl1-T}}$ and I_{Cl2} completely inactivated as after IP₃ injection (Fig. 1*a*). This cell is representative of six cells.

fluorescence or membrane current. Levels of the IP₃R were lowered by injection of 60 ng of the IP₃R antisense primer (AACTAGACATCTT-GTCTGACATGCTGAG) one day before the experiment as described by Kume *et al.* (25). The reverse sense primer (CTGCAGCAATGTCA-GACAAGATGTCTAGTT) was injected at the same level as a control.

RESULTS

Ca²⁺ Transient and Cl⁻ Currents Activated by High Concentrations of IP₃—The protocol used to measure Ca²⁺-activated Cl⁻ currents in *Xenopus* oocytes in response to IP₃ or AdA injection while simultaneously measuring cytosolic Ca²⁺ with confocal microscopy and Ca-green dextran is shown in Fig. 1. About 30 min after injection of Ca-green dextran, the oocytes were voltage-clamped at 0 mV and stepped to +40 mV every 15 s to monitor $I_{\text{Cl1-S}}$. $I_{\text{Cl1-S}}$ (current at the end of the +40 mV pulse, Fig. 1*b*) is an outward current at depolarizing potentials that is activated quickly (~10 s) after IP₃ injection by Ca²⁺ released from intracellular stores (16, 24, 26). In addition, once every 2.25 min, the oocyte was also stepped to -140 mV to monitor I_{Cl2} and then to +40 mV to monitor $I_{\text{Cl1-T}}$. I_{Cl2} (current at the end of the -140 mV pulse, Fig. 1*c*) is an inward current that is activated by Ca²⁺ entry through SOCs driven by the negative membrane potential. $I_{\text{Cl1-T}}$ is a transient outward current (peak outward current during the +40 mV pulse, Fig. 1*c*) that was activated by a depolarizing pulse preceded by a hyperpolarizing pulse to stimulate Ca²⁺ influx. The -140 mV pulse was given only once every 2.25 min to minimize Ca²⁺ influx (and store refilling) during the experiment. For a more detailed discussion of the Cl⁻ currents see Hartzell and co-workers (24, 27, 28).

Fig. 1, *a-c*, shows the response of Cl⁻ currents after injection of large amounts of IP₃ (estimated intra-oocyte concentration ~20 μM). When saturating levels of IP₃ were injected, $I_{\text{Cl1-S}}$ (filled squares) was activated immediately. As the stores became depleted of Ca²⁺ and SOCE developed, $I_{\text{Cl1-T}}$ (open triangles) and I_{Cl2} (open circles) were activated. Injection of IP₃ caused a large increase in Ca²⁺ fluorescence at all potentials (Fig. 1*d*) because of Ca²⁺ release from stores. Before the peak fluorescence was reached, the fluorescence was the same at all potentials, but afterward the fluorescence during the -140 mV pulse became greater than the fluorescence during the +40 mV pulse. The difference between the fluorescence at -140 mV and +40 mV is the voltage-dependent Ca²⁺ fluorescence, which we have shown is related to Ca²⁺ entry through SOCs (28).

Ca²⁺ Waves Stimulated by AdA Are Very Slow—Injection of large amounts of AdA (estimated intra-oocyte concentration ~2 μM ; note that AdA is 10–100 times more potent than IP₃ (13)) produced rather similar effects on the Cl⁻ currents to those produced by IP₃ (Fig. 2, *a-c*). There was a striking difference, however, in the kinetics of the Ca²⁺ fluorescence change produced by IP₃ and by AdA. The Ca²⁺ fluorescence did not begin to increase for several min and peaked ~8 min after injection of AdA (Fig. 2*d*). By comparison, after IP₃ injection, the Ca²⁺ fluorescence peaked in less than 2 min (Fig. 1*d*).

It may seem surprising in Fig. 2 that the Ca²⁺ wave peaked so much more slowly than $I_{\text{Cl1-S}}$. It should be noted that the 1-mm diameter oocyte is on the stage of an inverted microscope and that the injection takes place at the top, whereas the confocal image plane is <30 μm from the bottom. Cl⁻ currents,

which are measured from the entire surface of the oocyte, increase as soon as Ca²⁺ is released from stores near the injection site. However, the slow increase in Ca²⁺ fluorescence partly reflects the very slow transit time of the Ca²⁺ wave from the injection site to the confocal image plane ~1 mm away. There is some variability in the lag period between AdA injection and the increase in Ca²⁺ fluorescence. This variability is most likely related to the depth and position of the injection pipette in the oocyte.

