Potentiation and inhibition of Ca^{2+} release-activated Ca^{2+} channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP_3 receptors

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1. The effects of the IP_3-receptor antagonist 2-aminoethyldiphenyl borate (2-APB) on the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) in Jurkat human T cells, DT40 chicken B cells and rat basophilic leukaemia (RBL) cells were examined.

2. 2-APB elicited both stimulatory and inhibitory effects on Ca^{2+} influx through CRAC channels. At concentrations of 1–5 μM, 2-APB enhanced Ca^{2+} entry in intact cells and increased I_{CRAC} amplitude by up to fivefold. At levels $\geqslant$ 10 μM, 2-APB caused a transient enhancement of I_{CRAC} followed by inhibition.

3. 2-APB altered the kinetics of fast Ca^{2+}-dependent inactivation of I_{CRAC}. At concentrations of 1–5 μM, 2-APB increased the rate of fast inactivation. In contrast, 2-APB at higher concentrations ($\geqslant$ 10 μM) reduced or completely blocked inactivation.

4. 2-APB inhibited Ca^{2+} efflux from mitochondria.

5. 2-APB inhibited I_{CRAC} more potently when applied extracellularly than intracellularly. Furthermore, increased protonation of 2-APB at low pH did not affect potentiation or inhibition. Thus, 2-APB may have an extracellular site of action.

6. Neither I_{CRAC} activation by passive store depletion nor the effects of 2-APB were altered by intracellular dialysis with 500 μg ml^{-1} heparin.

7. I_{CRAC} is present in wild-type as well as mutant DT40 B cells lacking all three IP_3 receptor isoforms. 2-APB also potentiates and inhibits I_{CRAC} in both cell types, indicating that 2-APB exerts its effects independently of IP_3 receptors.

8. Our results show that CRAC channel activation does not require physical interaction with IP_3 receptors as proposed in the conformational coupling model. Potentiation of I_{CRAC} by 2-APB may be a useful diagnostic feature for positive identification of putative CRAC channel genes, and provides a novel tool for exploring the physiological functions of store-operated channels.

Depletion of Ca^{2+} from the endoplasmic reticulum (ER) activates Ca^{2+} entry across the plasma membrane in a variety of cell types, a process known as store-operated Ca^{2+} entry. Imaging and patch-clamp studies indicate that the store-operated Ca^{2+} entry mechanism exists in a wide range of tissues (Parekh & Penner, 1997; Putney, 1997; Lewis, 1999). Nevertheless, detailed information about the properties of the channels underlying store-operated Ca^{2+} entry is available only for a few cell types such as Jurkat T cells, mast cells and RBL cells. The store-operated current in these cells is highly selective for Ca^{2+} and is called the calcium release-activated Ca^{2+} current, or I_{CRAC}. The hallmarks of I_{CRAC} include an extremely high selectivity for Ca^{2+} over monovalent cations, an extremely low apparent unitary conductance for Ca^{2+}, voltage-independent gating, inward rectification and multiple modes of inactivation by Ca^{2+} (reviewed by Parekh & Penner, 1997; Lewis, 1999). CRAC channels play essential roles in controlling gene expression during the activation of T cells by antigen (Partiseti *et al.* 1994; Fanger *et al.* 1995) and in triggering secretion of inflammatory mediators from mast cells stimulated by allergens (Zhang & McCloskey, 1995).

Despite the wealth of electrophysiological data on the properties of I_{CRAC}, neither the mechanism(s) that links store depletion to activation of the current nor the molecular identity of the channels is well understood. Hypotheses presented to explain the mechanism of activation of I_{CRAC} by store depletion have included activation by calcium influx factor, vesicle-mediated insertion of active channels in the membrane and

physical coupling with IP_3 receptors in the ER membrane (for reviews, see Putney & McKay, 1999; Prakriya & Lewis, 2001*a*). The last hypothesis, known as the conformational coupling hypothesis (Berridge, 1995), has received support from observations that activation of Ca^{2+} entry requires close apposition of the ER, where IP_3 receptors are localized, and the plasma membrane (Boulay *et al.* 1999; Patterson *et al.* 1999; Ma *et al.* 2000). Further evidence comes from reports of binding and functional interactions between transient receptor potential (TRP)3 channels and IP_3 receptors (Kiselyov *et al.* 1998, 1999*a*; Boulay *et al.* 1999). Although the evidence that *trp3* encodes a store-operated channel is controversial (Zitt *et al.* 1997; Hofmann *et al.* 1999; Ma *et al.* 2000), it has been reported that an IP_3 receptor antagonist, 2-aminoethyldiphenyl borate (2-APB), blocks not only TRP3-mediated but also native store-operated Ca^{2+} influx in HEK cells (Ma *et al.* 2000).

2-APB has been described as a non-competitive antagonist of the IP_3 receptor and has been shown to inhibit IP_3 receptor-induced $[Ca^{2+}]_i$ elevations in a variety of cell types (Sugawara *et al.* 1997; Ma *et al.* 2000). Inhibition of store-operated Ca^{2+} entry by 2-APB has been taken as evidence for a role of IP_3 receptors in the activation of store-operated channels and therefore as proof for conformational coupling between store-operated channels and IP_3 receptors (Ma *et al.* 2000; van Rossum *et al.* 2000). However, 2-APB is not yet well characterized in terms of its possible sites of action, and more recent work suggests that 2-APB may inhibit I_{CRAC} in RBL cells independently of the IP_3 receptor (Braun *et al.* 2001). A more thorough characterization of the effects of 2-APB on I_{CRAC} and other Ca^{2+} transport pathways is needed to test the potential utility of this compound in studying the activation and regulation of store-operated channels such as the CRAC channel.

To explore the possible role of IP_3 receptors in the induction and maintenance of I_{CRAC}, we examined the effects of 2-APB in Jurkat T cells, DT40 B cells and RBL cells. We found that 2-APB has complex effects on I_{CRAC}. At low concentrations ($\leqslant 5\ \mu$M), 2-APB enhances the size of I_{CRAC} and speeds up fast Ca^{2+}-dependent inactivation of the channels. At higher concentrations, 2-APB inhibits I_{CRAC} while also removing Ca^{2+}-dependent inactivation. These complex effects on I_{CRAC} also occur in mutant DT40 cells that lack IP_3 receptors. Thus, our results indicate that 2-APB elicits multiple effects on I_{CRAC} gating by a mechanism that is independent of IP_3 receptors. These results have been reported previously in abstract form (Prakriya & Lewis, 2001*b*).

METHODS

Cells

Jurkat T cells (Jurkat E6-1 cell line) were grown in medium consisting of RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and penicillin–streptomycin. DT40 B cells and DT40 IP_3 receptor triple-knockout cells (RIKEN Cell Bank, Ibaraki, Japan) were maintained in a medium containing RPMI 1640 supplemented with 10% FCS, 5% chicken serum, 4 mM glutamine, 50 μM β-mercaptoethanol and penicillin–streptomycin. RBL-2H3 cells were grown in DMEM supplemented with 10% FCS, 4 mM glutamine and penicillin–streptomycin. All cells were maintained in log-phase growth at 37 °C in 6% CO_2.

Solutions and chemicals

The extracellular solution contained (mM): 155 NaCl, 4.5 KCl, 1 $MgCl_2$, 10 D-glucose and 5 Na-Hepes (pH 7.4) with 2 mM $CaCl_2$ in Ca^{2+} imaging experiments, and 20 mM $CaCl_2$ or 20 mM $BaCl_2$ in patch-clamp experiments. Ca^{2+}-free solutions contained 1 mM EGTA and 3 mM $MgCl_2$. The intracellular (pipette) solution contained (mM): 150 caesium methanesulfonate, 10 Cs-Hepes, 10 EGTA and 3 $MgCl_2$ (pH 7.3). In some experiments, a weakly buffered internal solution containing 1.2 mM EGTA was used. To isolate I_{CRAC} from inward rectifier K^+ currents in RBL-2H3 cells, 10 mM $CsCl_2$ was added to the standard extracellular solution. In experiments involving caged IP_3, 15 μM NPE-IP_3 (Calbiochem) was included in the patch pipette, and the internal solution contained 1.2 mM EGTA + 0.705 mM $CaCl_2$ (calculated free $[Ca^{2+}] = 150$ nM) to prevent store depletion prior to IP_3 release.

2-APB was the kind gift of Dr K. Mikoshiba (Tokyo University, Japan). In some experiments, 2-APB purchased from Sigma Chemical Co. (St Louis, MO, USA) was used; the drugs from both sources had identical effects on I_{CRAC}. Stock solutions of 2-APB, thapsigargin (TG; Sigma) and oligomycin (Sigma) were prepared in DMSO at concentrations of 20, 1 and 2 mM respectively. Antimycin A1 (Sigma) was dissolved in ethanol at 2 mM. The drugs were diluted to the concentrations indicated in the figure legends and applied to the cells using a multibarrel local perfusion pipette with a common delivery port. The solution exchange time was measured to be < 2 s, based on the rate at which the K^+ current reversal potential changed when the external solution was switched from 2 mM K^+ to 150 mM K^+.

$[Ca^{2+}]_i$ imaging

Cells were loaded for 15 min at 37 °C with 1 μM fura-2 AM (Molecular Probes, OR, USA) dissolved in Jurkat medium, then were washed with Jurkat or DT40 medium and stored in the dark until ready for use. Fura-2-loaded cells were plated on glass coverslips coated with poly-D-lysine and were preincubated for several minutes in an external solution containing 0.5 mM Ca^{2+}. Coverslips were mounted on the stage of a Zeiss Axiovert 35 microscope equipped with a ×40 Achrostigmat objective (NA 1.3). Imaging protocols and equipment were as described previously (Hoth *et al.* 1997). Briefly, cells were alternately illuminated at 350 and 380 nm using band-pass filters (Chroma Technology) and the fluorescence emissions at wavelengths greater than 480 nm were captured with an intensified CCD camera (Hamamatsu, Japan) and digitized and analysed using a VideoProbe imaging system (ETM Systems, Irvine, CA, USA). The 350 nm/380 nm ratio images were recorded at intervals of 5 s. $[Ca^{2+}]_i$ was calculated from the relationship $[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R)$, where the values of K, R_{min} and R_{max} were determined from an *in situ* calibration of fura-2 in Jurkat T cells loaded by intracellular dialysis (Lewis & Cahalan, 1989). R_{min} and R_{max} are the fluorescence ratios determined with internal solutions containing 10 mM EGTA and 10 mM $CaCl_2$, respectively; K was determined using an internal solution containing 180 nM free $[Ca^{2+}]_i$.

Patch-clamp recording

Patch-clamp experiments were conducted in the standard whole-cell recording configuration using recording electrodes that were pulled from 100 μl pipettes, coated with Sylgard and fire polished to a final resistance of 2–5 MΩ. Data were collected with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, USA) interfaced to an ITC-16 input/output board (Instrutech, Port Washington, NY, USA)

and a Macintosh G3 computer. Stimulation, data acquisition and analysis were performed using in-house software developed on the Igor Pro platform (Wavemetrics, Lake Oswego, OR, USA). The command voltage protocol usually consisted of a 100 ms step from the holding potential to −100 mV followed immediately by a 100 ms ramp from −100 to +60 mV, applied every 1–2 s. The holding potential was +30 mV unless otherwise indicated. Current was sampled at a rate of 5 kHz and filtered at 500 Hz offline. All data were corrected for leak currents that were collected using the same voltage stimulus in a Ca^{2+}-free bath solution, after I_{CRAC} induction and before data collection. Unless otherwise indicated, all average results are presented as the mean value ± S.E.M.

