

Calcium-dependent Potentiation of Store-operated Calcium Channels in T Lymphocytes

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ABSTRACT The depletion of intracellular Ca^{2+} stores triggers the opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in the plasma membrane of T lymphocytes. We have investigated the additional role of extracellular Ca^{2+} (Ca_o^{2+}) in promoting CRAC channel activation in Jurkat leukemic T cells. Ca^{2+} stores were depleted with 1 μM thapsigargin in the nominal absence of Ca_o^{2+} with 12 mM EGTA or BAPTA in the recording pipette. Subsequent application of Ca_o^{2+} caused I_{CRAC} to appear in two phases. The initial phase was complete within 1 s and reflects channels that were open in the absence of Ca_o^{2+} . The second phase consisted of a severalfold exponential increase in current amplitude with a time constant of 5–10 s; we call this increase Ca^{2+} -dependent potentiation, or CDP. The shape of the current-voltage relation and the inferred single-channel current amplitude are unchanged during CDP, indicating that CDP reflects an alteration in channel gating rather than permeation. The extent of CDP is modulated by voltage, increasing from $\sim 50\%$ at +50 mV to $\sim 350\%$ at -75 mV in the presence of 2 mM Ca_o^{2+} . The voltage dependence of CDP also causes I_{CRAC} to increase slowly during prolonged hyperpolarizations in the constant presence of Ca_o^{2+} . CDP is not affected by exogenous intracellular Ca^{2+} buffers, and Ni^{2+} , a CRAC channel blocker, can cause potentiation. Thus, the underlying Ca^{2+} binding site is not intracellular. Ba^{2+} has little or no ability to potentiate CRAC channels. These results demonstrate that the store-depletion signal by itself triggers only a small fraction of capacitative Ca^{2+} entry and establish Ca^{2+} as a potent cofactor in this process. CDP confers a previously unrecognized voltage dependence and slow time dependence on CRAC channel activation that may contribute to the dynamic behavior of I_{CRAC} . **Key words:** I_{CRAC} • patch clamp • Ca21 channel • capacitative calcium entry • thapsigargin

INTRODUCTION

In many cell types, the generation of inositol 1,4,5-trisphosphate (IP_3)¹ triggers a biphasic increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), consisting of a transient release of Ca^{2+} from the endoplasmic reticulum followed by sustained Ca^{2+} influx across the plasma membrane (Berridge, 1993; Clapham, 1995). This fundamental Ca^{2+} signaling mechanism is used in a cell- and agonist-specific fashion to control such basic functions as growth and metabolism, secretion, muscle contraction, and gene expression. The sustained phase

of the Ca^{2+} signal appears to result from activation of Ca^{2+} entry by the depletion of intracellular Ca^{2+} stores, a process originally termed capacitative Ca^{2+} entry (Putney, 1986; Putney, 1990). This hypothesis has received strong support from the ability of thapsigargin (TG), a specific inhibitor of microsomal Ca^{2+} -ATPases, as well as ionomycin, a lipophilic Ca^{2+} ionophore, to deplete stores and evoke Ca^{2+} entry without generating significant amounts of IP_3 (Putney and Bird, 1993). Store-operated Ca^{2+} channels (SOCs) in the plasma membrane form the basis of capacitative Ca^{2+} entry, and several types of SOC channels have been characterized using patch-clamp techniques. The most extensively studied SOC, known as the Ca^{2+} release-activated Ca^{2+} (CRAC) channel, is expressed in mast cells (Hoth and Penner, 1992), RBL cells (Fasolato et al., 1993; Zhang and McCloskey, 1995), and T lymphocytes and related T cell lines (Lewis and Cahalan, 1989; McDonald et al., 1993; Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994). CRAC channels are distinguished by their high selectivity for Ca^{2+} over other di-

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¹ Abbreviations used in this paper: $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; Ca_o^{2+} , extracellular Ca^{2+} ; CDP, Ca^{2+} -dependent potentiation; CRAC, Ca^{2+} release-activated Ca^{2+} ; IP_3 , inositol 1,4,5-trisphosphate; SOC, store-operated Ca^{2+} channel; TG, thapsigargin.

valent and monovalent cations, voltage-independent gating, rapid inactivation during hyperpolarizing voltage pulses, and an extremely small unitary conductance, estimated from nonstationary fluctuation analysis to be 10–30 fS (Hoth and Penner, 1992; Hoth and Penner, 1993; Zweifach and Lewis, 1993; Partiseti et al., 1994; Premack et al., 1994; Hoth, 1995; Zweifach and Lewis, 1995a). Other, less Ca^{2+} -selective SOC's have been described in epithelial (Lückhoff and Clapham, 1994) and endothelial cells (Vaca and Kunze, 1994), suggesting that the SOC's comprise a diverse family of related channel types whose expression patterns may be tissue specific.

The mechanisms that regulate SOC channel activity are the subject of intense interest but as yet are only partially understood. In its simplest form, the capacitative Ca^{2+} entry hypothesis holds that Ca^{2+} entry is activated by store depletion and is terminated by subsequent store refilling, thus creating a self-regulating feedback loop to control Ca^{2+} entry. These basic predictions are supported by experimental data obtained with Ca^{2+} imaging and patch-clamp techniques (Putney and Bird, 1993; Zweifach and Lewis, 1995b). However, the link between store depletion and channel activation, which in principle could involve a diffusible messenger or direct physical coupling between channels and stores, is still not established in any cell type (for reviews, see Putney and Bird, 1993; Fasolato et al., 1994; Berridge, 1995). Furthermore, the simplest form of the capacitative Ca^{2+} entry hypothesis does not explain store-independent modes of negative regulation of I_{CRAC} by intracellular Ca^{2+} . For example, Ca^{2+} influx driven by membrane hyperpolarization causes a rapid inactivation of CRAC channels occurring within milliseconds, the result of Ca^{2+} binding to sites located within several nanometers of the pore (Zweifach and Lewis, 1995a). In addition, a global rise of $[\text{Ca}^{2+}]_i$ causes slow inactivation of I_{CRAC} over tens of seconds, partly as a result of store refilling, but also because of a store-independent mechanism (Zweifach and Lewis, 1995b).

In this study we have investigated the role of extracellular Ca^{2+} in the gating of CRAC channels in Jurkat T lymphocytes. We find that, contrary to the most basic predictions of the capacitative Ca^{2+} entry hypothesis, I_{CRAC} is not fully activated by store depletion in the absence of extracellular Ca^{2+} (Ca_o^{2+}). The addition of Ca^{2+} to cells with depleted stores evokes as much as a fourfold increase in CRAC channel activity through Ca^{2+} binding to a voltage-dependent site probably on the channel itself. The involvement of extracellular Ca^{2+} in I_{CRAC} gating contributes to the apparent divalent ion selectivity of the current and confers a voltage and time dependence to CRAC channel activation that may contribute to the dynamic behavior of I_{CRAC} . A pre-

liminary report of a portion of these results has been presented (Lewis et al., 1996).

METHODS

Cells and Materials

Jurkat E6-1 human leukemic T cells were maintained in complete medium containing RPMI 1640 and 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 25 mM HEPES, in a 6% CO_2 humidified atmosphere at 37°C. Log-phase cells ($0.2\text{--}1.2 \times 10^6/\text{ml}$) were used in all experiments. TG (LC Pharmaceuticals, Woburn, MA) was prepared as a 1-mM stock in DMSO.