The Ca²⁺ waves induced by injection of smaller amounts of AdA moved even more slowly. In Fig. 3*a*, typical traces of Ca²⁺ fluorescence at +40 mV in response to injection of large amounts of IP₃ (~20 μM, filled squares), large amounts of AdA (~2 μM, open circles), and small amounts of AdA (~5 nM, open triangles) are superimposed. In the case of low concentrations of adenophostin, the time-to-peak of the Ca²⁺ fluorescence was ~20 min. Fig. 3*b* shows averages of the time-to-peak of the Ca²⁺ fluorescence to these injections. The time-to-peak for large concentrations of AdA was >2 times slower than for large concentrations of IP₃, and the time-to-peak for small concentrations of AdA was >7 times slower than for large concentrations of IP₃. It was not possible to measure the time-to-peak for small IP₃ concentrations because small IP₃ concentrations produced oscillating Ca²⁺ waves that exhibited no clear peak. The slowness of the Ca²⁺ wave is illustrated in a different way in the images in Fig. 3*c*. After injection of AdA, the spread of the Ca²⁺ fluorescence is very slow relative to the spread of the IP₃-induced wave of Ca²⁺ release.

These data confirm our earlier suggestion that AdA causes release of Ca²⁺ from stores much more slowly than IP₃ does. These findings support the idea that small concentrations of AdA do not stimulate I_{Cl1-S} because slow release of Ca²⁺ from stores does not elevate Ca²⁺ in the vicinity of the Cl⁻ channels to an activating level. This could occur if efflux and/or local Ca²⁺ buffering removes free Ca²⁺ as rapidly as it is released, so that an effective Ca²⁺ concentration is not attained.

Small Concentrations of AdA Completely Deplete Ca²⁺ Stores—Although Fig. 3 shows that low concentrations of AdA release Ca²⁺ from stores, the question remains whether the stimulation of Ca²⁺ entry by low concentrations of AdA is because of depletion of stores. For example, the AdA-stimulated Ca²⁺ release might be so slow that the stores refill. To examine this question, we measured the effects of low concentrations of AdA (~5 nM) that did not activate I_{Cl1-S} on Ca²⁺ store depletion. Fig. 4 shows the results of a typical experiment. Injection of 10 nl of 0.5 μM AdA did not detectably stimulate I_{Cl1-S} (Fig. 4, *a-c*), but both I_{Cl1-T} and I_{Cl2} developed robustly. I_{Cl1-T} and I_{Cl2} were dependent on extracellular Ca²⁺, and their activation corresponded to the activation of SOCE (16). Ca²⁺ fluorescence began to increase about 5 min after AdA injection and continued to increase for 20 min (Fig. 4*d*). Voltage-dependent Ca²⁺ fluorescence (open circles), which reflects SOCE, developed shortly after Ca²⁺ release and remained at a high level for the duration of the experiment. To test whether stores were depleted of Ca²⁺, ionomycin in Ca²⁺-free Ringer was applied to release Ca²⁺ from any remaining stores. Ionomycin had only a very small effect on I_{Cl1-S} and had no effect on the Ca²⁺ fluorescence at +40 mV. This showed that the stores had been virtually completely depleted of Ca²⁺ by AdA.

This result contrasts to that observed when small amounts of IP₃ were injected (Fig. 5). Concentrations of IP₃ that stimulated Ca²⁺ influx, as determined by the presence of voltage-dependent Ca²⁺ fluorescence and activation of I_{Cl1-T} and I_{Cl2}, inevitably stimulated I_{Cl1-S}. In some cells, as in Fig. 5, the increase in I_{Cl1-S} was not accompanied by a significant increase