Two methods were typically employed to deplete intracellular Ca^{2+} stores and activate I_{CRAC}. With the first method, stores were passively depleted by dialysing the cells with an internal recording solution containing either 10 or 1.2 mM EGTA. With the second method, cells were preincubated in the recording chamber with Ca^{2+}-free Ringer solution containing 1 μM TG for 5–10 min before seal formation. In the experiments of Fig. 9*C*, I_{CRAC} was activated by the release of caged IP_3; NPE-IP_3 was photolysed by a 10 s exposure to unattenuated light from a xenon light source (Lambda-10, Sutter Instruments) passed through a 360 nm interference filter (Chroma Technology).

RESULTS

Inhibition of store-operated Ca^{2+} influx and mitochondrial Ca^{2+} efflux by 2-APB

The effects of 2-APB on Ca^{2+} influx through CRAC channels were first examined in Ca^{2+} imaging experiments using fura-2-loaded Jurkat T cells. TG (1 μM), a membrane-permeant inhibitor of the SERCA Ca^{2+}-ATPase in the ER (Thastrup *et al.* 1989), was applied for ∼500 s in a Ca^{2+}-free solution to deplete intracellular Ca^{2+} stores and activate CRAC channels (Fig. 1). Subsequent restoration of Ca^{2+} to the bath produces a rapid elevation of $[Ca^{2+}]_i$ due to influx through open CRAC channels, followed by a gradual decline to a high steady-state $[Ca^{2+}]_i$ plateau, as the rates of Ca^{2+} influx and Ca^{2+} clearance reach a new balance (Hoth *et al.* 1997). As shown in Fig. 1*A*, application of 40 μM 2-APB rapidly abolished the $[Ca^{2+}]_i$ elevation and brought $[Ca^{2+}]_i$ back to baseline within 120 s, similar to its reported effects on store-operated Ca^{2+} entry in other cell types (Ma *et al.* 2000; van Rossum *et al.* 2000). In addition, application of 40 μM 2-APB in a Ca^{2+}-free solution eliminated the subsequent rise in $[Ca^{2+}]_i$ upon Ca^{2+} re-addition (data not shown), indicating that the inhibitory effect required neither external Ca^{2+} nor elevated $[Ca^{2+}]_i$. Thus, these results confirmed that 2-APB very effectively blocks $[Ca^{2+}]_i$ elevations produced by Ca^{2+} influx through CRAC channels.

High doses of 2-APB reduced $[Ca^{2+}]_i$ relatively rapidly to baseline values, even when preceded by a long, large $[Ca^{2+}]_i$ elevation (Fig. 1*A*). Previous work has shown that mitochondria take up a substantial amount of Ca^{2+} during such extended periods of influx, such that when influx is terminated (e.g. by removal of extracellular Ca^{2+}), $[Ca^{2+}]_i$ falls to a low plateau value that reflects a balance between Ca^{2+} pumping across the plasma membrane and Ca^{2+} efflux from the loaded mitochondria (marked with a dashed line in Fig. 1*B*; Hoth *et al.* 1997). This plateau declines with a time constant of hundreds of seconds as the mitochondria slowly unload their Ca^{2+}. The lack of this low $[Ca^{2+}]_i$ plateau in the presence of 2-APB suggests that the drug might prevent Ca^{2+} release from mitochondria. To test this idea, we applied 2-APB in Ca^{2+}-free solution after a period of prolonged $[Ca^{2+}]_i$ elevation. Addition of 50 μM 2-APB during the mitochondria-dependent Ca^{2+} plateau immediately caused $[Ca^{2+}]_i$ to decline back to baseline, consistent with an acute inhibition of mitochondrial Ca^{2+} export (Fig. 1*B*). In principle, this result could also be produced by an increased rate of Ca^{2+} extrusion across the plasma membrane; however, 2-APB did not alter the initial rate of $[Ca^{2+}]_i$ decline when Ca^{2+}_o was first removed (data not shown), demonstrating that the drug does not detectably affect Ca^{2+} pumps in the plasma membrane. Washout of the drug allowed Ca^{2+} to recover to the plateau level, often with a small overshoot. This behaviour would be expected from the resumption of mitochondrial export and a delayed approach to a new equilibrium between mitochondrial Ca^{2+} export and Ca^{2+} clearance across the plasma membrane. A lower concentration of 2-APB (5 μM) caused less-complete inhibition of mitochondrial export (Fig. 1*B*). Because a Na^+–Ca^{2+} exchanger in the mitochondrial inner membrane is believed to be responsible for carrying out a significant proportion of Ca^{2+} export (Gunter *et al.* 2000), we speculate that 2-APB may inhibit the mitochondrial Na^+–Ca^{2+} exchanger. We did not explore the mechanism of this mitochondrial effect further, but instead focused on the effects of 2-APB on Ca^{2+} influx.

Potentiation of store-operated Ca^{2+} entry by 2-APB

An unexpected observation was that the inhibition of Ca^{2+} influx was always preceded by a transient elevation of $[Ca^{2+}]_i$ (Fig. 1*A*, arrow). When the concentration of 2-APB was reduced to 10 μM, the $[Ca^{2+}]_i$ rise induced by 2-APB became much more pronounced and sustained (Fig. 1*C*). Interestingly, this potentiation of Ca^{2+} influx occurred only after store depletion; no effects were seen after application of dilute 2-APB to resting T cells (Fig. 1*C*). These results indicate that, in addition to its known ability to inhibit store-operated Ca^{2+} influx, 2-APB can also potentiate Ca^{2+} entry in Jurkat T cells.

The progression from potentiation to inhibition of Ca^{2+} entry as 2-APB concentration is increased is seen most clearly in the dose–response experiment of Fig. 2. Whereas low concentrations of 2-APB ($\leqslant$ 5 μM) elicited $[Ca^{2+}]_i$ elevations that were largely sustained during the 400 s of drug application, high concentrations ($\geqslant$ 10 μM) produced a transient rise followed by a progressive fall in $[Ca^{2+}]_i$. The rate of both the rise and the decay of $[Ca^{2+}]_i$ were accelerated as the concentration of 2-APB was increased.

Potentiation and blockade of I_{CRAC} by 2-APB

Several mechanisms could in principle account for the effects of 2-APB on $[Ca^{2+}]_i$ in the imaging experiments

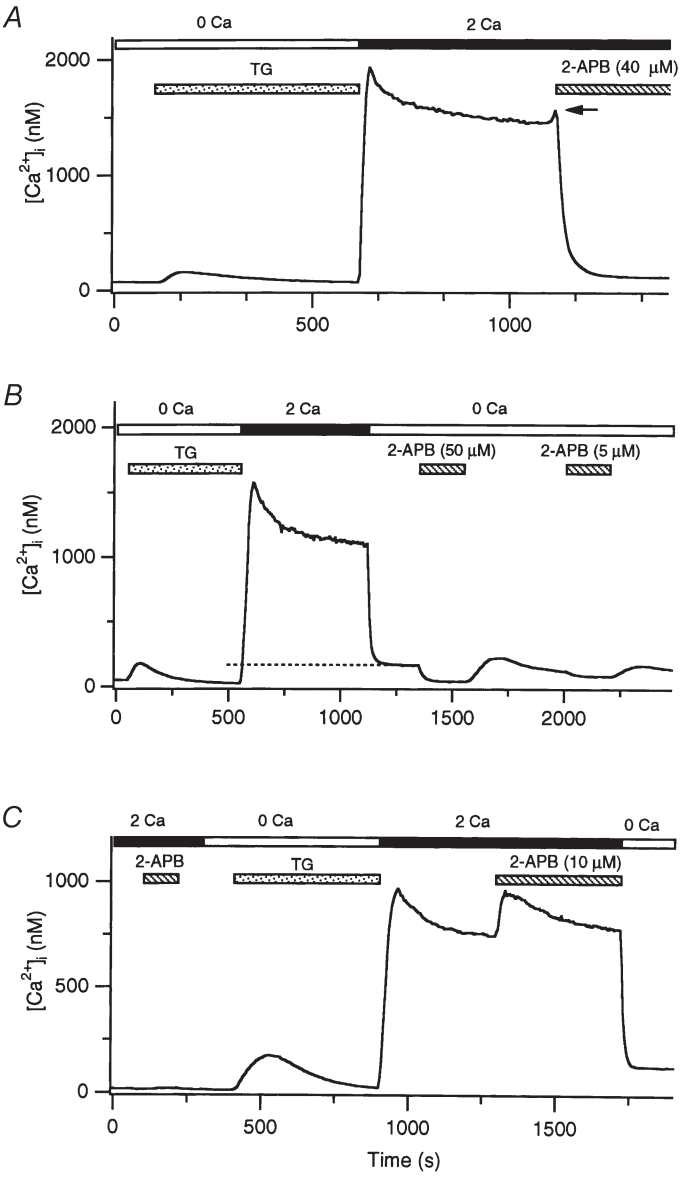

Figure 1. Effects of 2-APB on Ca^{2+} entry through CRAC channels and on Ca^{2+} release from mitochondria

In each panel, TG (1 μM; stippled bar) was applied in Ca^{2+}-free Ringer solution to irreversibly deplete stores and activate CRAC channels in Jurkat T cells. Subsequent addition of 2 mM Ca^{2+} to the bath (filled bar) elicited a rise in $[Ca^{2+}]_i$. Each trace represents the average of 150–200 cells. *A*, a high concentration of 2-APB (40 μM; hatched bar) inhibited Ca^{2+} influx through CRAC channels. The arrow marks a transient enhancement of $[Ca^{2+}]_i$ prior to inhibition. *B*, 2-APB inhibits Ca^{2+} release by mitochondria. After Ca^{2+} influx through CRAC channels was terminated by removing extracellular Ca^{2+}, $[Ca^{2+}]_i$ fell to a low elevated plateau that reflected the export of Ca^{2+} from mitochondria (dashed line). Addition of 50 μM 2-APB to the Ca^{2+}-free solution rapidly and reversibly reduced $[Ca^{2+}]_i$ to baseline due to inhibition of mitochondrial Ca^{2+} export. A lower concentration of 2-APB (5 μM) produced a smaller effect. *C*, a low concentration of 2-APB (10 μM) did not elevate $[Ca^{2+}]_i$ in resting cells before internal stores had been depleted by TG. However, after I_{CRAC} activation, 2-APB enhanced the $[Ca^{2+}]_i$ rise resulting from influx through CRAC channels.