Patch-Clamp Recording

Patch-clamp experiments were conducted in the standard whole-cell recording configuration (Hamill et al., 1981). Extracellular Ringer's solution contained (in mM): 155 NaCl, 4.5 KCl, 1 MgCl_2 , 1–42 CaCl_2 as indicated, 10 D-glucose, and 5 Na-HEPES, pH 7.4. Ca^{2+} -free Ringer's contained 3 mM MgCl_2 . Internal solutions contained (in mM): 140 Cs aspartate, 10 Cs-HEPES, pH 7.2, and either 0.66 CaCl_2 , 11.68 EGTA, and 3.01 MgCl_2 (12 mM EGTA solution); or 0.066 CaCl_2 , 1.2 EGTA, and 2.01 MgCl_2 (1.2 mM EGTA solution); or 0.90 CaCl_2 , 12 BAPTA, and 3.16 MgCl_2 (BAPTA solution). Free $[\text{Ca}^{2+}]$ in these solutions as measured with indo-1 (see Zweifach and Lewis, 1995a) was 5 nM; free $[\text{Mg}^{2+}]$ was calculated to be 2 mM. Recording electrodes were pulled from 100- μl pipettes (VWR Scientific Corp., Philadelphia, PA), coated with Sylgard (Dow Corning Corp., Midland, MI) near their tips, and fire polished to a resistance of 2–6 M Ω when filled with Cs aspartate pipette solution. The patch-clamp output (Axopatch 200; Axon Instruments, Foster City, CA) was filtered at 1.5 kHz with an eight-pole Bessel filter (Frequency Devices, Inc., Haverhill, MA) and digitized at 2–5 kHz. Stimulation and recording was performed with an Apple Macintosh computer and a data interface (ITC-16; Instrutech, Elmont, NY) using custom software extensions (available from R. Lewis upon request) to Igor Pro (WaveMetrics, Inc., Lake Oswego, OR). Command potentials were corrected for the –12 mV junction potential that exists between the aspartate-based pipette solutions and Ringer's solution. All data (except for the traces shown in Figs. 1 A and 9 B) are corrected for leak and residual capacitative current evoked by the equivalent voltage stimuli in the absence of Ca_o^{2+} . Leak conductance ranged from 20 to 100 pS. Series resistance compensation was not used, since the series resistance (4–25 M Ω) produced voltage errors of <3 mV. All experiments were conducted at 22–25°C.

Solution Exchange

Cells were allowed to settle onto but not firmly adhere to glass coverslip chambers shortly before each experiment. External solutions were changed by lifting cells off the coverslip and positioning them ~1 mm inside one barrel of a perfusion tube array through which the desired solutions flowed (<0.1 ml/min). The speed of solution exchange with this system was estimated from the response time of the K^+ current to a change in extracellular $[\text{K}^+]$. Voltage-dependent type n K^+ current was recorded in response to 50-ms pulses from –80 mV to 0 mV every 0.5 s with a pipette solution containing (in mM): 140 K aspartate, 0.1 CaCl_2 ,

1.1 EGTA, 2 MgCl₂, and 10 K-HEPES, pH 7.2. Upon switching the cell from normal Ringer's to K⁺ Ringer's (170 mM K⁺), the K⁺ current reached a steady-state value within two pulses. This result places an upper limit of 1 s on the time required for solution exchange.

RESULTS

Exposure to Extracellular Ca²⁺ Elicits a Biphasic Increase in I_{CRAC}

In its simplest form, the capacitative Ca²⁺ entry hypothesis postulates that CRAC channels are activated solely by a signal produced by depleted Ca²⁺ stores, and hence should be maximally activated when the stores are completely empty. Thus, the hypothesis predicts that exposing a cell with empty stores to extracellular Ca²⁺ should cause I_{CRAC} to attain its maximal amplitude instantaneously. This prediction does not hold; exposure to Ca²⁺ causes I_{CRAC} to increase with a biphasic time course. In the experiment shown in Fig. 1, Ca²⁺ stores were emptied by incubating cells in Ca²⁺-free Ringer's solution supplemented with 1 μM TG for 3

min while dialyzing the cell's interior with a pipette solution containing 5 nM buffered free Ca²⁺. I_{CRAC} was monitored during 100-ms voltage steps from the holding potential of -25 mV to -80 mV followed by voltage ramps from -80 mV to +50 mV (Fig. 1 A). This stimulus was chosen to allow accurate measurement of the steady-state current amplitude during the step (Fig. 1 B), as well as a rapid determination of the current-voltage relation during the ramp (Fig. 1 C). After store emptying was complete, exposure of the cell to 22 mM Ca_o²⁺ elicited a biphasic increase in inward current (Fig. 1 B). The first phase occurred within the time needed for the solution change (1 s; see Methods), and was followed by a further 2.5-fold increase that developed slowly with an exponential time course (time constant = 5.3 s). Similar results were obtained in experiments in which the Ca²⁺-free Ringer's solution was supplemented with 1 mM EGTA (free [Ca²⁺] < 10⁻⁹ M), demonstrating that the response is not affected by the 5–10 μM Ca²⁺ present in Ca²⁺-free Ringer's.

The initial current (observed immediately after Ca_o²⁺ addition) was identified as I_{CRAC} based on a number of

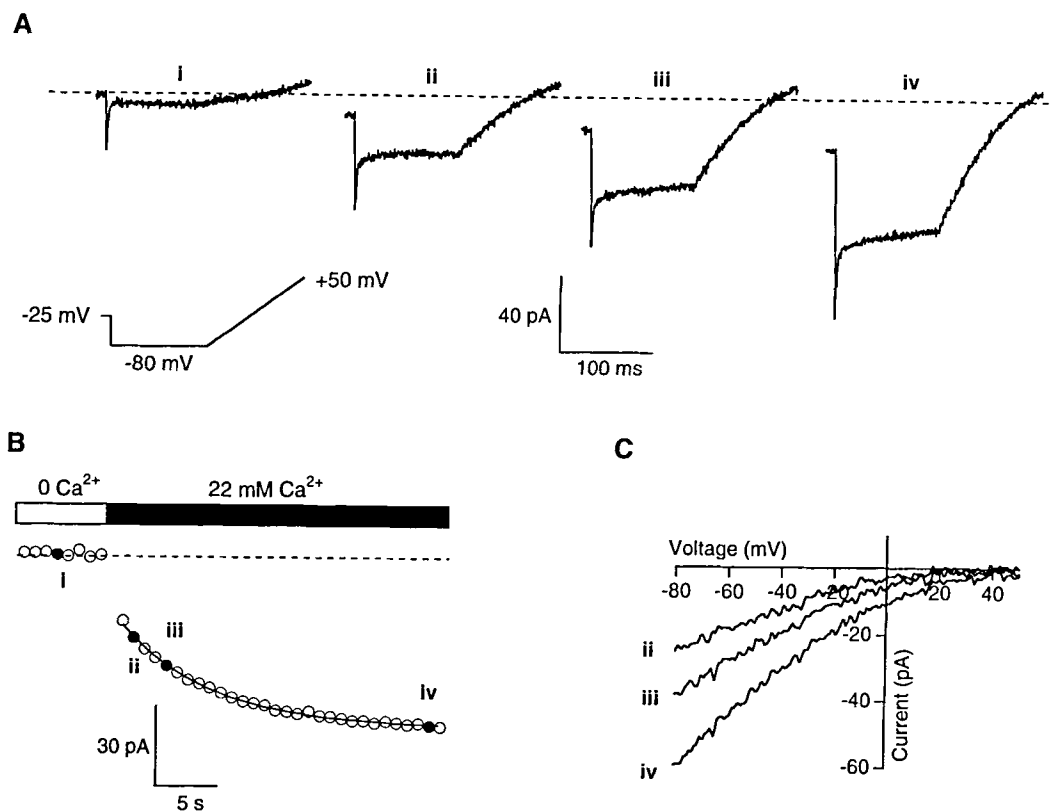


FIGURE 1. Application of extracellular Ca²⁺ causes a biphasic increase in I_{CRAC}. The cell was pretreated for 3 min with 1 μM TG in Ca²⁺-free Ringer's to deplete stores. Internal solution contained 12 mM BAPTA. (A) Measurement of I_{CRAC} with a step-ramp protocol. A 100-ms step from the holding potential (-25 mV) to -80 mV is followed by a 100-ms ramp to +50 mV, delivered at a rate of 1.5 Hz. Traces show responses before (i) and after (ii–iv) addition of 22 mM Ca²⁺ to the cell. Times of data collection are shown in B. (B) Time course of I_{CRAC} in response to Ca_o²⁺ addition. 22 mM Ca²⁺ Ringer's was applied

during the time shown by the bar. Each point is the average current measured during the last 5 ms of the steps to -80 mV shown in A. The dashed line shows the current measured in the absence of Ca_o²⁺ (-6.0 pA). An exponential curve has been fitted and superimposed on the data ($\tau = 5.3$ s). (C) Representative ramp currents collected at various times after Ca_o²⁺ addition. Currents were elicited by ramps from -80 mV to +50 mV as shown in A and are corrected for the response in 0 Ca_o²⁺. Labels ii–iv refer to the traces in A collected at the times indicated in B. The shape of the current-voltage relation does not change with time, consistent with an increase in I_{CRAC}.

characteristic properties (for reviews, see Fasolato et al., 1994; Lewis and Cahalan, 1995), including a high selectivity for Ca^{2+} over monovalent cations, rapid inactivation during hyperpolarizing pulses, an inwardly rectifying current-voltage relation without a clear reversal potential up to +50 mV, and a lack of voltage-dependent gating and significant current noise. Two arguments indicate that the slow increase in current reflects an increase in I_{CRAC} rather than the Ca^{2+} -dependent activation of another current. First, we observed a similar slow rise in other recordings with 12 mM EGTA or 12 mM BAPTA in the pipette, conditions that prevent global rises in $[\text{Ca}^{2+}]_i$ on this time scale (Zweifach and Lewis, 1995b). Second, as the current's amplitude increased, its basic properties did not change, including its dependence on Ca_o^{2+} (see Fig. 3 A), low noise (data not shown), and Ca^{2+} selectivity as shown by the shape of the current-voltage relation (Fig. 1 C). Therefore, based on this set of characteristics, we conclude that the slow increase in current amplitude is due to a time-dependent enhancement of I_{CRAC} evoked by an increase in extracellular $[\text{Ca}^{2+}]_o$.