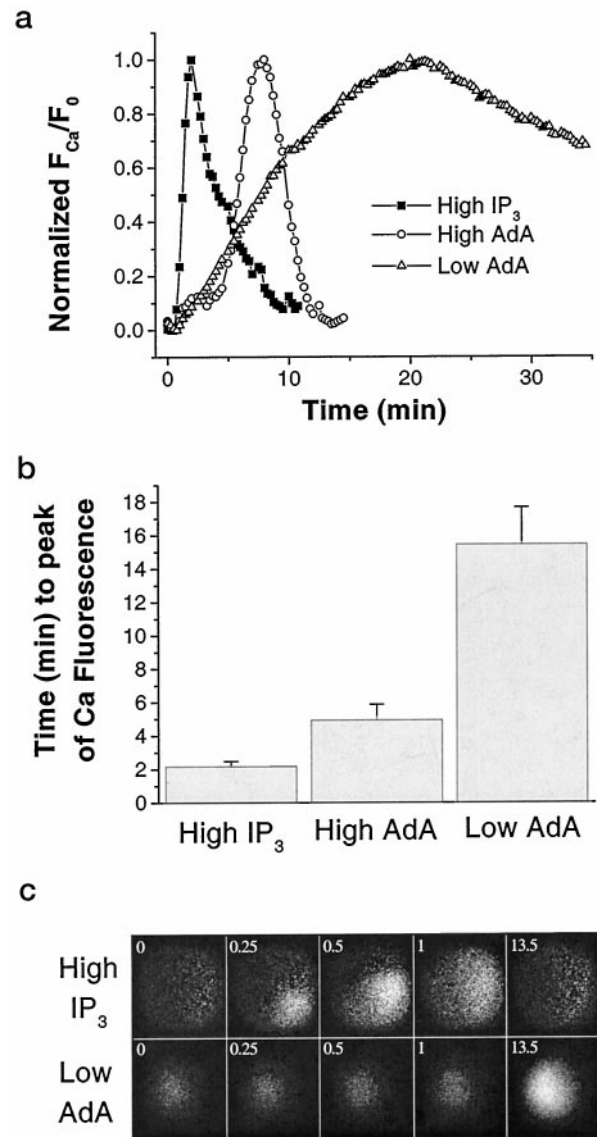


FIG. 3. Comparison of the velocity of Ca²⁺ waves in response to IP₃ and AdA injection. *a*, Ca²⁺ fluorescence at +40 mV in response to injection of 23 nl of 1 mM IP₃ (filled circles), 23 nl of 100 μM AdA (open circles), and 10 nl of 0.5 μM AdA (open triangles). *b*, time-to-peak of Ca²⁺ waves measured from time of injection of 23 nl of 1 mM IP₃ (High IP₃, *n* = 6), 23 nl of 100 μM AdA (High AdA, *n* = 5), and 10 nl of 0.5 μM AdA (Low AdA, *n* = 7). The speed of the Ca²⁺ release wave after IP₃ or AdA injection was estimated by calculating the time required for the Ca²⁺-dependent fluorescence to reach its maximal value. It was not possible to perform the same analysis on cells injected with low levels of IP₃, because in many instances, such IP₃ injections lead to repetitive Ca²⁺ waves that oscillate and not a single wave that sweeps through the entire oocytes as observed when the oocyte is injected with high IP₃ levels or AdA. The speed of the wave was significantly slower (*p* < 0.006) between high IP₃ and AdA injections and between high and low AdA injections. *c*, images of Ca-green-1 fluorescence in response to injection of 23 nl of 1 mM IP₃ (High IP₃) and 10 nl of 0.5 μM AdA (Low AdA). The times in min at which the confocal images were taken are indicated in the top left corner of each image. IP₃ or AdA were injected at time 0.

in Ca²⁺ fluorescence, because the IP₃ effect was local and did not propagate into the region of the oocyte that was imaged. Both voltage-dependent Ca²⁺ fluorescence and I_{Cl1-T} and I_{Cl2} eventually declined to base line. Application of ionomycin at the end of the experiment evoked a large increase in Ca²⁺ fluorescence and in I_{Cl1-S}, showing that the stores were not completely depleted of Ca²⁺.