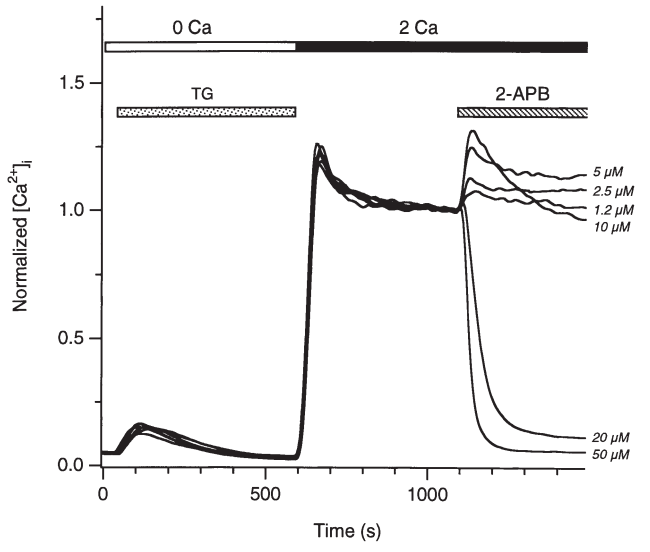

Figure 2. Potentiation and inhibition of Ca^{2+} entry by 2-APB are concentration dependent

Six representative experiments with increasing concentrations of 2-APB are superimposed. In each experiment, TG followed by re-addition of 2 mM Ca^{2+} was used to elicit a high $[Ca^{2+}]_i$ plateau. As [2-APB] was increased, sustained potentiation of the $[Ca^{2+}]_i$ rise gave way to transient potentiation followed by inhibition. Data were normalized to the $[Ca^{2+}]_i$ measured immediately before addition of 2-APB, to allow better comparison between the different experiments. The average $[Ca^{2+}]_i$ at this time was 1342 $\pm$ 20 nM (mean $\pm$ S.E.M.).

shown in Figs 1 and 2. These include potentiation and block of CRAC channels as well as mechanisms unrelated to CRAC channels, such as alteration of the membrane potential and driving force for Ca^{2+} entry by modulation of K^+ channels. To distinguish between these possibilities, we directly examined the effects of 2-APB on I_{CRAC} in whole-cell recordings. I_{CRAC} was induced in these experiments by passively depleting internal Ca^{2+} stores through intracellular dialysis with 10 mM EGTA in the patch pipette (Hoth & Penner, 1992; Zweifach & Lewis, 1993), and the peak amplitude of I_{CRAC} was measured during repetitive hyperpolarizing pulses to −100 mV.

The effects of 2-APB on the current paralleled those on $[Ca^{2+}]_i$. Thus, 5 μM 2-APB evoked a large sustained increase in the amplitude of the inward current at −100 mV (Fig. 3*A*, top trace), whilst 20 μM 2-APB induced an initial increase in current followed by a slow decline in I_{CRAC} amplitude (Fig. 3*A*, middle trace). Increasing the 2-APB concentration to 40 μM increased the rate of potentiation and the subsequent blockade of I_{CRAC} (Fig. 3*A*, bottom trace). The decay of I_{CRAC} followed an approximately single-exponential time course. No effects were seen in control experiments in which the corresponding concentration of DMSO was applied (data not shown). Therefore, these results suggest that whereas high concentrations of 2-APB block I_{CRAC}, low concentrations activate an additional inward current.

Several lines of evidence indicate that the additional inward current activated by 2-APB is I_{CRAC} itself rather than an unrelated current. First, the I–V profile and prominent inward rectification of the current in 2-APB were identical to that of I_{CRAC} (Fig. 3*B*). Second, consistent with the high Ca^{2+} selectivity of I_{CRAC}, the inward current was absent in nominally Ca^{2+}-free external solutions (Fig. 3*C*). Third, the 2-APB-induced current was completely blocked by low concentrations of La^{3+} (1.5 μM; Fig. 3*D*) and SKF 96365 (not shown), two known inhibitors of I_{CRAC} (Franzius *et al.* 1994; Aussel *et al.* 1996). Finally, 2-APB only induced inward current subsequent to store depletion and activation of I_{CRAC} by EGTA-containing internal solutions. In the experiment shown in Fig. 3*E*, brief application of 2-APB (5 μM) before

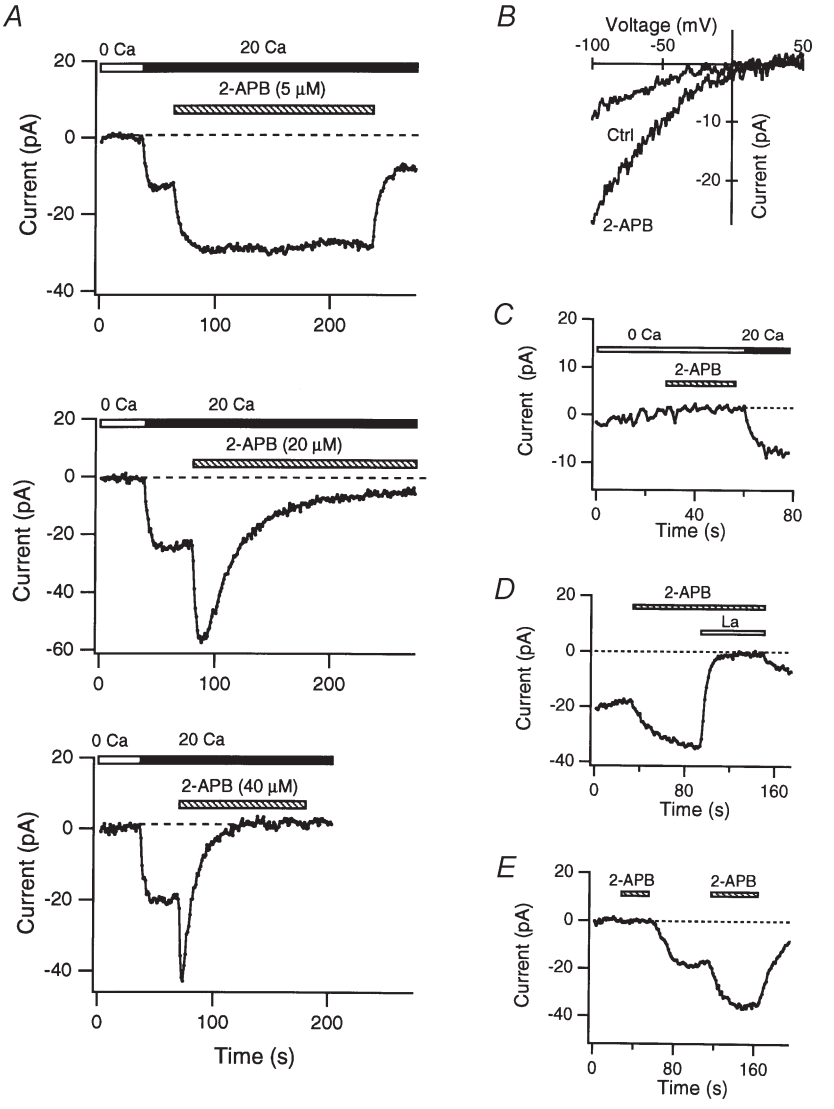

Figure 3. Potentiation and inhibition of I_{CRAC} by 2-APB

A, effects of 2-APB on I_{CRAC}. Current was activated by depleting Ca^{2+} stores passively with 10 mM EGTA in the recording pipette. 2-APB at 5 μM (top) enhanced I_{CRAC} amplitude over the duration of drug application, whereas 20 μM (middle) and 40 μM 2-APB (bottom) initially enhanced, then inhibited, I_{CRAC}. Shown here are the peak Ca^{2+} currents elicited during 200 ms steps to −100 mV from a holding potential of +10 mV. *B*–*E*, evidence that the additional inward current activated by 2-APB is I_{CRAC}. *B*, the current–voltage relation of the inward current activated by store depletion before and after 2-APB (10 μM). *C*, 2-APB (10 μM) did not activate inward current in Ca^{2+}-free Ringer solution. I_{CRAC} was induced passively as described above. *D*, the inward current activated by 2-APB (10 μM) is blocked by extracellular La^{3+} (1.5 μM). *E*, 2-APB (5 μM) does not elicit inward current before I_{CRAC} is activated. After the delayed induction of I_{CRAC} by intracellular EGTA (1.2 mM), application of 2-APB potentiated I_{CRAC} robustly.

the passive induction of I_{CRAC} had no effect, but after I_{CRAC} was activated, the same dose caused robust potentiation. Taken together with the evidence that a low dose of 2-APB elevated $[Ca^{2+}]_i$ only after stores were depleted (Fig. 1*C*), these data indicate that the transient inward current activated by 2-APB is I_{CRAC} itself. Thus, we conclude that 2-APB elicits both excitatory and inhibitory effects on I_{CRAC}.

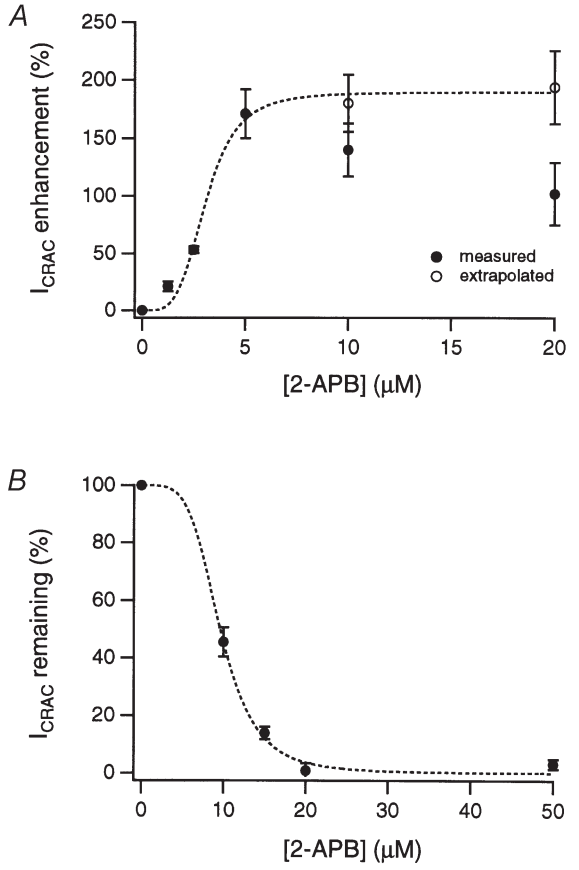

Figure 4. Concentration dependence of potentiation and inhibition of I_{CRAC} by 2-APB

A, extent of I_{CRAC} potentiation as a function of 2-APB concentration. Filled circles show peak currents measured from experiments similar to those in Fig. 3. To correct for the overlap of potentiation and inhibition at 2-APB concentrations $\geqslant 10\ \mu$M, peak currents were also estimated by back-extrapolation (open circles; see text). The dashed line indicates a fit to the Hill equation $Y = Y_{max}/(1 + ([2\text{-APB}]/K_{½})^{n_H})$, where Y_{max} is the maximal potentiation, $K_{½}$ is the concentration that yields half-maximal potentiation, and n_H is the apparent cooperativity of the process. The best fit was obtained with $K_{½} = 3.1\ \mu$M and $n_H = 4.1$. *B*, degree of I_{CRAC} inhibition as a function of 2-APB concentration. Inhibition was calculated from the steady-state current level relative to the peak current after addition of 2-APB. The dashed line is a fit of the Hill equation, with $K_{½} = 9.6\ \mu$M and $n_H = 4.2$. Experimental conditions were as described for Fig. 3; holding potential = +30 mV. In *A* and *B*, each point represents the mean ± S.E.M. of three to five cells.