We considered several explanations for the slow increase in I_{CRAC} , such as enhanced activation due to additional store depletion via Ca^{2+} -induced Ca^{2+} release, inefficient exchange of extracellular Ca^{2+} , or a Ca^{2+} -dependent effect on CRAC channel activity. The increase in current amplitude did not result from increased store depletion after Ca^{2+} influx, as it occurred in the presence of 1 μM TG, which fully depletes stores in Jurkat cells (Premack et al., 1994, Zweifach and Lewis, 1995b). The current's increase is significantly

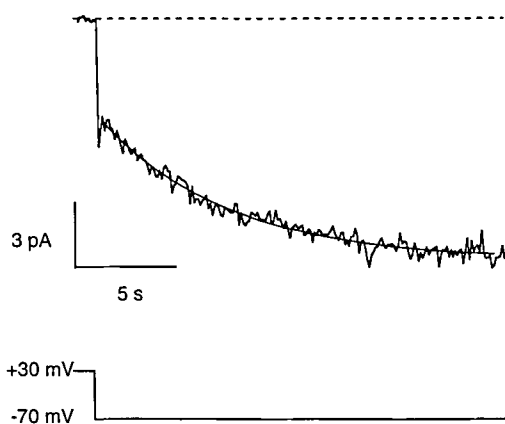


FIGURE 2. I_{CRAC} increases slowly during prolonged hyperpolarization. After depletion of stores as described in Fig. 1, the cell was exposed to 2 mM Ca_o^{2+} at a holding potential of +30 mV. In the constant presence of Ca_o^{2+} , a hyperpolarizing voltage step to -70 mV causes a biphasic increase in inward current with kinetics similar to those observed in response to Ca^{2+} addition (cf. Fig. 1 B). An exponential curve with a time constant of 6.3 s has been fitted to the data. The trace shown has been leak subtracted using the response to an identical voltage step applied in the absence of Ca_o^{2+} .

slower than the solution exchange time of < 1 s (see Methods), suggesting that poor exchange is also not a likely explanation. This conclusion is further supported by the observation that increasing the driving force for Ca^{2+} entry by hyperpolarization evokes a similar slow relaxation of I_{CRAC} . As illustrated in Fig. 2, a long hyperpolarizing voltage step from +30 mV to -70 mV in the constant presence of 2 mM Ca_o^{2+} caused a biphasic increase in current similar in time course and amplitude to that produced by a sudden increase in $[\text{Ca}^{2+}]_o$ (Fig. 1). These results are consistent with the conclusion, discussed below, that extracellular Ca^{2+} potentiates the activity of CRAC channels, and that this process is enhanced by membrane hyperpolarization.

Potentiation of I_{CRAC} Involves a Change in Gating Rather than Permeation

In principle, the potential of I_{CRAC} could result from an increase in the current through individual CRAC channels, i . Although it is difficult to measure i directly because of the low estimated unitary conductance of ~ 10 –30 fS (Zweifach and Lewis, 1993), we can exploit another property of I_{CRAC} , fast inactivation, to track changes in i . Ca^{2+} entering through open CRAC channels binds to intracellular sites within several nanometers of the pore to cause fast inactivation of I_{CRAC} (Hoth and Penner, 1993; Zweifach and Lewis, 1995a). The extent of fast inactivation that occurs during brief hyperpolarizing voltage steps is determined by i (Zweifach and Lewis, 1995a). In the experiment shown in Fig. 3, fast inactivation of I_{CRAC} during 50-ms pulses to -132 mV was measured repeatedly after exposing the cell to elevated $[\text{Ca}^{2+}]_o$. Even though exposure to 2 mM Ca_o^{2+} slowly potentiated I_{CRAC} , leading to an approximate doubling of its initial magnitude, the extent of fast inactivation during each pulse remained constant. This result suggests that the slow increase in I_{CRAC} is not due to a change in the unitary current, i . To confirm that a doubling of i would in fact cause a detectable increase in fast inactivation, we next exposed the cell to 22 mM Ca_o^{2+} . This caused an immediate doubling of the current, along with an easily detectable increase in fast inactivation (Fig. 3). Again, while current amplitude increased further over the next 10 s, the extent of fast inactivation remained constant. Identical results were obtained during a switch from 0 Ca_o^{2+} directly to 22 mM Ca_o^{2+} (Fig. 3). These results verify that the extent of fast inactivation is a sensitive indicator of the unitary CRAC current. Therefore, the fact that fast inactivation is constant as I_{CRAC} becomes potentiated supports the conclusion that potentiation is due to an increase in the number of activatable CRAC channels (N) or their open probability (P_o) rather than to an increase in unitary current.

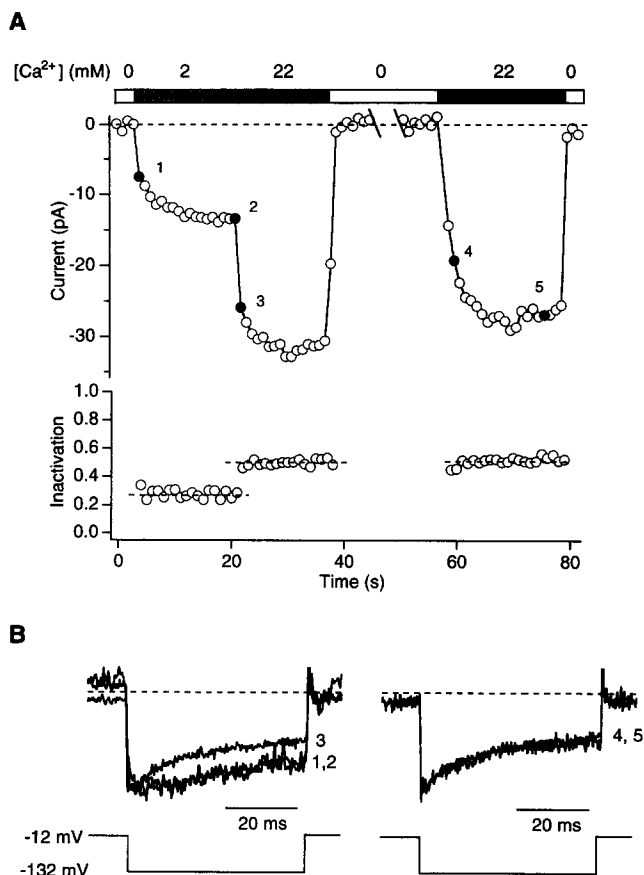


FIGURE 3. CDP reflects a change in CRAC channel gating rather than unitary current. In this experiment, the fast inactivation of I_{CRAC} during hyperpolarizing pulses is used to monitor changes in the unitary CRAC current during CDP (see text). A pipette solution containing 12 mM EGTA rather than BAPTA was used to maximize fast inactivation. (A) Fast inactivation of I_{CRAC} is constant during CDP. 50-ms pulses to -132 mV were delivered at 1 Hz from a holding potential of -12 mV. The upper graph shows peak I_{CRAC} amplitude measured 3–4 ms after the beginning of each pulse as the cell was exposed to the indicated $[\text{Ca}^{2+}]_o$. The lower graph shows the extent of inactivation measured from the ratio of the initial and final values of current during each pulse. Despite time-dependent changes in I_{CRAC} due to CDP, fast inactivation is constant and determined only by $[\text{Ca}^{2+}]_o$. (B) Representative current traces (1–5) collected at the times indicated in A. Traces have been leak subtracted and scaled to the same peak amplitude to facilitate comparisons of inactivation rates; the dashed line indicates 0 current level.