To obtain a more quantitative measure of the extent of store

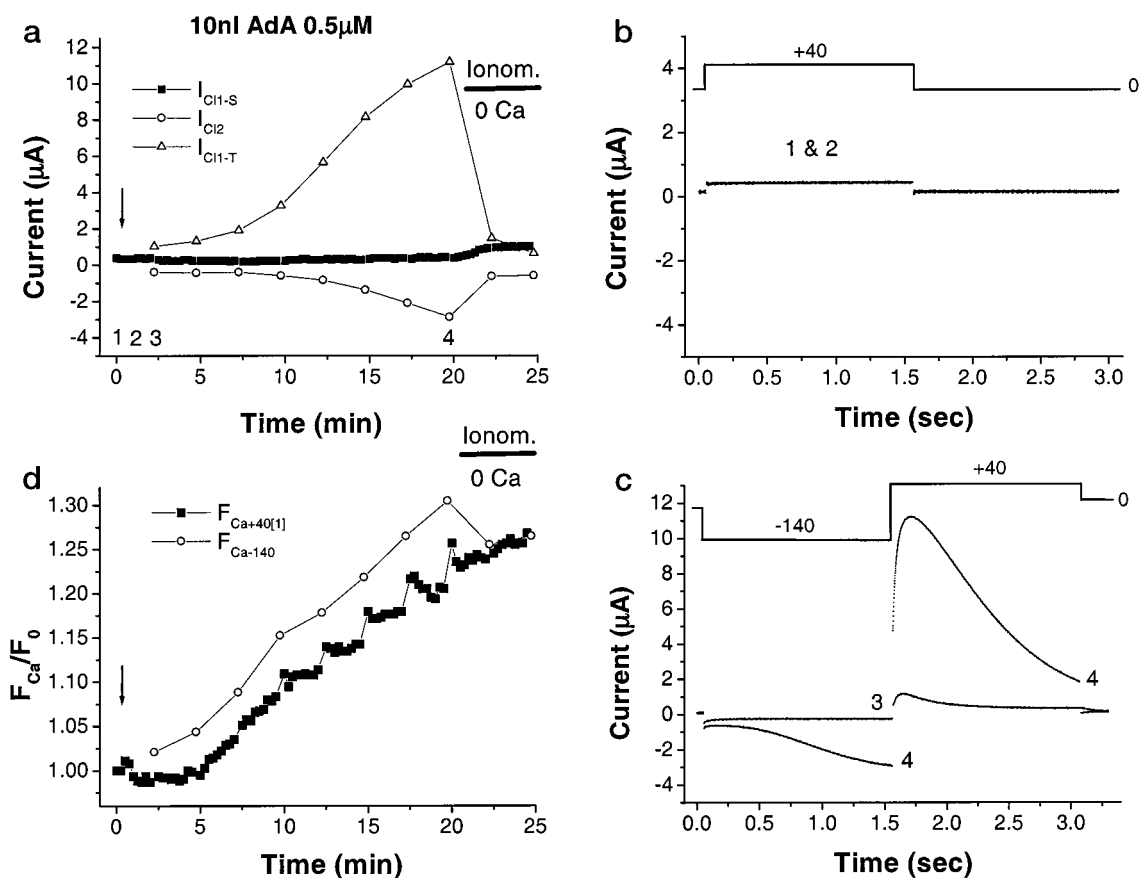


FIG. 4. Effect of small amount of AdA on Cl⁻ currents and Ca²⁺ fluorescence in *Xenopus* oocyte. The conditions were identical to Fig. 1 except that 10 nl of 0.5 μM AdA was injected at the arrow. *a*, summary of Cl⁻ current amplitudes before and after AdA injection. Filled squares, I_{C11-S}; open circles, I_{C12}; and open triangles, I_{C11-T}. *b*, current traces corresponding to the +40 mV pulses labeled 1 and 2 in *a*. *c*, current traces corresponding to the -140 mV/+40 mV pulse combination labeled 3 and 4 in *a* and *d*. Ca²⁺ fluorescence during the +40 mV pulses from the 0-mV holding potential (F_{Ca+40}; filled squares) and at -140 mV (F_{Ca-140}; open circles) every 10th pulse. At the end of the experiment, the oocyte was exposed to 14 μM ionomycin in calcium-free Ringer (Ionom. 10 Ca²⁺) to release any residual Ca²⁺ from stores (see Fig. 6). This cell is representative of eight cells.

depletion after injection of IP₃ or AdA, we calculated the ratio of ionomycin-induced Ca²⁺ release to IP₃- or AdA-induced Ca²⁺ release. This ratio gives a measure of the level of residual Ca²⁺ in intracellular stores after IP₃R agonist injection. The results from these experiments are shown in Fig. 6. Injections of high IP₃, high AdA, or low AdA all left the stores largely depleted of Ca²⁺. In contrast, low IP₃ concentrations were less effective in depleting the stores. These data show that concentrations of AdA that did not noticeably activate I_{C11-S} were capable of depleting intracellular Ca²⁺ stores to similar levels as high concentrations of IP₃.

Thus, we conclude that AdA stimulates SOCE as a consequence of depletion of internal Ca²⁺ stores and not by some direct effect on SOCs. Furthermore, previous conclusions, based on Ca²⁺-activated Cl⁻ current activation, which suggested that low concentrations of AdA stimulate SOCE without releasing Ca²⁺ from stores (17), can be explained by the observation that slow release of Ca²⁺ from stores is often insufficient to activate I_{C11-S}.

Effect of AdA on SOCE Requires Active IP₃R—If this conclusion is correct, the effects of AdA on SOCE should depend on the ability of the IP₃R to release Ca²⁺. Thus, treatments that suppress IP₃R function should inhibit the effects of AdA injections. We suppressed IP₃R function either by injecting the competitive inhibitor heparin (Fig. 7) or by reducing IP₃R expression by injection of antisense oligonucleotides to the *Xenopus* IP₃R (Fig. 8).

Injection of heparin to block the IP₃R significantly reduced

I_{C12} and I_{C11-T} currents induced by small AdA injections (Fig. 7, *a–b*). In a similar fashion, heparin blocked the Cl⁻ current response induced by IP₃ (Fig. 7, *c–d*). Reducing IP₃R levels by antisense oligonucleotides as described previously by others (25, 29) also reduced the effects of IP₃ and AdA treatments on I_{C11-T} and I_{C12} (Fig. 8). The effects of antisense treatment were less pronounced than the effects of heparin, but it was clear that antisense had a significant effect. Note that although antisense treatment inhibited I_{C12} and I_{C11-T} in response to IP₃ injection, there was no decrease in levels of I_{C11-S} (Fig. 8*d*). Actually, I_{C11-S} was slightly potentiated as compared with sense-injected cells (Fig. 8*c*). This observation could be explained if one assumes there are two distinct subpopulations of IP₃ receptors with differential turnover rates of the IP₃R. If we postulate the existence of subsets of stores, one close to the I_{C11-S} Cl⁻ channels containing IP₃R with a very slow turnover rate and a second located further from the Cl⁻ channels containing an IP₃R population that turns over rapidly, then injection of antisense IP₃ oligonucleotides will reduce the levels of IP₃R in the latter subset faster, resulting in sufficient Ca²⁺ release after IP₃ injection to activate I_{C11-S} but insufficient release from most of the stores to induce significant SOCE.