The potentiating and inhibitory effects of 2-APB on I_{CRAC} differed in two ways. First, although potentiation of I_{CRAC} was rapidly reversible (Fig. 3*A*, top trace; average time constant of recovery, $\tau_{rec} = 6.2 \pm 1.3$ s, $n = 6$), inhibition of I_{CRAC} in Jurkat cells reversed extremely slowly following removal of the drug (Fig. 3*A*, bottom trace and Fig. 6*A*). In four cells exposed to 40 μM 2-APB, washout of the drug caused the current to recover by an average of 22 % after 160 s, suggesting a τ_{rec} of $>$ 600 s. A second difference was in the concentration dependence of the two effects. Current potentiation was evident at lower drug concentrations than those that inhibited I_{CRAC}. Unfortunately, an overlap in the effective concentrations for potentiation and inhibition complicated efforts to estimate separately the $K_{½}$ values for the two processes. The concentration dependence of I_{CRAC} enhancement measured directly from the peak current amplitudes before and after exposure to 2-APB is shown in Fig. 4*A* (filled circles). Potentiation peaked around 5 μM, then decreased with further increases in 2-APB concentration, presumably because of increasing current inhibition. To attempt to correct for the effect of inhibition and estimate the true extent of potentiation by 2-APB at these concentrations, we fitted a single-exponential function to the current decay induced by 2-APB and extrapolated the current amplitude back to the time the drug was first applied (Fig. 4*A*, open circles). The estimated potentiation at different concentrations of 2-APB was then fitted with a Hill equation, yielding an apparent $K_{½}$ of ~3 μM for potentiation. By contrast, the apparent $K_{½}$ for I_{CRAC} blockade as measured by inhibition of the peak current was ~10 μM (Fig. 4*B*). This sensitivity of CRAC channels to inhibition by 2-APB is identical to that reported by Ma *et al.* (2000) for inhibition of TRP3-mediated Sr^{2+} entry in transfected HEK 293 cells. However, it is significantly lower than the IC_{50} for inhibition of Ca^{2+} release from IP_3 receptors (25–42 μM; Maruyama *et al.* 1997; Ma *et al.* 2000).

Low concentrations of 2-APB enhance fast inactivation of I_{CRAC}

Ca^{2+} entering through CRAC channels causes them to inactivate over tens of milliseconds during large hyperpolarizing pulses (Hoth & Penner, 1993; Zweifach & Lewis, 1995*b*; Fierro & Parekh, 1999*a*). Fast inactivation is a biexponential process and is believed to result from Ca^{2+} binding to multiple intracellular sites located within several nanometers of the channel pore (Zweifach & Lewis, 1995*b*). Interestingly, 2-APB altered fast inactivation in parallel with its effects on the amplitude of I_{CRAC}. At low concentrations that caused potentiation, 2-APB increased the rate and extent of fast inactivation (Fig. 5). Enhancement of fast inactivation by dilute 2-APB was observed across a wide range of test potentials, where the predominant effect was to accelerate the fastest

exponential component while increasing its amplitude. As shown in Fig. 5*A*, this effect developed with a similar time course to the increase in peak I_{CRAC} (potentiation), suggesting that the two phenomena may be related. At long times (> 150 s), the inactivation rate actually decreased (not shown), perhaps due to the slow removal of inactivation at these low 2-APB concentrations (see below). There are several possible explanations for the speeding of I_{CRAC} inactivation by dilute 2-APB; a likely possibility is that by increasing the activity of CRAC channels, 2-APB enhances the accumulation of Ca^{2+} at the inactivation sites. This and other alternative mechanisms are discussed below (see Discussion).

High concentrations of 2-APB remove fast inactivation of I_{CRAC}

In addition to inhibiting I_{CRAC}, a dramatic effect of high concentrations of 2-APB was to eliminate fast inactivation. In the example shown in Fig. 6*A*, 40 μM 2-APB evoked a rapid transient increase in I_{CRAC} followed by a slow and complete inhibition of the current over the next 40 s. As can be seen in the lower traces, the extent of inactivation during brief voltage pulses from +10 to −100 mV decreased over the duration of exposure to the drug. The removal of inactivation (open circles) appeared to occur in parallel with inhibition of the peak current (filled circles). The exponential time constants for the two effects were similar (Fig. 6*B*; $n = 5$ cells), suggesting that the two could be coupled. The simplest hypothesis would be that the inhibition of current through CRAC channels reduces inactivation by lessening the accumulation of intracellular Ca^{2+}. This seems unlikely, however. In Fig. 6*A*, traces *a* (before 2-APB was applied) and *c* (collected after 2-APB had potentiated and inhibited I_{CRAC}) show identical peak current amplitudes even though inactivation was almost completely absent in *c*. Hence, while the removal of inactivation and drug-induced inhibition of the current may be related (see Discussion), inactivation did not disappear because of reduced Ca^{2+} flux through CRAC channels.

The removal of inactivation by 2-APB measured during drug applications lasting 150 s or longer was dose dependent, increasing in extent from 5 to 50 μM (Fig. 6*C*). The threshold concentration for this effect appears to be ~5 μM, as judged from the small delayed decline in the extent of inactivation at this dose of 2-APB (see above).

The potentiation of I_{CRAC} by 2-APB is not due to removal of fast inactivation

Given that 2-APB was able to inhibit fast Ca^{2+}-dependent inactivation of I_{CRAC} (Fig. 6), we considered the possibility that a reduction of steady-state inactivation might contribute to potentiation of the current. As a first test, we measured the degree of inactivation under steady-state conditions in the presence and absence of 2-APB (Fig. 7). Following a 1 s conditioning step to varying potentials, I_{CRAC} peak amplitude was measured in response to a step to −100 mV. I_{CRAC} amplitude was roughly constant following conditioning steps to +20 mV and above, but declined following more negative conditioning steps to a level of ~40 % at −80 mV and below. A dose of

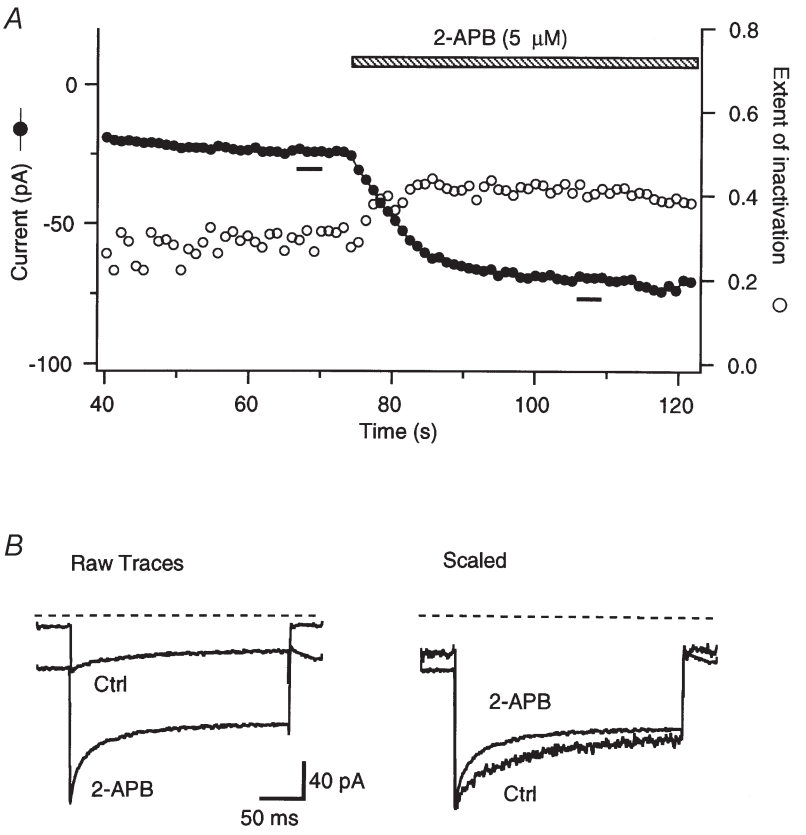

Figure 5. Low concentrations of 2-APB accelerate fast inactivation of I_{CRAC}

A, peak I_{CRAC} (●) and the extent of inactivation (○) measured during steps to −100 mV. To monitor changes in the speed of inactivation, the extent of inactivation was measured as $(I_{peak} - I_t)/I_{peak}$, where I_{peak} is the peak current and I_t is the current measured 50 ms after each step to −100 mV. Addition of 5 μM 2-APB enhanced the peak current amplitude and increased the extent of inactivation. *B*, the time course of inactivation in the presence and absence of 2-APB. Traces on the left show the averages of five leak-corrected responses to hyperpolarizations given in *A* (bars in *A*) in 20 mM Ca_o^{2+} with or without 5 μM 2-APB. The right plot shows the same traces normalized to their peak current. Note that the rate of current decay was enhanced in the presence of 2-APB. Holding potential = +30 mV.

2-APB (5 μM) that caused a severalfold potentiation of I_{CRAC} reduced this inactivation by only ~20% at the most negative potentials. Therefore, it seems unlikely that the modest effect of dilute 2-APB on inactivation makes a large contribution to potentiation. This conclusion was tested further by investigating whether 2-APB could potentiate I_{CRAC} under conditions that prevented fast inactivation. The degree of potentiation by 2-APB was similar at holding potentials of −50 or +50 mV (Fig. 7*B*), even though steady-state inactivation was prominent at −50 mV but absent at +50 mV (see Fig. 7*A*). A third test exploited the fact that Ba^{2+} currents through CRAC channels do not show fast inactivation (Zweifach & Lewis, 1995*b*). 2-APB potentiated Ba^{2+} currents through CRAC channels to at least as great a degree as it did Ca^{2+} currents (Fig. 7*C* and *D*). These results show clearly that the removal of inactivation does not explain the potentiation of I_{CRAC} by 2-APB.