Potentiation of I_{CRAC} Is Calcium Dependent and Is Enhanced by Hyperpolarization

The results presented in Figs. 1 and 2 illustrate the slow enhancement of I_{CRAC} by increasing $[\text{Ca}^{2+}]_o$ or by membrane hyperpolarization, respectively. To determine the relationship between these two forms of potentiation, we measured the enhancement of CRAC channel activity produced by elevation of $[\text{Ca}^{2+}]_o$ at a variety of holding potentials. Cells with depleted stores were

switched repeatedly from 0 Ca^{2+} to 2 mM Ca^{2+} at holding potentials of -75 to $+50$ mV. I_{CRAC} was monitored during 50-ms pulses delivered once per second to a fixed potential of -75 mV to indicate the level of CRAC channel activation. The holding potential has a powerful effect on the extent of potentiation by Ca^{2+} . As shown in Fig. 4 A, at a holding potential of $+50$ mV, I_{CRAC} amplitude is relatively constant from 2 to 20 s after the addition of Ca^{2+} . In contrast, when the cell was held at -50 mV, the current increased dramatically during the same time period. The effects of holding potentials from -50 mV to $+50$ mV on the potentiation of I_{CRAC} are summarized for this cell in Fig. 4 B. Each point represents the current amplitude at the end of pulses to -75 mV like those shown in Fig. 4 A. The initial amplitude of the current in response to addition of Ca^{2+} is relatively independent of the holding potential, but the degree of time-dependent potentiation increases in a graded fashion with hyperpolarization. Interestingly, the fact that the initial current amplitude is independent of holding potential demonstrates that the potentiation process is not driven by hyperpolarization alone; instead, hyperpolarization appears to accentuate the effect of Ca^{2+} . For this reason, we refer to the time-dependent enhancement of I_{CRAC} as Ca^{2+} -dependent potentiation, or CDP.

The time course of CDP is well fit by a simple exponential relation of the form

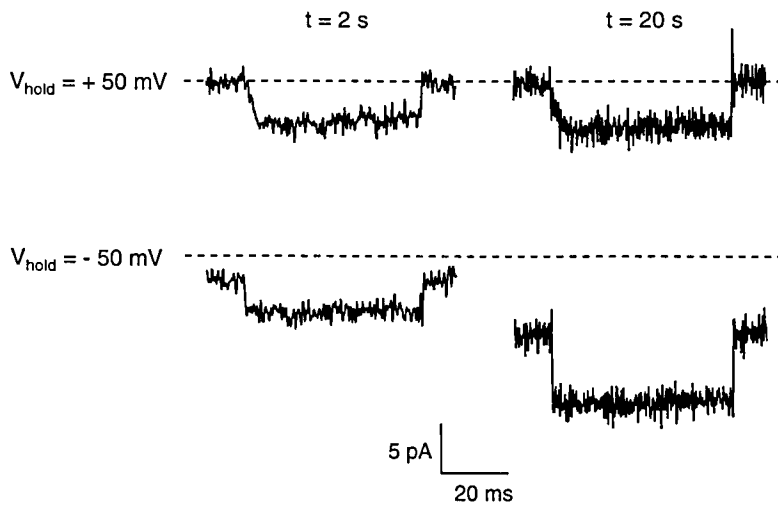
$$I(t) = I_{\infty} + (I_0 - I_{\infty}) e^{-t/\tau}, \quad (1)$$

where $I(t)$ is the current magnitude at time t , I_{∞} is the extrapolated steady-state current, I_0 is the current's initial value immediately after exposure at Ca^{2+} , and τ is the time constant of potentiation. The extent of CDP can be defined as the ratio of steady-state to initial current, I_{∞}/I_0 . As shown in Fig. 5 A by data pooled from a number of cells, the voltage dependence of CDP is well fit by a form of the Boltzmann equation,

$$\text{CDP} \equiv \frac{I_{\infty}}{I_0} = \frac{\text{CDP}_{\text{max}}}{1 + e^{\frac{z\delta F(V - V_{1/2})}{RT}}}, \quad (2)$$

where F , R , and T have their usual meanings and $V_{1/2}$ is the voltage at which CDP is half of its maximal value, CDP_{max} . For the pooled data, CDP_{max} was 5.2, $V_{1/2}$ was -12 mV, and the fitted value for $z\delta$, which we interpret only as an empirical measure of the voltage dependence of the CDP process, was 0.87 (Fig. 5 A), corresponding to an e -fold change every 71 mV. Neither the kinetics nor the extent of CDP changed when the frequency of hyperpolarizing test pulses was halved, confirming that the pulses were brief enough that they did not bias the measurement of CDP. Also, the extent of CDP did not change as I_{CRAC} slowly declined during prolonged experiments (data not shown). Thus, CDP is independent of the number of open channels, and it is

A



B

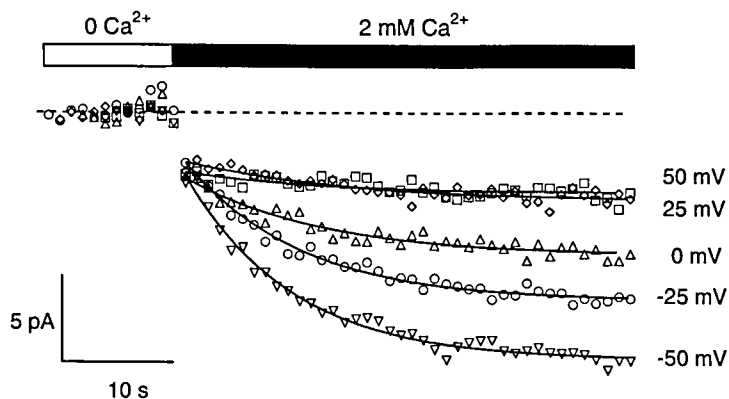


FIGURE 4. The extent of CDP is voltage dependent. (A) I_{CRAC} is potentiated by Ca^{2+} at -50 mV but not $+50$ mV. Currents were evoked by steps to -75 mV from a holding potential of $+50$ mV (top) or -50 mV (bottom) at 2 or 20 s after addition of 2 mM Ca_0^{2+} . All traces are corrected for leak currents evoked by the same voltage pulse in 0 Ca_0^{2+} . At the positive holding potential, I_{CRAC} does not increase much between 2 and 20 s after Ca^{2+} addition. In contrast, at $V_{\text{hold}} = -50$ mV, I_{CRAC} increases by approximately threefold over the same time period. (B) Ca^{2+} -dependent potentiation of CRAC channels at different holding potentials in a single cell. I_{CRAC} was measured as a function of time after addition of 2 mM Ca_0^{2+} during the last 5 ms of hyperpolarizing voltage steps as shown in A. The experiment was repeated five times at holding potentials of $+50$ to -50 mV to generate each set of data points. The initial current amplitude after Ca^{2+} addition is relatively independent of holding potential, but potentiation of I_{CRAC} by Ca^{2+} is enhanced by hyperpolarization. Exponential functions have been fitted to each set of data points.

therefore valid to pool data from cells with different levels of I_{CRAC} as in Fig. 5 A.

In contrast to the extent of CDP, the time course of this process is not obviously dependent on holding potential. Whether evoked by $[\text{Ca}^{2+}]_0$ elevation at potentials of $+25$ to -75 mV or by long hyperpolarizations of -40 mV to -120 mV at a constant $[\text{Ca}^{2+}]_0$ of 2 mM, CDP developed with a time constant of 5–10 s (Fig. 5 B). Accurate values for the time constant of CDP were difficult to obtain at holding potentials $>+25$ mV, because of the small extent of CDP under these conditions.