DISCUSSION

In many cell types, release of Ca²⁺ from endoplasmic reticulum stores stimulates Ca²⁺ influx into the cytosol from the extracellular space through SOCs by a process termed SOCE. The mechanisms by which release of Ca²⁺ from stores stimu-

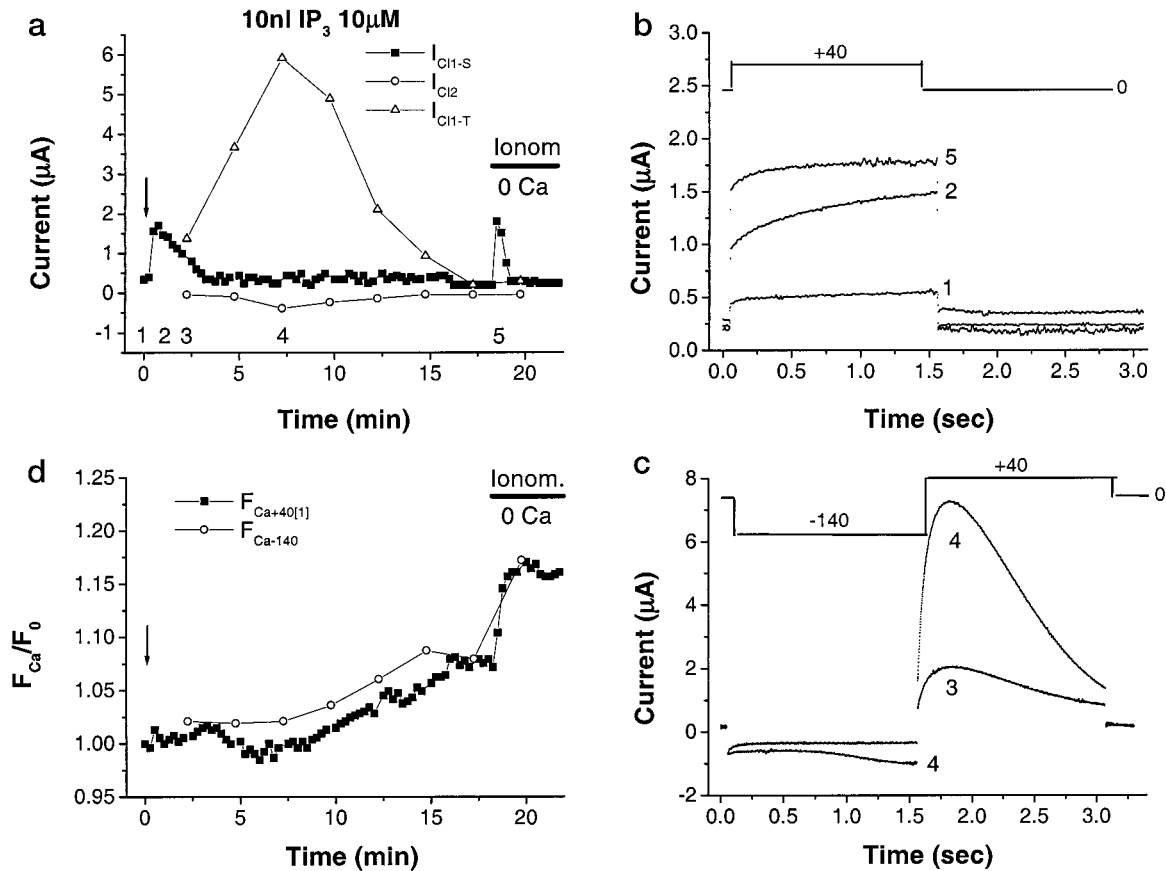


FIG. 5. Effect of small amounts of IP₃ on Cl⁻ currents and Ca²⁺ fluorescence in *Xenopus* oocyte. The conditions were identical to Fig. 1 except that 10 nl of 10 μ M IP₃ was injected at the arrow. *a*, summary of Cl⁻ current amplitudes before and after IP₃ injection. *Filled squares*, I_{Cl1-S} ; *open circles*, I_{Cl2} ; and *open triangles*, I_{Cl1-T} . *b*, current traces corresponding to the +40 mV pulses labeled 1, 2, and 5 in *a*. *c*, current traces corresponding to the -140 mV/+40 mV pulse combination labeled 3 and 4 in *a*. *d*, Ca²⁺ fluorescence during the +40 mV pulses from the 0-mV holding potential (F_{Ca+40} (filled squares)) and at -140 mV (F_{Ca-140} (open circles)) every 10th pulse. At the end of the experiment, the oocyte was exposed to 14 μ M ionomycin in calcium-free Ringer (*Ionom./0 Ca²⁺*) to release any residual Ca²⁺ from stores (see Fig. 6). This cell is representative of 14 cells.