Inhibition and potentiation of I_{CRAC} by 2-APB is not cell specific

Although 2-APB has been reported to block I_{CRAC} in RBL cells (Braun *et al.* 2001), no information exists about whether it potentiates I_{CRAC} in any cells other than Jurkat

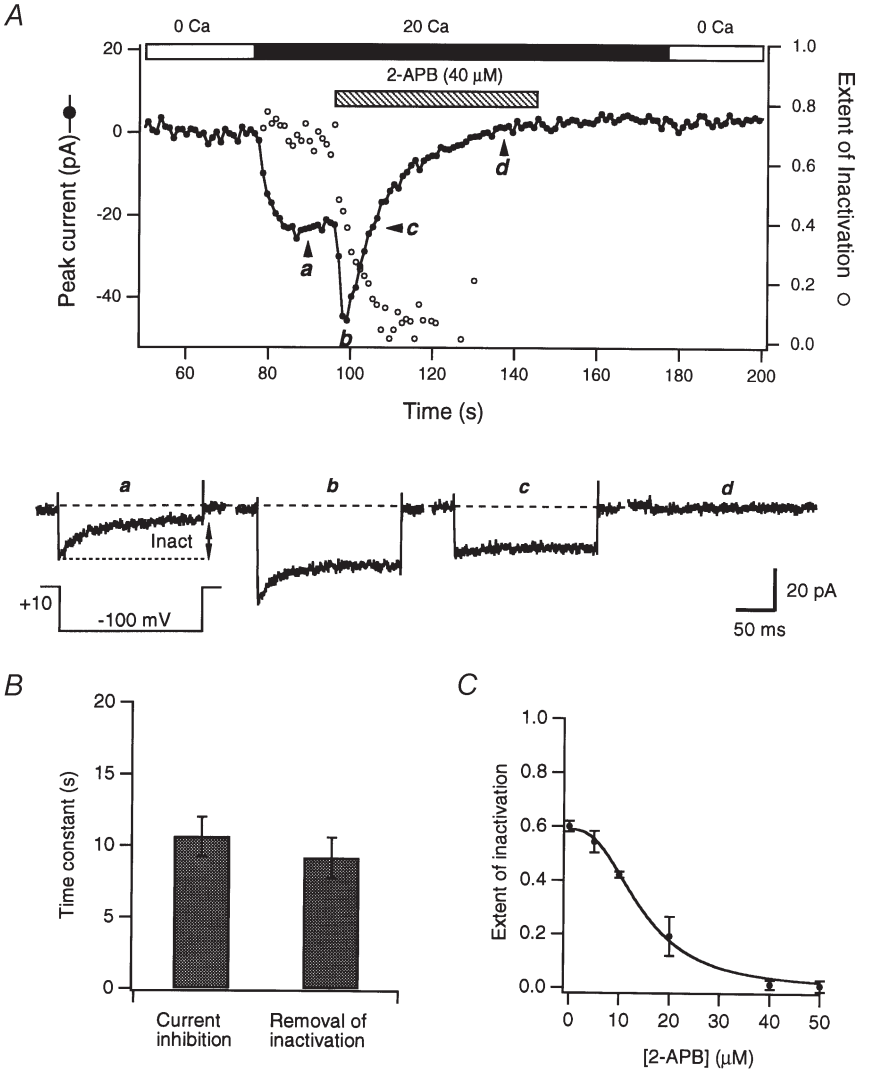

Figure 6. High concentrations of 2-APB eliminate fast inactivation of I_{CRAC}

A, plot of peak I_{CRAC} (●) and the extent of inactivation (○) during pulses to −100 mV before and after exposure to 40 μM 2-APB (hatched bar). The extent of inactivation was measured at the end of the 250 ms test pulses (I_t = 250 ms; see legend to Fig. 5). Examples of currents at various time points are shown below to illustrate the removal of fast inactivation. *B*, inhibition of I_{CRAC} and removal of fast inactivation occur at a similar rate. The time courses of I_{CRAC} inhibition and reduction in the extent of inactivation were fitted with single-exponential functions and the time constants from the resulting fits are shown (40 μM 2-APB; n = 5). *C*, dose dependence of the removal of inactivation by 2-APB. The extent of inactivation was measured 100 ms after steps to −100 mV, at a time when the effects of 2-APB had reached steady state. Each point represents the mean of three to six cells.

human T cells. Therefore, we tested the effects of low and high doses of 2-APB on I_{CRAC} in RBL cells and DT40 cells, a chicken pre-B cell line. TG-treated DT40 cells exhibited inwardly rectifying currents with reversal potentials $> +30$ mV (Fig. 8*A* and *B*). The pharmacological hallmarks of this current were also similar to I_{CRAC} in Jurkat T cells: it was effectively blocked by 20 μM La^{3+} as well as by 30 μM SKF 963615 (data not shown).

The effects of 2-APB on I_{CRAC} in RBL and DT40 cells were similar to those observed in Jurkat T cells. In both cell types, high doses of 2-APB elicited a transient potentiation of I_{CRAC}, which was then followed by a slow and progressive inhibition that was largely irreversible (Fig. 8). The degree of potentiation in RBL cells tended to be somewhat smaller than that observed in either Jurkat or DT40 cells: 20 μM 2-APB elicited only a 28 ± 8% increase in I_{CRAC} current amplitude in RBL cells ($n = 4$ cells), compared with ~100% in Jurkat cells (see Fig. 4*A*). As in Jurkat cells, the high concentration of 2-APB removed fast inactivation of I_{CRAC} in RBL and DT40 cells. Thus, these results indicate that the complex effects of 2-APB on CRAC channel gating were not specific to a single cell type or species.

CRAC channel activation and the effects of 2-APB are independent of IP_3 receptors

2-APB has been reported to be a selective antagonist of the IP_3 receptor (Maruyama *et al.* 1997). The inhibition of store-operated Ca^{2+} influx by 2-APB has therefore been

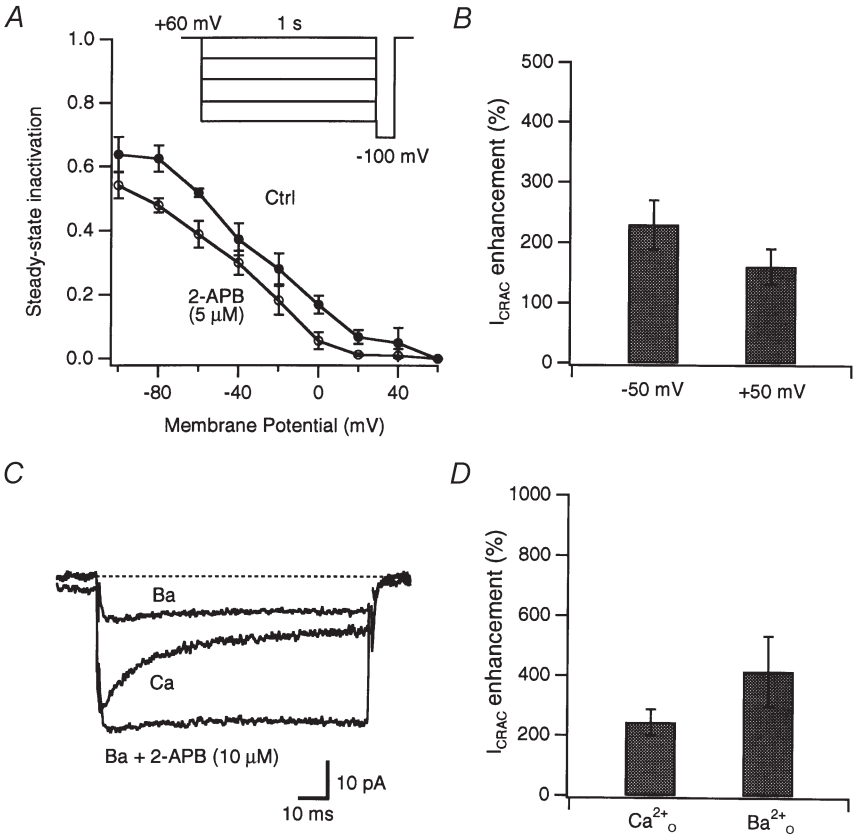

Figure 7. Potentiation of I_{CRAC} by 2-APB does not result from the removal of fast inactivation

A, 2-APB alters the extent of steady-state inactivation of I_{CRAC}. The extent of inactivation was manipulated by applying a 1 s conditioning step to various potentials (inset) followed by a test pulse to −100 mV where I_{CRAC} amplitude was measured. Steady-state inactivation at each potential, Inact(V), was calculated from the relation $\text{Inact}(V) = 1 - (I_{cond}/I_{60\,mV})$, where I_{cond} is the peak current during a test pulse to −100 mV after a conditioning step to potential V, and $I_{60\,mV}$ is the peak current at −100 mV following a conditioning step to +60 mV. Holding potential = +60 mV; $n = 4$ cells. *B*, potentiation of I_{CRAC} by 5 μM 2-APB is only marginally reduced at positive potentials that remove resting inactivation. Peak I_{CRAC} amplitude was measured during 50 ms test pulses to −100 mV from the indicated holding potential; $n = 5$ cells. *C*, potentiation of I_{CRAC} by 2-APB persists when Ba^{2+} is used as the charge carrier. I_{CRAC} was activated by internal dialysis with 10 mM EGTA. Traces show sequential recordings of current from one cell during test pulses to −100 mV in the presence of 20 mM Ca^{2+}_o (Ca), then shortly after 20 mM Ba^{2+} was substituted for Ca^{2+}_o (Ba), and finally after 10 μM 2-APB was applied in the presence of Ba^{2+} (Ba + 2-APB). Even though fast inactivation did not occur in Ba^{2+}_o, 2-APB induced robust potentiation. *D*, summary of potentiation elicited by 10 μM 2-APB in 20 mM Ca^{2+}_o ($n = 9$ cells) or Ba^{2+}_o ($n = 5$ cells).

attributed to the inhibition of IP_3 receptors, and has been used to support the notion that store-operated channels are activated through conformational coupling to IP_3 receptors (Ma *et al.* 2000). Therefore, a critical question is whether I_{CRAC} is dependent on IP_3 receptors, and whether the complex effects of 2-APB on I_{CRAC} are due to the drug acting through IP_3 receptors as opposed to other targets, possibly the CRAC channel itself. We examined this issue using three independent approaches. First, we examined the effects of heparin, an IP_3 receptor antagonist, on I_{CRAC} to determine whether IP_3 binding to its receptor was necessary for I_{CRAC} activation or the effects of 2-APB. Heparin potently inhibits IP_3 binding with a K_i of 2–10 μg ml^{-1} (Supattapone *et al.* 1988; Parys *et al.* 1992), and previous reports have indicated that it does not affect passive induction of I_{CRAC} in mast and RBL cells (Hoth & Penner, 1993; Fierro & Parekh, 1999*b*). Consistent with these reports, we found that, in Jurkat T cells dialysed through the recording pipette with 10 mM EGTA to activate I_{CRAC}, heparin (500 μg ml^{-1}) had no effect on either the current amplitude or time course of induction (Fig. 9*A* and *B*). Under these conditions, heparin was clearly effective in inhibiting IP_3 receptors because it did block the rapid induction of I_{CRAC} by uncaging intracellular IP_3 (Fig. 9*C*). Importantly, intracellular heparin also failed to affect the ability of 2-APB to either potentiate or inhibit I_{CRAC} (Fig. 9*D* and *E*). These results suggest that the binding of background levels of IP_3 is not necessary for activation of I_{CRAC} through passive store depletion, nor is it required for the effects of 2-APB on I_{CRAC}.