The simplest explanation consistent with the results presented above is that CDP is a Ca^{2+} -dependent process, and that hyperpolarization enhances its Ca^{2+} sensitivity. To test this idea, we determined the Ca^{2+} sensitivity of CDP at two holding potentials. As described above, I_{CRAC} was measured during repetitive pulses to -75 mV delivered from a holding potential of either

$+25$ mV or -25 mV, as the cell was exposed to a variety of extracellular Ca^{2+} concentrations to induce CDP (Fig. 5 C). CDP was markedly Ca^{2+} dependent at a holding potential of $+25$ mV. At this potential, the relationship between $[\text{Ca}^{2+}]_0$ and the extent of CDP could be described by a single-site Michaelis-Menten model with $K_{\text{in}} = 3.3$ mM. In contrast, increasing $[\text{Ca}^{2+}]_0$ from 1 to 42 mM at a holding potential of -25 mV evoked only a small increase in CDP if any, suggesting that it is nearly saturated under these conditions. Importantly, CDP at $+25$ and -25 mV appears to approach the same asymptotic value in the limit of high $[\text{Ca}^{2+}]_0$. This result suggests that hyperpolarization increases the Ca^{2+} sensitivity of CDP. However, the asymptotic CDP values in Fig. 5 C (~ 4) are somewhat lower than the maximal values plotted in Fig. 5 A (~ 4.7). Whereas this difference is not statistically significant, it illustrates variability in the extent of CDP among individual cells

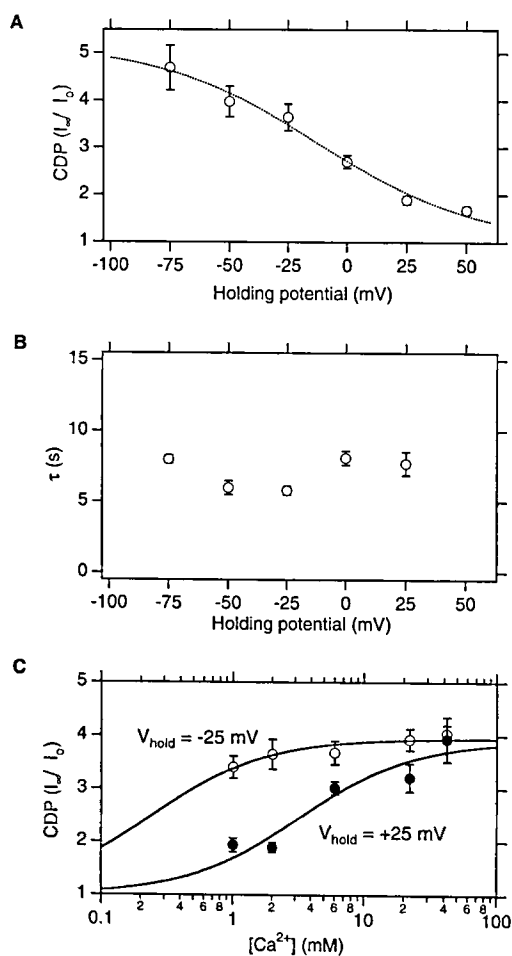


FIGURE 5. The dependence of CDP on $[Ca^{2+}]_o$ and voltage. (A) The steady-state value of CDP is enhanced by hyperpolarization. CDP is quantified as the ratio of the steady-state I_{CRAC} level (I_{∞}) to the initial I_{CRAC} level (I_0) immediately after Ca^{2+} addition. I_{∞} and I_0 were measured from monoexponential fits to data like those in Fig. 4 B. Each point is the mean \pm SEM value obtained from 4–10 cells using an internal solution containing 12 mM BAPTA. A Boltzmann curve with $\alpha\delta = 0.87$ has been fitted to the data (see text). (B) The kinetics of CDP as a function of holding potential. Data were obtained from the same cells as in A by fitting monoexponential functions to the time course of I_{CRAC} after Ca^{2+} addition (see Fig. 4 B). (C) Voltage shifts the sensitivity of CDP to $[Ca^{2+}]_o$. CDP evoked by the addition of 1–42 mM Ca^{2+}_o was measured at holding potentials of -25 mV (\circ) and $+25$ mV (\bullet). A single binding site Michaelis-Menten equation has been fitted by least-squares to the data with maximum = 3.8 and $K_m = 3.3$ mM ($+25$ mV).

that could mask a small difference in the asymptotic levels of CDP seen at $+25$ and -25 mV. Thus, we cannot entirely exclude some direct voltage dependence of CDP.

CRAC Channels Are Nonconducting at Very Depolarized Potentials

In addition to the slow voltage-dependent enhancement of I_{CRAC} that results from the voltage sensitivity of

CDP, we also found evidence for rapid voltage-dependent gating of these channels. In response to hyperpolarization from positive holding potentials ($+25$ or $+50$ mV), I_{CRAC} develops over several milliseconds, whereas the same hyperpolarization from more negative holding potentials (<0 mV) evokes an immediate increase in I_{CRAC} to its maximal amplitude (Fig. 6 A). These results suggest that CRAC channels are in a nonconducting state at very depolarized potentials ($\geq +25$ mV) in 2 mM Ca^{2+}_o , and only upon repolarization can the channels conduct. Further supporting this conclusion is the finding that I_{CRAC} activation becomes faster with increasing levels of hyperpolarization. The time constant for activation decreased from 13 ms at -40 mV to 1.3 ms at -120 mV (Fig. 6 B).

Two possible sources of artifact were considered to explain this phenomenon. We first considered whether it occurs because of an error in leak or capacitive current subtraction. Small changes in series resistance in the time between collection of the leak current (in 0 Ca^{2+}_o) and the test current (in 2 mM Ca^{2+}_o) could introduce an apparent time dependence to the leak-subtracted traces. However, this artifact would cause a symmetrical current relaxation after the onset and termination of the voltage step, and this is clearly not the case. It is also unlikely that a series resistance artifact would create the graded responses to holding potential and step potential seen in Fig. 6, A and B. A second possibility is that the current relaxation arises from the activation or deactivation of a contaminating current. For a second current to make a contribution, its activity must differ between the Ca^{2+} -free and 2 mM Ca^{2+} conditions such that it is not removed by the leak subtraction procedure. Furthermore, to offset I_{CRAC} at the beginning of the pulse, the current must increase in size instantaneously in response to hyperpolarization before rapidly deactivating. This would imply that the channels are open at depolarized potentials before the pulse and closed immediately after the end of the pulse. Thus, a difference in total current before and after the pulse would be predicted, but such an offset is not observed (Fig. 6). Therefore, we conclude that the current relaxation is due to rapid changes in CRAC channel activity. In the presence of 2 mM Ca^{2+} , CRAC channels are either blocked or closed at very positive potentials and only become functional at potentials $< +25$ mV.

An intriguing parallel exists between the conditions that favor conduction by CRAC channels and those that promote the CDP process. Under conditions where CDP is minimal ($\geq +25$ mV, 2 mM Ca^{2+}_o), CRAC channels appear to be nonconducting, whereas at more negative voltages both CDP and conduction occur (see Figs. 6 A and 4 B). This correlation suggests that CRAC channels must be in a conducting state for Ca^{2+} to be able to potentiate their activity.

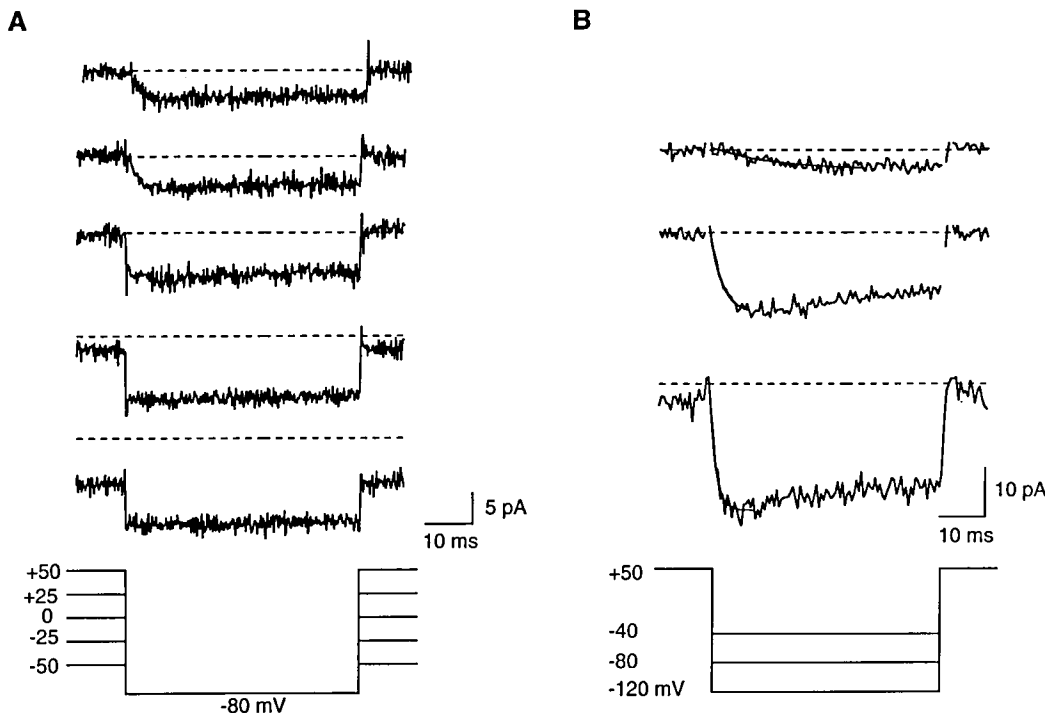


FIGURE 6. CRAC channels are nonconductive at very depolarized potentials. (A) The effect of holding potential on I_{CRAC} evoked by hyperpolarizing pulses. Responses to a voltage step to -75 mV delivered from holding potentials of $+50$ to -50 mV are shown. At $V_{hold} = +25$ mV or above, CRAC channels are initially closed or blocked, and open within 5 ms of the hyperpolarization. At more negative holding potentials, the current reaches its maximal value immediately upon hyperpolarization, indicating that CRAC channels are conductive. 2 mM Ca_0^{2+} ,