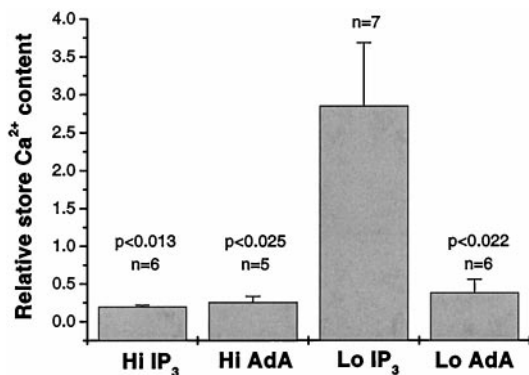


FIG. 6. The extent of store depletion after IP₃ or AdA injection. The relative Ca²⁺ content remaining in the internal stores after injection of IP₃ or AdA was assessed in Figs. 1, 2, 4, and 5 by exposure of the oocyte to 14 μ M ionomycin. The relative amount of Ca²⁺ remaining in the store was expressed as the ratio of the peak amplitude of the Ca²⁺ fluorescence at +40 mV produced by ionomycin exposure to the peak Ca²⁺ fluorescence at +40 mV produced by IP₃ or AdA injection. *Hi IP₃*, 23 nl of 1 mM IP₃; *Hi AdA*, 23 nl of 10 μ M AdA; *Lo IP₃*, 10 nl of 10 μ M IP₃; *Lo AdA*, 10 nl of 0.5 μ M AdA. The bars show mean ratio \pm S.E. The number of cells used for this analysis is indicated on top of each bar. The level of Ca²⁺ in intracellular stores after high IP₃, high AdA, or low AdA treatments was not significantly different ($p > 0.32$). However, all three treatments were significantly different ($p < 0.025$) than injection of low concentrations of IP₃.

lates SOCE is unknown, but one hypothesis states that the endoplasmic reticulum releases a diffusible chemical messenger that opens SOCs. The search for such a calcium influx

factor has so far not been very fruitful, and the putative calcium influx factors that have been discovered have not found universal acceptance (9, 10). When it was suggested that AdA could stimulate Ca²⁺ influx without stimulating Ca²⁺ release from stores (17), some hope was raised that clues to the structure of calcium influx factors would be learned from AdA. The suggestion that AdA could stimulate Ca²⁺ entry without depleting Ca²⁺ from stores was based on the observation that low concentrations of AdA did not stimulate Ca²⁺-activated Cl⁻ currents in the absence of extracellular Ca²⁺ and therefore did not release Ca²⁺ from stores but did stimulate Ca²⁺-activated Cl⁻ currents in the presence of Ca²⁺ influx. The present studies using Ca²⁺ imaging demonstrate, however, that even very low amounts of AdA (calculated oocyte concentration \sim 5 nM) released Ca²⁺ from stores. Under these conditions, even though I_{Cl1-S} was not activated, the stores were completely depleted of Ca²⁺ as demonstrated by the inability of ionomycin to increase Ca²⁺ fluorescence. We believe that the Ca²⁺ released from stores by low concentrations of AdA is unable to activate I_{Cl1-S} because of its significantly slower rate of Ca²⁺ release (\sim 7 times slower than IP₃).

How do the kinetics of Ca²⁺ release from stores determine the response of the Cl⁻ channels? It has been suggested that I_{Cl1-S} responds to the rate-of-change of cytosolic Ca²⁺ (20) because the peak activation of I_{Cl1-S} corresponds to the maximum rate of change of cytosolic Ca²⁺ and because the amplitude of I_{Cl1-S} does not correlate with the steady-state levels of cytosolic Ca²⁺. However, we have shown (27) that the turn-off of I_{Cl1-S} is not explained by inactivation of the current as previously sug-