Next, we investigated whether 2-APB preferentially acts upon I_{CRAC} from the inside or the outside of the cell. A previous report (Braun *et al.* 2001) showed that 2-APB inhibits I_{CRAC} in RBL cells more effectively when applied outside, and we found a similar result in Jurkat cells. Applying 2-APB intracellularly by including a high concentration of the drug (80 μM) in the patch pipette did not significantly inhibit either the induction or the maintenance of I_{CRAC} (Fig. 10*A* and *B*). In contrast, subsequent exposure of the cells to 40 μM 2-APB extracellularly caused significant inhibition (Fig. 10*A*). The greater potency of extracellular 2-APB appears to support the idea that the site of action is extracellular and therefore independent of IP_3 receptors. However, the interpretation of this experiment is complicated by the fact that 2-APB is membrane permeant. It is well established that lipophilic compounds that are known to block ion channels from an intracellular site often act more effectively when applied from the outside (DeCoursey, 1995). This paradox results from the fact that when membrane-permeant compounds are applied through the recording pipette, their concentration at the membrane may be lower than expected because diffusion out of the cell is faster than diffusion out of the pipette (DeCoursey, 1995). The membrane permeability and protonation state of 2-APB under our experimental conditions was not known, but a reduction of extracellular pH would have been expected to increase the protonation of 2-APB and reduce the concentration of the more membrane-permeant, non-protonated form. If the site of action were intracellular, then increased protonation of 2-APB would impair access to the site and result in a reduced effect. Therefore, we examined the effects of extracellular pH on potentiation and inhibition by bath-applied 2-APB. Reducing extracellular pH from 7.4 to 6.4 partially inhibited I_{CRAC} (Fig. 10*C*; see also Malayev &

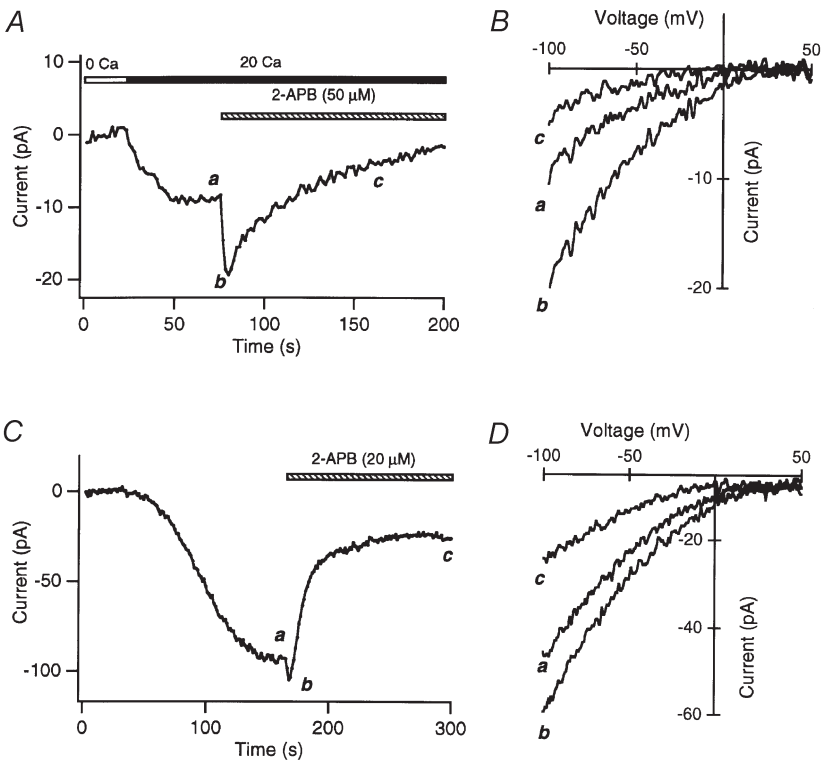

Figure 8. 2-APB potentiates and inhibits I_{CRAC} in DT40 B cells and RBL cells

A, effects of 50 μM 2-APB on I_{CRAC} in a DT40 cell. I_{CRAC} was induced by exposing the cell to 1 μM TG for 5 min prior to seal formation; I_{CRAC} during pulses to −100 mV is shown. *B*, the current–voltage relation of Ca^{2+} currents before and after exposure to 2-APB, collected at the times shown in *A*. *C*, effects of 20 μM 2-APB on I_{CRAC} in an RBL cell. I_{CRAC} was induced by passive depletion of internal stores by 10 mM EGTA in the patch pipette. Note the smaller extent of I_{CRAC} enhancement by 2-APB. *D*, current–voltage profile of I_{CRAC} in the presence and absence of 2-APB, collected at the times shown in *C*.

Nelson, 1995). However, the degrees of potentiation and inhibition of I_{CRAC} by 2-APB were identical at the two pH values (Fig. 10*D*). Therefore, these data support the idea that the site of action of 2-APB is extracellular rather than intracellular.

An extracellular site of action raises the possibility that 2-APB does not exert its effects on I_{CRAC} through the intracellular IP_3 receptor. To provide a more direct test of this idea, we examined the effects of 2-APB on I_{CRAC} in mutant DT40 cells in which all three isoforms of the IP_3 receptor (IP_3R) were knocked out by homologous recombination (Sugawara *et al.* 1997). These cells have been shown to lack functional type I, II and III IP_3 receptors as assayed by their failure to release Ca^{2+} in response to stimulation of phospholipase C-linked B-cell receptors or muscarinic receptors, and Northern analysis confirmed the absence of the IP_3 receptor mRNA sequence in regions including and immediately surrounding the knockout sites (Sugawara *et al.* 1997). In addition, recent measurements showed that, unlike their wild-type counterparts, the DT40 mutant cells bind essentially no ^3H-IP_3 (Broad *et al.* 2001), suggesting that the amino-terminal cytoplasmic domain of the IP_3 receptor, which has been proposed to couple to store-operated channels, is also missing. Despite the lack of IP_3 receptors, store-operated Ca^{2+} entry in these cells was shown to be normal (Sugawara *et al.* 1997). Consistent with these results, we found that the $IP_3R^{-/-}$ DT40 mutants exhibited CRAC currents that were virtually indistinguishable from those of control DT40 cells in terms of their size, *I–V* relationship and Ca^{2+}-dependent fast inactivation (Fig. 11*A–C*). Thus, I_{CRAC} activation does not appear to require any of the known isoforms of the IP_3 receptor.

Application of 50 μM 2-APB to the $IP_3R^{-/-}$ mutant DT40 cells produced a transient elevation of I_{CRAC} followed by a slow inhibition (Fig. 11*A*). The magnitudes of both the

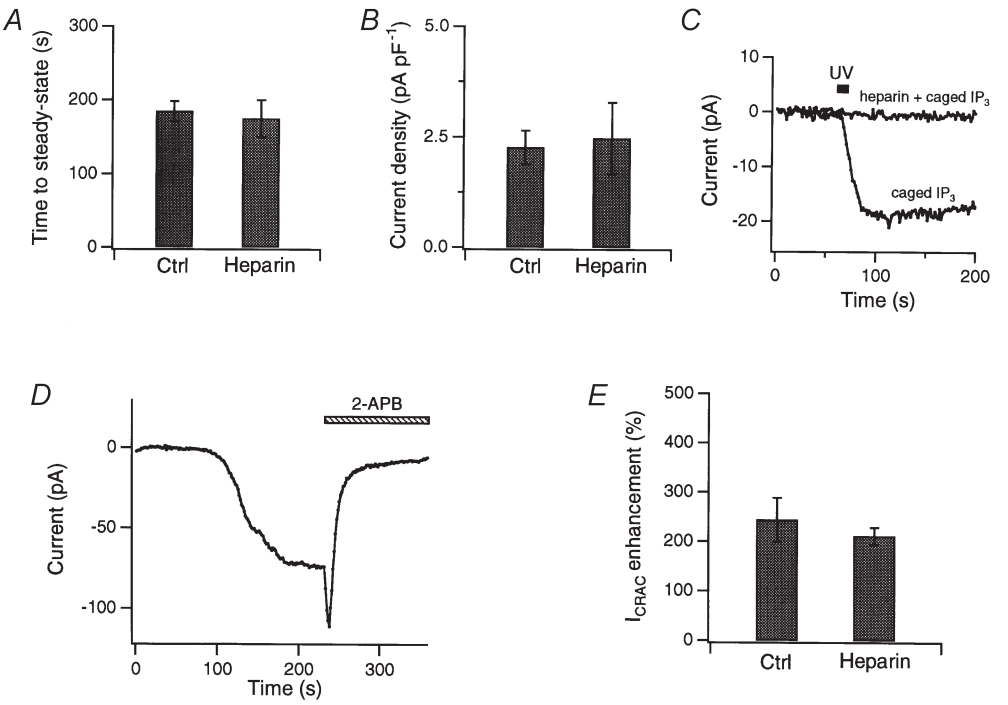

Figure 9. Heparin does not interfere with the activation of I_{CRAC} by passive store depletion or with the effects of 2-APB

In all experiments except as noted, 500 μg ml^{-1} heparin was included in the recording pipette, and I_{CRAC} was induced through passive depletion of internal stores by 10 mM EGTA in the patch pipette. *A* and *B*, heparin affects neither the rate of I_{CRAC} activation (*A*) nor its maximal amplitude at steady state (*B*). The time to steady state was measured as the time to reach 95% of the maximal current amplitude. Current amplitude was normalized to cell capacitance (average $C_m = 9.95$ pF). Heparin-treated cells ($n = 6$) are compared with matched control cells ($n = 7$). *C*, heparin loading via the patch pipette effectively inhibited IP_3 receptors. In control Jurkat cells lacking heparin, I_{CRAC} was activated by a 10 s UV flash to release caged IP_3 (15 μM). With 500 μg ml^{-1} heparin in the pipette, uncaging IP_3 failed to activate I_{CRAC}. Results are representative of four cells loaded with caged IP_3 alone and three cells with caged IP_3 and heparin. *D*, 40 μM 2-APB first potentiated and then inhibited I_{CRAC} in a Jurkat cell loaded with heparin via the patch pipette. Shown here are the peak currents during steps to −100 mV from +30 mV. *E*, summary of the potentiating effects of 5 μM 2-APB in heparin-treated ($n = 6$) and matched control ($n = 5$) cells.

peak potentiation and the steady-state inhibition caused by 2-APB were identical to those seen in the control DT40 and Jurkat T cells (Fig. 11*D*). In addition, 2-APB diminished the extent of fast inactivation over time, just as it does in control DT40 cells (data not shown). Thus, we conclude that the complex stimulatory and inhibitory effects of 2-APB on CRAC channels occur through a mechanism that is independent of IP_3 receptors.

DISCUSSION

In this study, we have described complex effects of 2-APB on the amplitude and kinetic behaviour of I_{CRAC}. These include an increase of current amplitude (potentiation) and speeding of inactivation by low concentrations of 2-APB ($< 5\ \mu$M), as well as inhibition of the current and removal of fast inactivation by higher concentrations ($\geqslant 5\ \mu$M). Importantly, none of these effects appeared to occur through the IP_3 receptor, as previously postulated by the conformational coupling hypothesis for store-operated Ca^{2+} entry. In the sections that follow, we discuss some possible underlying mechanisms and implications of these phenomena.