12 mM BAPTA. (B) The kinetics of CRAC channel opening are dependent on membrane potential. Responses are shown to voltage steps to -40 , -80 , and -120 mV from a holding potential of $+50$ mV. In each case, the CRAC channels are initially nonconductive (see A), but the current increases with time during hyperpolarizing pulses. Monoexponential curves with time constants of 13 ms (-40 mV), 2.6 ms (-80 mV), and 1.3 ms (-120 mV) have been fitted and superimposed on the data. Traces shown in A and B are the averages of two to three sweeps and are leak subtracted.

Properties of the Ca^{2+} Binding Site for CDP

We examined the properties of the CDP site, with the aim of determining its ion selectivity and location. The strategy behind these experiments was to replace Ca_0^{2+} with permeant or impermeant divalent cations for a period of time and to assess the level of potentiation they induced by measuring the time-dependent enhancement of I_{CRAC} that was evoked by a subsequent return to Ca_0^{2+} .

To interpret this type of experiment, it is essential to know how quickly CDP is reversed after Ca_0^{2+} is withdrawn. Reversal of CDP is illustrated in Fig. 7 A. CDP was first induced by addition of 2 mM Ca^{2+} at a holding potential of 0 mV. Subsequent removal of Ca_0^{2+} for 10 s reversed the potentiation, as indicated by the fact that a second addition of Ca^{2+} evoked the same CDP response as the first. The time-dependent relaxation of current in 0 Ca^{2+} may reflect the decay of currents carried by Na^+ (Hoth and Penner, 1993; Zhang and McCloskey, 1995) or Mg^{2+} through CRAC channels that are slowly depotentiating. Na^+ or Mg^{2+} are unlikely to contribute significantly to I_{CRAC} in the presence of millimolar Ca_0^{2+} because of the high selectivity of CRAC channels for Ca^{2+} under such conditions (Hoth and Penner, 1993; Hoth, 1995). To resolve the kinetics of CDP reversal, we used changes in the holding potential

to modulate the extent of CDP while monitoring CRAC channel activity with brief hyperpolarizing voltage steps. As shown in Fig. 7 B, changing the holding potential from $+50$ to -25 mV elicited an increase in channel activity characteristic of CDP (see Fig. 2 for a similar example using a long voltage step). Upon returning to a holding potential of $+50$ mV, I_{CRAC} amplitude decayed to its initial level with a time constant of 1.7 s. Normalized results from six cells are summarized in Fig. 7 C. Taken together, these results demonstrate the rapid reversal of CDP when the driving force for Ca^{2+} entry is reduced, either by removing Ca_0^{2+} or by depolarizing the membrane.

Ba^{2+} was unable to substitute effectively for Ca^{2+} in maintaining CRAC channel potentiation (Fig. 8). At a holding potential of 0 mV, substitution of Ba^{2+} for Ca^{2+} caused channel activity to decline with a time course similar to that of CDP reversal in Ca^{2+} -free conditions (see Fig. 7 A). A subsequent exposure to Ca_0^{2+} elicited the slow relaxation characteristic of CDP, indicating that CRAC channels had depotentiating in the presence of Ba_0^{2+} . In seven trials from four cells, CRAC channel potentiation reversed by $74 \pm 8\%$ after a 10 – 15 -s exposure to Ba^{2+} . It should be noted that these experiments were conducted at a holding potential of 0 mV; given the voltage dependence of CDP, it is possible that po-

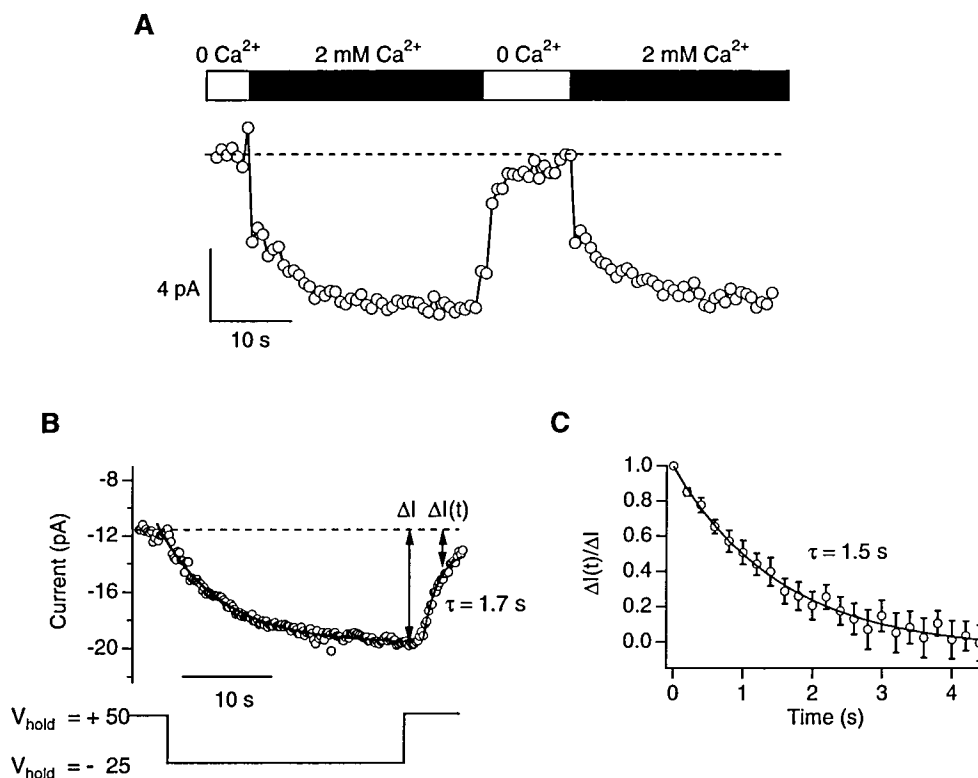


FIGURE 7. The kinetics of CDP reversal by Ca_o^{2+} removal or depolarization. (A) Removal of extracellular Ca^{2+} reverses the effect of CDP. After induction of CDP by 2 mM Ca_o^{2+} , Ca_o^{2+} was replaced by Mg^{2+} for 10 s. Upon readdition of 2 mM Ca_o^{2+} , the time course of I_{CRAC} was similar to that observed during the first exposure to Ca^{2+} , indicating that CDP had been reversed during the incubation in Ca^{2+} -free Ringer's. I_{CRAC} was measured during pulses to -75 mV from a holding potential of $+50$ mV. (B) The kinetics of reversal of CDP in response to changes in holding potential. Each point represents I_{CRAC} evoked in the presence of 2 mM Ca_o^{2+} by a 30-ms step to -75 mV, delivered every 200 ms from a holding potential of $+50$ or -25 mV. At $V_{\text{hold}} = -25$ mV, I_{CRAC} increased with a time constant of 7.1 s, indicative of CDP. After V_{hold} was returned to $+50$ mV, the level of I_{CRAC} evoked by the test pulses decreased with a time constant of 1.7 s, demonstrating the time-dependent reversal of CDP. (C) Average time course of CRAC channel depolarization at $+50$ mV, measured as described in B. I_{CRAC} is plotted relative to its maximum level (at -25 mV) after the return of the holding potential to $+50$ mV. Points show the mean \pm SEM values from six cells, and an exponential curve with a time constant of 1.5 s is superimposed.

tentiation by Ba^{2+} could be revealed at more negative holding potentials.