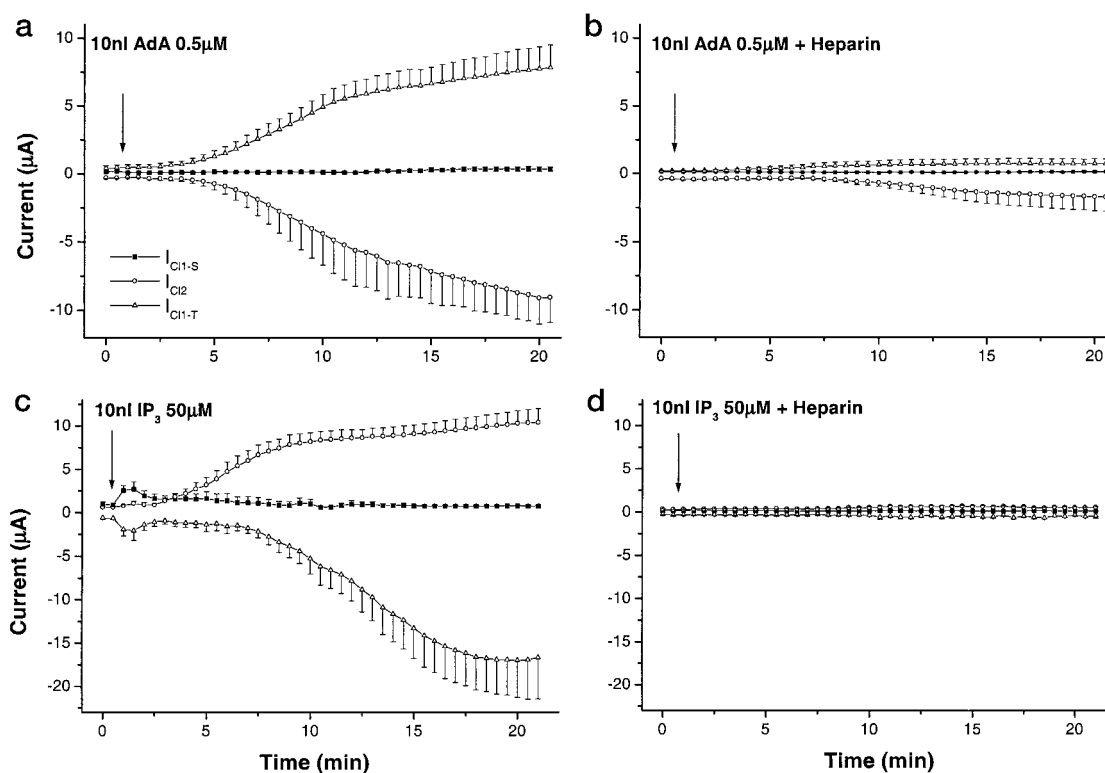


FIG. 7. Heparin blocks both IP_3 - and AdA-dependent store depletion. *a-b*, cells were injected with 10 nl of 0.5 µM AdA alone (*a*; $n = 5$) or preinjected with 92 nl of 100 mg/ml heparin before AdA injection (*b*; $n = 6$). *c-d*, cells were injected with 10 nl of 50 µM IP_3 alone (*c*; $n = 5$) or preinjected with 92 nl of 100 mg/ml heparin before IP_3 injection (*d*; $n = 3$). The site of injection is indicated by the arrow. Cl^- currents are measured as described in Fig. 1.