2-APB exerts complex effects on the amplitude and kinetics of I_{CRAC}

We were surprised to find that 2-APB had much more complex effects on I_{CRAC} than only the slow inhibition reported previously (Braun *et al.* 2001). Low doses (1–5 μM) reversibly enhanced the amplitude of I_{CRAC} by up to fivefold and slightly increased the rate of fast Ca^{2+}-dependent inactivation. At higher doses ($\geqslant 5\ \mu$M) of the drug, the slow inhibition of I_{CRAC} also became apparent, along with the disappearance of fast inactivation. All of these effects were seen in Jurkat, RBL and DT40 cells; thus, they appear to be independent of the cell type (T cells, mast cells and B cells) and species (human, rat and chicken), suggesting that they may apply to CRAC

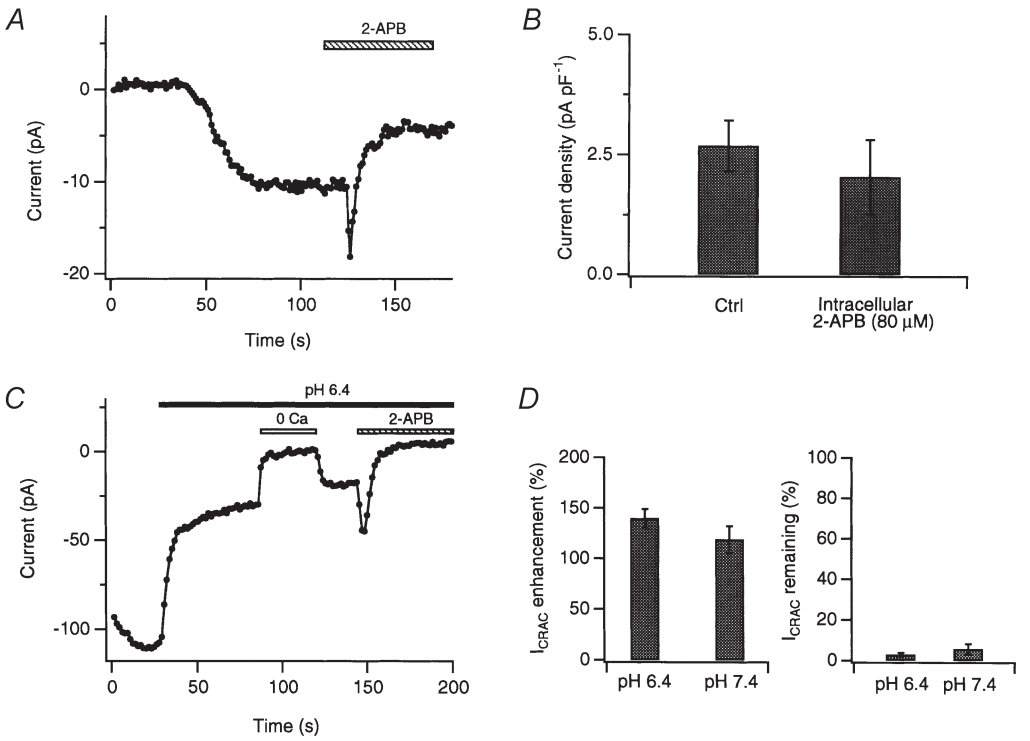

Figure 10. 2-APB may have an extracellular site of action

A, intracellular 2-APB is ineffective at inhibiting I_{CRAC}. I_{CRAC} was activated by dialysis with 10 mM EGTA in the presence of 80 μM 2-APB in the recording pipette. Peak I_{CRAC} amplitudes during repeated steps to −100 mV are shown. After I_{CRAC} amplitude reached steady state, application of 40 μM 2-APB via the bath elicited the normal sequence of potentiation and inhibition. *B*, comparison of the steady-state current amplitudes of I_{CRAC} (normalized to cell capacitance) in cells treated with intracellular 2-APB (80 μM; $n = 5$ cells) and matched controls ($n = 7$ cells) in which I_{CRAC} was activated by intracellular EGTA only. *C*, lowering extracellular pH does not alter the ability of 2-APB to potentiate or inhibit I_{CRAC}. I_{CRAC} was activated by treatment with 1 μM TG immediately prior to seal formation. After allowing the I_{CRAC} amplitude to reach steady state, the external pH was lowered from 7.4 to 6.4, which dramatically reduced the current amplitude. Application of 40 μM 2-APB potentiated and inhibited I_{CRAC} in a manner similar to that at pH 7.4. *D*, summary of the extent of potentiation and inhibition of I_{CRAC} induced by 40 μM 2-APB at pH 7.4 ($n = 5$ cells) and pH 6.4 ($n = 5$ cells).

channels in general. Thus, the reason why potentiation was not observed in a previous study of RBL cells (Braun *et al.* 2001) probably stems not from the cell type but from a combination of other factors. The amplitude of potentiation was less pronounced in RBL cells than in Jurkat or DT40 cells (compare Fig. 8*C* with Figs 8*A* and 3*A*), and this smaller effect may have been exacerbated in other studies by the use of high concentrations of 2-APB (100 μM), which produce a predominant inhibitory effect, and by pre-application of 2-APB rather than acute application after I_{CRAC} had been activated.

The degree to which these 2-APB effects are specific for CRAC channels is mostly unknown. The inhibitory effects of 2-APB seem to be relatively non-specific, as they apply to IP_3 receptors (Sugawara *et al.* 1997), endogenous store-operated channels in HEK 293 cells (Ma *et al.* 2000) and the mitochondrial Ca^{2+} release machinery (Fig. 1*B*) in addition to I_{CRAC}. However, to our knowledge, 2-APB is the first example of a pharmacological agent that can enhance the activation of I_{CRAC} beyond the level achieved by complete store depletion. Because Ca^{2+} entry through CRAC channels is a key requisite step for the activation of T cells by antigen, potentiation of I_{CRAC} may be useful as a novel therapeutic approach to boost activation of immune cells in immunodeficient individuals. In addition, potentiation by 2-APB may prove to be a useful diagnostic tool for testing putative CRAC channel genes and probing the physiological functions of CRAC channels *in vivo*.

Possible mechanisms for the potentiation of I_{CRAC} by 2-APB

In resting cells in which Ca^{2+} stores were full and which displayed no Ca^{2+} influx through CRAC channels, low doses of 2-APB were ineffective in raising $[Ca^{2+}]_i$ or activating I_{CRAC}. Thus, rather than activating I_{CRAC} by itself, 2-APB appears to boost the activity of CRAC channels once they are activated by store depletion (Fig. 3*E*). This potentiation does not result from an increased emptying of Ca^{2+} stores, since 2-APB increases I_{CRAC} severalfold even after treatment with TG, which is known to deplete the ionomycin-releasable stores in Jurkat cells by $> 90\,\%$ (see Fig. 1*A* of Hoth *et al.* 1997).

Although 2-APB does reduce the level of Ca^{2+}-dependent fast inactivation at doses as low as 5 μM, this effect is not responsible for potentiation of the current. Conditions that limit or prevent fast inactivation, including holding potentials more positive than +30 mV or the use of Ba^{2+} as the charge carrier, did not reduce the amount of potentiation by 2-APB (Fig. 7). In principle, 2-APB could

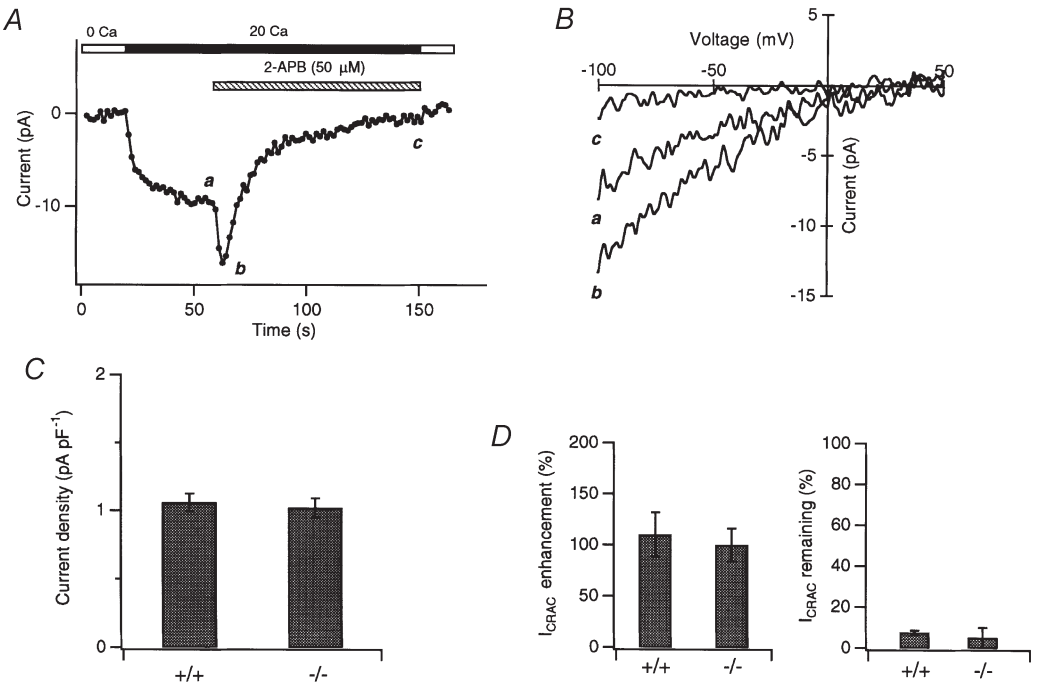

Figure 11. Effects of 2-APB are retained in mutant DT40 cells lacking IP_3 receptors

A, I_{CRAC} was induced in a mutant DT40 $IP_3R^{-/-}$ cell by application of 1 μM TG 5 min prior to seal formation. After recording traces in 0 and 20 mM Ca_o^{2+} solutions, the cell was exposed to 50 μM 2-APB. Shown here are peak current amplitudes recorded during steps to −100 mV. *B*, responses to voltage ramps recorded in the absence or presence of 2-APB at the times shown in *A*. *C*, I_{CRAC} amplitude (at −100 mV, normalized to cell capacitance) in $IP_3R^{-/-}$ mutant (−/−; $n = 6$) and wild-type (+/+; $n = 5$) DT40 cells. *D*, comparison of potentiation and inhibition of I_{CRAC} by 50 μM 2-APB in wild-type ($n = 5$) and $IP_3R^{-/-}$ mutant ($n = 6$) DT40 cells.

act by interfering with other known forms of I_{CRAC} inactivation, such as slow Ca^{2+}-dependent inactivation (Zweifach & Lewis, 1995*a*) or kinase-dependent inactivation (Parekh & Penner, 1995). However, this seems unlikely, as both processes would be minimized by our recording conditions (10 mM EGTA and 0 ATP in the recording pipette).

In general, potentiation of I_{CRAC} could occur through increases in the number of activatable channels (N), the single-channel current (i) or the open probability of the channels (P_o). Without single-channel recordings, it is difficult to discriminate between these possible mechanisms, but we can make some educated guesses by exploiting the changes in fast inactivation that accompany I_{CRAC} potentiation. Previous work has shown that I_{CRAC} inactivation is influenced by the local $[Ca^{2+}]_i$ around individual CRAC channels (Zweifach & Lewis, 1995*b*). 2-APB at low concentrations does not induce inactivation by itself, since Ba^{2+} currents do not inactivate even in the presence of the drug (Fig. 7*C*); instead, a reasonable conclusion is that it increases the speed of fast Ca^{2+}-dependent inactivation, possibly by producing an increase in the local $[Ca^{2+}]_i$ around CRAC channels. Fast inactivation is strongly dependent on the amplitude of i but not on N (Zweifach & Lewis, 1995*b*). Thus, if potentiation were due to an increase in i, then one would expect a significant increase in the extent of inactivation, particularly under conditions where the current increases significantly (e.g. by a factor > 2; see Fig. 5). This was not observed, suggesting that i is probably not affected by 2-APB. In addition, because CRAC channels inactivate independently (Zweifach & Lewis, 1995*b*), an increase in N would not be expected to affect inactivation. It might be argued that overlap of Ca^{2+} microdomains between adjacent CRAC channels might increase with N, undermining channel independence and increasing inactivation. To test this possibility, we reduced the current with SKF 96365, reasoning that this would reduce the overlap of microdomains and therefore reduce the inactivation if overlap existed. We found that, in the presence of a potentiating dose of 2-APB, a half-blocking dose of SKF 96365 did not affect the rate of inactivation (data not shown), making the overlap of domains unlikely.