Interestingly, extracellular Ni^{2+} is able to potentiate CRAC channel activity even though it blocks I_{CRAC} . In the experiment shown in Fig. 9, after determining the extent of CDP in 2 mM Ca^{2+} , the cell was washed with Ca^{2+} -free Ringer's for 10 s to allow CDP to reverse. Ni^{2+} was next applied for 40 s at a concentration (2 mM) sufficient to block CRAC channels fully (Hoth and Penner, 1993; Zweifach and Lewis, 1993). In many experiments, Ni^{2+} also reduced the leak conductance in a reversible manner, as shown by a positive shift in the current under these Ca^{2+} -free conditions. Upon reexposing the cell to 2 mM Ca^{2+} , I_{CRAC} appeared immediately at a magnitude close to the fully potentiated level. Consistent with these results, I_{CRAC} was potentiated to $77 \pm 2\%$ of its maximal value by exposure to 2 mM Ni^{2+} for ~ 30 s (10 trials from five cells). The effect of Ni^{2+} is not due to the trapping of Ca^{2+} in a site within the pore, because we allowed channels to fully depotentiate in 0 Ca_o^{2+} before exposure to Ni^{2+} . Thus, Ni^{2+} itself appears to be able to potentiate the activity of CRAC channels. The cause of the slow relaxation observed after replacement of Ni^{2+} with Ca^{2+} (Fig. 9 A) is not

known, but may indicate that Ni^{2+} is not quite as effective as Ca^{2+} in potentiating I_{CRAC} . Alternatively, the residual slow relaxation may reflect a slow reversal of the effect of Ni^{2+} on the leak current.

The observation that Ni^{2+} , a CRAC channel blocker, can cause potentiation suggests that the binding site for CDP is not intracellular. This conclusion is sup-

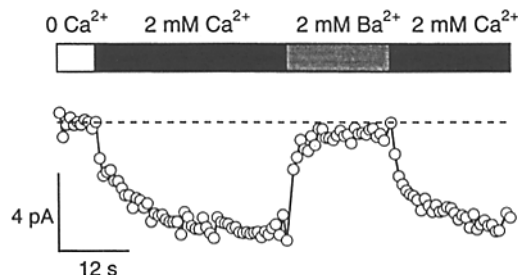
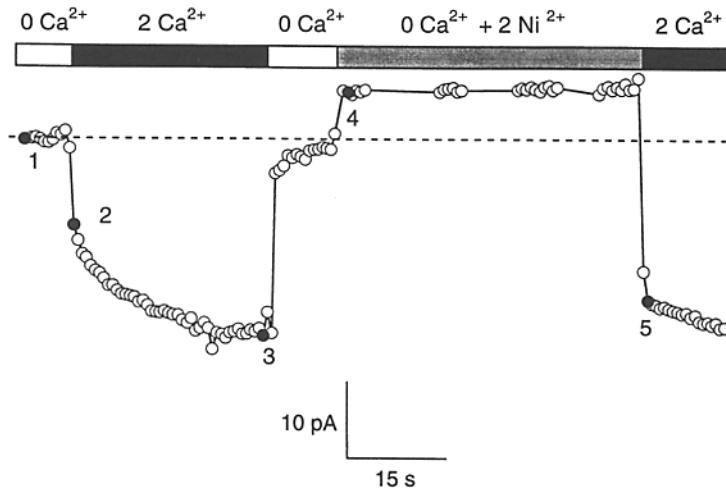


FIGURE 8. Ba^{2+} is relatively ineffective at potentiating CRAC channels. CDP was measured as described in Fig. 7 A. After induction of CDP, Ca_o^{2+} was replaced by 2 mM Ba^{2+} for ~ 15 s. A subsequent return to 2 mM Ca_o^{2+} evokes the same relaxation seen during the initial exposure to Ca_o^{2+} , indicating that Ba^{2+} was unable to maintain the channels in a potentiated state.

A



B

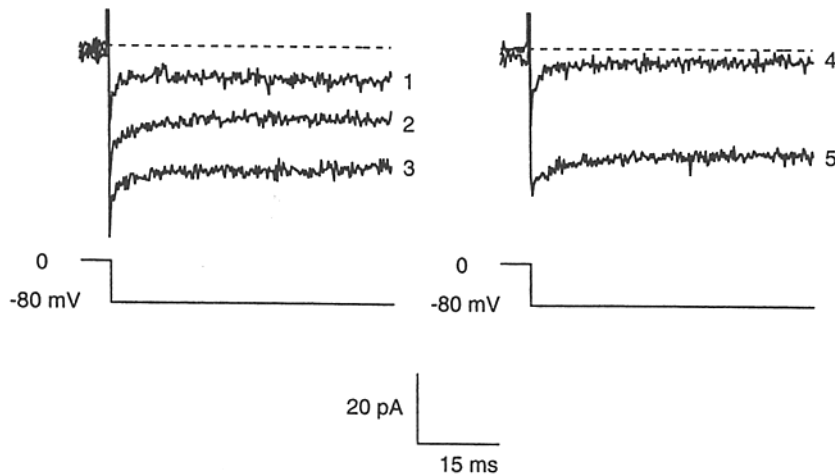


FIGURE 9. Extracellular Ni^{2+} causes CRAC channel potentiation. CDP was measured as described in Fig. 7 A. (A) Effect of Ni^{2+} on I_{CRAC} and potentiation. The cell was first exposed to 2 mM Ca_o^{2+} to demonstrate the extent of CDP. Channels were then depotentiated by a 10-s exposure to Ca^{2+} -free Ringer's before exposure to 0 Ca^{2+} + 2 mM Ni^{2+} for 40 s. Upon returning the cell to 2 mM Ca_o^{2+} , I_{CRAC} appeared at a value close to the maximally potentiated level seen during the first application of Ca^{2+} . (B) Individual sweeps collected at the times indicated in A.

ported by the insensitivity of CDP to pipette-applied Ca^{2+} chelators. The voltage dependence, kinetics, and extent of CDP were similar in the presence of 1.2 or 12 mM intracellular EGTA. Moreover, the maximal extent of CDP at negative potentials (Π_{max}) was slightly enhanced ($\sim 20\%$) in experiments using 12 mM intracellular BAPTA relative to those with intracellular EGTA. This effect is opposite to that expected if the Ca^{2+} binding site driving CDP were intracellular, but could be related to the ability of BAPTA, a rapid Ca^{2+} chelator, to inhibit the Ca^{2+} -dependent inactivation of I_{CRAC} (Zweifach and Lewis, 1995a). Based on these results, we conclude that CDP does not result from the accumulation and binding of Ca^{2+} at an intracellular site but, rather, involves a site located extracellularly or within the pore of the CRAC channel as discussed below.

DISCUSSION

Calcium Greatly Potentiates the Activation of CRAC Channels by Store Depletion

In its most basic form, the capacitive Ca^{2+} entry hypothesis postulates that the activity of store-operated channels is controlled by changes in the content of intracellular Ca^{2+} stores (Putney, 1990). Thus, the role of calcium in regulating CRAC channels is expected to derive solely from its transit into and out of the stores. Recent studies have shown additional store-independent roles for intracellular Ca^{2+} in driving both rapid and slow inactivation of CRAC channels by distinct mechanisms (Hoth and Penner, 1993; Zweifach and Lewis, 1995a; Zweifach and Lewis, 1995b). In this study, we demonstrate a potent effect of extracellular Ca^{2+} in en-

hancing the activation of CRAC channels. In cells with fully depleted stores, CRAC channels are $\sim 20\%$ as active in the nominal absence of Ca^{2+} as in its presence. Because this process of Ca^{2+} -dependent potentiation, or CDP, is increased by membrane hyperpolarization, it introduces a previously unrecognized voltage dependence into I_{CRAC} activation. Taken together, these results reveal new complexity in the positive and negative regulation of CRAC channels that challenges the notion that capacitative Ca^{2+} entry is controlled simply by store content.