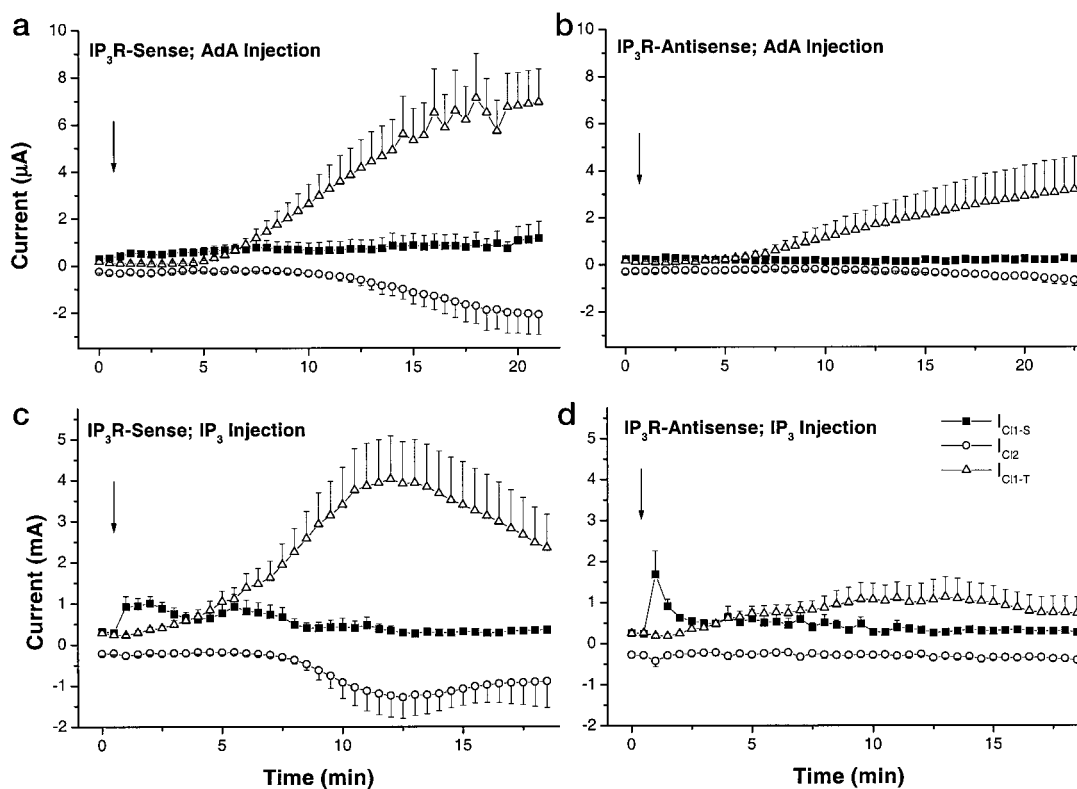


FIG. 8. Lowering IP_3R levels blocks AdA and IP_3 induced SOCE. Oocytes were injected with 60 ng of sense (*a* and *c*) or antisense (*b* and *d*) IP_3R oligonucleotides (25) and incubated for 1–2 days. Oocytes were injected with 10 nl of 0.5 µM AdA (*a* and *b*) or with 23 nl of 10 µM IP_3 (*c* and *d*) as indicated by the arrow in each panel. Filled squares, I_{Cl1-S} ; open circles, I_{Cl2} ; and open triangles, I_{Cl1-T} . $n = 6-7$ oocytes/panel.

gested (20). Furthermore, we have found that the Ca^{2+} concentration measured by cytosolic Ca^{2+} dyes (such as Ca^{2+} -green dextran) does not reflect the concentration of Ca^{2+} just below

the plasma membrane (measured by lipophilic Ca^{2+} dyes such as Ca -green C18) (28). We have presented evidence that the subplasmalemmal Ca^{2+} concentration changes much more

quickly than does the Ca²⁺ concentration deeper in the cytosol because plasma membrane Ca²⁺ efflux systems can rapidly clear Ca²⁺ from the subplasmalemmal space. Consequently, we would predict that the subplasmalemmal Ca²⁺ concentration would depend on the relative rates of Ca²⁺ release from stores and cytosolic Ca²⁺ buffering and Ca²⁺ efflux from the oocyte. If Ca²⁺ release is slow, the concentration of Ca²⁺ in the subplasmalemmal space may not rise sufficiently to activate Ca²⁺-activated Cl⁻ channels.

The different kinetics of Ca²⁺ release produced by AdA and IP₃ are probably related to differences between AdA and IP₃ activation of IP₃Rs. First, the apparent diffusion coefficient of AdA or IP₃ in the cytosol will depend on the fraction of molecules (κ) that are bound to the IP₃R at any one time ($D_{\text{obs}} = D/\kappa$). Because AdA has a 100-fold higher affinity for the IP₃R than IP₃ does, AdA diffusion will be slower because a larger fraction of the total AdA (compared with IP₃) will be bound to IP₃Rs. Second, AdA exhibits a higher cooperativity in activating IP₃Rs than IP₃ does. Hirota *et al.* 1995 (13) have shown that IP₃ has a Hill coefficient of 1.8 for Ca²⁺ release by the type 1 IP₃R, whereas the Hill coefficient for AdA was 3.9. This implies that at least 2 molecules of IP₃ and 4 molecules of AdA are needed to open an IP₃R. This factor will also contribute to the slow movement of the Ca²⁺ release wave in response to small amounts of AdA. Accordingly, the elementary Ca²⁺ release events ("Ca²⁺ puffs") induced by AdA have been shown by Marchant and Parker (30) to be smaller and faster than those induced by IP₃. Because Ca²⁺ waves are initiated by the summation of Ca²⁺ puffs, the smaller and faster puffs induced by AdA may contribute to the slower propagation of the AdA wave. However, the mechanisms by which AdA releases Ca²⁺ from stores remains to be fully elucidated.

Although low concentrations of AdA evoke a slow release of Ca²⁺ from stores and little or no I_{Cl1-S}, high concentrations release Ca²⁺ only about 2-fold more slowly than IP₃ and also evoke significant I_{Cl1-S}. This finding that AdA activates different I_{Cl1-S} responses depending upon the kinetics of Ca²⁺ release from intracellular stores is interesting because it provides another example of how the temporal features of a Ca²⁺ signal contribute to its physiological consequences. Different receptors can induce different Ca²⁺ release kinetics depending on factors including spatial localization of the receptor and/or IP₃-sensitive stores or the activation of different PLC isoforms

(31–36). These different release kinetics can then play an important role in determining which effectors are activated.

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