If 2-APB does not influence N or i, the most likely explanation is that 2-APB potentiates I_{CRAC} by increasing P_o. The resulting increase in accumulation of intracellular Ca^{2+} at the inactivation sites would be expected to enhance inactivation, though perhaps to a smaller degree than would be expected from a comparable increase in i. A mechanism of this sort has been proposed to underlie the speeding of inactivation of voltage-gated L-type Ca^{2+} channels by Bay K 8644, which increases the P_o of these channels (Noceti *et al.* 1998). Further experiments on I_{CRAC} at the single-channel level will be needed to resolve this issue.

Inhibition of I_{CRAC} and the removal of inactivation by 2-APB

At concentrations $\geqslant 5\ \mu$M, 2-APB slowly inhibited I_{CRAC} and removed fast inactivation. The reversal of these effects after 2-APB was washed out of the bath was even slower and was often incomplete. In this sense, the inhibition by 2-APB is similar to that by imidazole compounds like SKF 96365 or econazole (Franzius *et al.* 1994; Christian *et al.* 1996). It is not known whether 2-APB (or SKF 96365) acts by blocking the channel pore directly, or by altering channel gating. Our data suggest that 2-APB does not act through a simple 1:1 interaction with the CRAC channel pore. The on-rate for inhibition, calculated from the time constant of I_{CRAC} inhibition in Fig. 6*B*, and $K_{½}$ for current inhibition (10 μM, Fig. 4*B*) was $1/500\ \mu M^{-1}\ s^{-1}$. Thus, a simple 1:1 binding interaction would predict an off-rate of $\sim 1/50\ s^{-1}$, or a time constant of recovery of $\sim$50 s. This is much faster than the observed recovery time (22% recovery at 160 s; estimated recovery time constant of > 600 s). The discrepancy cannot be explained by slow washout of the drug, as the potentiation effect of 2-APB reversed rapidly after drug removal (Fig. 3*A*). Instead, these results suggest that the mechanism of inhibition is more complex than simple pore blockade, and that the recovery from inhibition is not limited by the dissociation rate of 2-APB from its target.

2-APB inhibited both the amplitude of I_{CRAC} and the extent of fast inactivation with similar time courses, raising the intriguing possibility that these two effects may be causally linked. It is possible, for example, that 2-APB at high concentrations interrupts the coupling between the CRAC channel and a component that confers fast inactivation, and the activity of the CRAC channel declines because this component is necessary for maintaining the channel in an activatable state. Such a mechanism might also explain the kinetic discrepancy discussed above, if recovery of the inactivation component were much slower than the unbinding of 2-APB. Further experiments on the molecular underpinnings of fast inactivation may give clues as to the mechanism of I_{CRAC} inhibition by 2-APB.

IP_3 receptors are not needed for I_{CRAC} activation or inactivation

The mechanism underlying the activation of store-operated Ca^{2+} channels has been an area of active debate and, despite considerable efforts, no consistent picture has emerged. The leading candidate hypotheses include activation by a diffusible factor released from depleted stores, fusion of store-operated channel-laden vesicles with the plasma membrane, and direct activation of store-operated channels by conformational coupling to IP_3 receptors in the ER membrane (for reviews, see Putney & McKay, 1999; Prakriya & Lewis, 2001*a*). The

conformational coupling hypothesis has received much recent support from evidence that IP_3 receptors bind directly to some members of the TRP family, thought by some to be the molecular correlates of CRAC channels (Kiselyov *et al.* 1998, 1999*a*; Boulay *et al.* 1999). Moreover, inhibitors of IP_3 receptors, such as 2-APB and heparin, also inhibit store-operated Ca^{2+} entry and the currents mediated by the putative store-operated channel TRP3 (Kiselyov *et al.* 1998; Ma *et al.* 2000). It has been suggested that fast inactivation of CRAC channels may arise through the inhibitory effects of Ca^{2+} on IP_3 receptors to which they are coupled (Berridge, 1995).

Our data are inconsistent with a necessary role for IP_3 receptors in the activation and inactivation of I_{CRAC}. First, we found that heparin failed to affect activation or the maintenance of I_{CRAC} in store-depleted cells, even at a concentration that completely prevented the ability of IP_3 to activate I_{CRAC}. A similar result was described recently in RBL cells (Broad *et al.* 2001). In contrast, currents mediated by recombinant TRP3 channels in excised patches from HEK 293 cells or endogenous 'CRAC-like' channels from A431 cells require addition of IP_3 and are blocked potently by heparin (Kiselyov *et al.* 1998, 1999*b*). Thus, the ability to activate I_{CRAC} through passive depletion, coupled with the inability of heparin to prevent this, suggests that CRAC channels differ from these other channels in not requiring even background levels of IP_3 for activation. Second, the activation and properties of I_{CRAC} were apparently normal in DT40 cells in which all three subtypes of IP_3 receptor had been knocked out by homologous recombination. These results confirm and extend earlier work by Sugawara *et al.* (1997) showing that TG was able to induce Ca^{2+} entry in these cells. Thus, our results show that CRAC channels do not require IP_3 receptors or IP_3 for their activation. However, these results do not rule out conformational coupling involving other proteins.

2-APB affects CRAC channel gating independently of IP_3 receptors

In the mutant DT40 cells lacking all three subtypes of IP_3 receptors, 2-APB elicited the same combination of I_{CRAC} potentiation, inhibition and changes in fast inactivation as in wild-type cells. Thus, the actions of 2-APB on I_{CRAC} are probably unrelated to any effect on IP_3 receptors. These results therefore show that inhibition of Ca^{2+} entry by 2-APB cannot be used to test the conformational coupling model for I_{CRAC} activation.

If IP_3 receptors are not involved, what then is the target for 2-APB's effects on CRAC channel activity? One possible site of action we considered was mitochondria, as 2-APB inhibited Ca^{2+} release from these organelles (Fig. 1*B*). In Jurkat T cells, uptake and release of Ca^{2+} by mitochondria is required to enable the cells to maintain a high rate of Ca^{2+} entry through CRAC channels (Hoth *et al.* 1997). To test the possibility that the effects of 2-APB on I_{CRAC} are an indirect consequence of modulating mitochondrial function, we treated cells with 1 μM antimycin A1 + 1 μM oligomycin to inhibit mitochondrial Ca^{2+} uptake. The inhibitors did not alter the ability of 2-APB to potentiate and inhibit I_{CRAC} (data not shown). Therefore, the effects of 2-APB on I_{CRAC} appear to be distinct from its effects on mitochondrial Ca^{2+} transport.

2-APB was more effective at modulating I_{CRAC} when applied extracellularly than when it was applied intracellularly through the recording pipette. A similar result was reported for RBL cells by Braun *et al.* (2001), who concluded that the drug's site of action was extracellular. The interpretation of this experiment is complicated by several factors. First, 2-APB may exist in multiple forms, including a protonated and unprotonated monomer, as well as a dimer (van Rossum *et al.* 2000), but the relative efficacy of these various compounds in affecting Ca^{2+} entry in general is unknown. Second, the ability of lipophilic compounds to interact with intracellular sites when applied intracellularly via a patch pipette is a function of their membrane permeability. Thus, when applied through the recording pipette, the concentration of a hydrophobic form of 2-APB near the plasma membrane may be much lower than expected, due to the fact that the rate-limiting diffusion step for such a hydrophobic compound may be across the tip of the pipette rather than across the plasma membrane. We reasoned that increasing the protonation of the drug should reduce permeability to some degree, and this should reduce the efficacy of extracellular drug if its site of action is intracellular. This approach has been applied previously in studies of membrane-permeant K^+ channel blockers. For example, methadone is known to block K^+ channels at an intracellular site, yet it is effective only when applied from outside the cell. Acidification of the extracellular medium was shown to inhibit the blocking action of methadone by reducing the concentration of the uncharged, lipophilic species (Horrigan & Gilly, 1996). In our experiments, reducing extracellular pH from 7.4 to 6.4 failed to decrease either potentiation or inhibition by 2-APB, implying that lowering the concentration of the unprotonated (and presumably membrane-permeant) form of 2-APB had no effect. The relative amounts of the protonated and unprotonated forms of 2-APB will depend on its pK_a, which is unknown, but is likely to be determined by its ethylamine group ($pK_a = 10.8$; Weast & Lide, 1989). Based on this pK_a value, one would predict that only 0.04 % of the 2-APB will be unprotonated at pH 7.4, but that this fraction will decrease by a factor of 10 at pH 6.4. Thus, the lack of an effect from lowering the pH (Fig. 10*C*) is consistent with an extracellular but not an intracellular site of action. However, given the various complications outlined above, a direct examination of this issue using membrane-impermeant variants of 2-APB

will be needed to identify firmly the active species of 2-APB and its site of action.

The opposing effects of 2-APB on I_{CRAC} are reminiscent of the effects of the plant alkaloid ryanodine on the sarcoplasmic reticulum Ca^{2+} release channel in cardiac muscle. At submicromolar (1–50 nM) concentrations, ryanodine stabilizes the Ca^{2+} release channel in a sub-conductance state with a dramatically higher P_o, whereas at micromolar (10–1000 μM) concentrations, ryanodine closes the channel (Buck *et al.* 1992; Humerickhouse *et al.* 1993). These effects are manifested as increases in Ca^{2+} flux and muscle contraction by dilute ryanodine and inhibition of Ca^{2+} flux and muscle contraction by concentrated ryanodine. Binding studies indicate that the Ca^{2+} release channel has a high-affinity site, thought to mediate the stimulatory effects of ryanodine, and one or more low-affinity sites that mediate channel closure (Sutko *et al.* 1997). Thus, by analogy, it is possible that 2-APB has two separate binding sites, a high-affinity site that mediates I_{CRAC} potentiation and a low-affinity site that produces inhibition. Alternatively, different species of 2-APB may be responsible for the different effects: monomers, which are more prevalent at low concentrations, may mediate potentiation, whilst dimers, which are favoured at higher concentrations, may be responsible for inhibition. Further studies will be needed to resolve these issues.

In summary, we found that 2-APB elicits complex effects on I_{CRAC}. Low concentrations of 2-APB reversibly enhanced I_{CRAC}, whereas high concentrations also inhibited I_{CRAC}. 2-APB also altered the gating of I_{CRAC} by removing fast Ca^{2+}-dependent inactivation. It was surprising to find that so many different properties of CRAC channels were affected, and an important question is whether the various effects of 2-APB are linked in some way or whether they are truly independent. Given that 2-APB affects the function of so many Ca^{2+} signalling proteins (IP_3 receptors, CRAC channels, TRP channels and the mitochondrial Ca^{2+} release mechanism), the drug clearly has limited utility as a specific inhibitor of store-operated Ca^{2+} entry. However, some effects of 2-APB (e.g. potentiation and removal of inactivation) may be sufficiently unique that identification of the parts of the molecule that are responsible for these effects and the proteins with which they interact could provide new clues to the mechanisms underlying activation, deactivation and inactivation of CRAC channels.

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