The process of CDP may help to explain several previous observations of the voltage and Ca^{2+} sensitivity of capacitative Ca^{2+} entry. Donnadieu et al. (1992) first proposed that gating of the capacitative Ca^{2+} entry pathway might be inhibited by depolarization, noting that Ca^{2+} influx in T cells depolarized to 0 mV is roughly half of that predicted by constant field theory based on the level of influx measured at -50 mV. The voltage sensitivity of CDP can account for this observation, as depolarization from -50 to 0 mV reduces CRAC channel activity by $\sim 50\%$ (Figs. 4 B and 5 A). CDP may also contribute to nonlinear behavior of capacitative Ca^{2+} entry in *Xenopus* oocytes as described by Petersen and Berridge (1994). The Ca^{2+} -activated Cl^- current in TG-treated oocytes was found to depend on voltage and $[\text{Ca}^{2+}]_o$ in a highly nonlinear fashion. To explain these results, the authors suggested that intracellular Ca^{2+} accumulation exerts positive feedback on the activity of depletion-activated Ca^{2+} channels. The voltage and Ca^{2+} dependence of CDP we have described in Jurkat T cells is qualitatively consistent with the results in oocytes, suggesting an alternative explanation independent of intracellular Ca^{2+} (see below); however, several quantitative differences exist. The effects in oocytes begin to appear as the membrane is hyperpolarized beyond -30 mV, a voltage range in which CDP in Jurkat cells is already near saturation (Fig. 5 A). Also, I_{Cl} appears to increase in size during consecutive hyperpolarizing pulses delivered every 60 s (Petersen and Berridge, 1994); such accumulation of potentiation is not predicted by the time constant for depotentiation of ~ 2 s we have measured in Jurkat cells (Fig. 7). These discrepancies may reflect cell-specific differences in the properties of CDP, differences in the recording conditions, or true facilitation of depletion-activated Ca^{2+} channels in oocytes by intracellular Ca^{2+} . Finally, a slow decrease in CRAC channel activity has been observed in mast cells after the removal of extracellular divalent cations (Hoth and Penner, 1993) and in mast cells, RBL cells, and Jurkat cells after replacement of Ca^{2+} with Ba^{2+} (Hoth, 1995). Based on these results, Hoth proposed that extracellular Ca^{2+} may be needed to keep CRAC channels functional (Hoth, 1995). These results can be explained by CDP, and the fact

that these effects were observed in several different cell types suggests that CDP is a general feature of CRAC channels. However, we do not know at present whether CDP is a feature of all SOCs or whether it is restricted to CRAC channels.

Several studies have also demonstrated critical effects of extracellular Ca^{2+} on the function of voltage- and ligand-operated ion channels. Removal of Ca^{2+}_o causes delayed rectifier K^+ channels to lose their ion selectivity and their voltage-dependent gating, as inferred from a parallel increase in nonselective leak conductance and decrease in K^+ conductance (Armstrong and Lopez-Barneo, 1987; Armstrong and Miller, 1990). Ca^{2+}_o removal also shifts the voltage dependence of Na^+ channel opening to more negative potentials; the similar dependences of the gating shift and channel blockade on $[\text{Ca}^{2+}]_o$ led to the suggestion that Ca^{2+} binds within the Na^+ channel pore to stabilize the closed conformation (Armstrong and Cota, 1991). Finally, extracellular Ca^{2+} enhances the current through neuronal nicotinic ACh receptors through an effect on their gating (Vernino et al., 1992). Like CDP, the effect of Ca^{2+} on ACh receptors is not intracellular and cannot be mimicked by Ba^{2+} or Mg^{2+} . Taken together with the effects of Ca^{2+} on CRAC channel gating, these studies highlight the essential role of calcium in maintaining the normal gating properties of channels that are not normally considered to be calcium dependent.

The Mechanism of CDP

In the following section we examine the basic properties of CDP with the aim of developing a hypothesis for its underlying mechanism.

Voltage modulates the Ca^{2+} sensitivity of CDP. As shown above, both an increase in $[\text{Ca}^{2+}]_o$ and membrane hyperpolarization are capable of evoking a slow increase in CRAC channel activity in cells with depleted stores. The effect of voltage appears to require extracellular Ca^{2+} , as the initial level of I_{CRAC} produced upon Ca^{2+} addition is independent of the holding potential (Fig. 4 B). One possible mechanism that would explain this result is that Ca^{2+} binding is required to permit subsequent voltage-dependent gating of CRAC channels. This type of mechanism would predict that, in the presence of a saturating amount of Ca^{2+} , the extent of CDP would be voltage dependent. However, this does not appear to be the case, as the levels of CDP observed at -25 or $+25$ mV in the presence of high $[\text{Ca}^{2+}]_o$ are not significantly different (Fig. 5 C). For this reason, we favor the interpretation that CDP is intrinsically Ca^{2+} dependent, and that the membrane potential merely modulates the Ca^{2+} sensitivity of the process.

The site for CDP is not intracellular. In principle, the enhancement of CDP by hyperpolarization could arise

from the increased binding of Ca^{2+} to a voltage-dependent site, or from accelerated influx and accumulation of Ca^{2+} at an intracellular location. However, the inability of exogenously supplied intracellular Ca^{2+} chelators to suppress CDP argues against an intracellular site, as does the ability of Ni^{2+} to promote potentiation (Fig. 9). At millimolar concentrations, Ni^{2+} blocks I_{CRAC} (Lewis and Cahalan, 1989; Hoth and Penner, 1993; Zweifach and Lewis, 1993; see Fig. 9), and it is not expected to accumulate intracellularly to any significant extent. Any Ni^{2+} that does manage to permeate the channels would be efficiently bound by intracellular BAPTA. Therefore, assuming that Ni^{2+} and Ca^{2+} bind to the same site to potentiate CRAC channels, these results argue strongly against an intracellular location for the binding site. This leaves two major possible sites for CDP. Ca^{2+} could bind within the channel pore to a site whose apparent affinity is influenced by the membrane electric field, or it could bind to an extracellular site whose affinity is determined by a voltage-driven allosteric mechanism.

Whereas we cannot at present rule out an extracellular binding site, several observations suggest that the sites that determine divalent ion permeation and CDP may be related. First, CDP is evoked by Ca^{2+} , which permeates CRAC channels, as well as Ni^{2+} , an ion that blocks Ca^{2+} flux possibly by binding within the pore (Fig. 9). In contrast, at the holding potential used in our experiments (0 mV), Ba^{2+} neither carries substantial current (Hoth, 1995) nor blocks Ca^{2+} flux through CRAC channels (Hoth and Penner, 1993), and it also fails to cause potentiation (Fig. 8). Thus, there appears to be a correlation between the strength of an ion's interaction with sites involved in permeation and its ability to potentiate CRAC channels. It should be noted that, at more negative potentials, Ba^{2+} is able to support current through CRAC channels (McDonald et al., 1993; Zweifach and Lewis, 1993; Hoth, 1995), but we have not tested its ability to cause potentiation at these potentials. Second, a correlation exists between the proportion of CRAC channels that are conductive under a given set of conditions and the extent of CDP. For example, in the presence of 2 mM Ca_o^{2+} , depolarization to +25 mV or above appears to shut or induce blockade of CRAC channels (Fig. 6), and at these potentials the addition of 2 mM Ca^{2+} evokes a minimal amount of CDP (Figs. 4 and 5). Hyperpolarization opens the channels and restores CDP in parallel (Figs. 5 and 6). These results raise the possibility that CRAC channels must be conductive to allow Ca^{2+} access to the site that underlies CDP. Whereas these results support the notion that the CDP site is within the channel pore, further experiments will be needed to identify its location definitively.

CDP is due to an increase in channel activity. Using the ex-

periment of fast inactivation during hyperpolarizing pulses to gauge the single-channel CRAC current (Zweifach and Lewis, 1995a), we found that the unitary CRAC current is constant as the whole-cell current increases due to CDP (Fig. 3). Thus, CDP appears to involve an increase in N or P_o rather than i . If, as discussed above, CRAC channels must first open before they can become potentiated, then an increase in P_o rather than N is most likely to account for CDP, as it is difficult to imagine how binding of Ca^{2+} to a site within the pore of one channel could render another channel activatable. Regardless of the exact mechanism, CDP involves a change in CRAC channel gating rather than permeation.

Kinetic models for CDP. Several classes of models could account for the main features of CDP, including its amplitude, slow kinetics, and voltage dependence. Perhaps the simplest model assumes that Ca^{2+} is required to maintain the channel in a fully functional conformation. In this type of scheme, the time course of CDP may reflect the rate at which channels return from an inactive conformation to either an active closed or open state after the rapid binding of Ca^{2+} to the CDP site. In a second type of model, Ca^{2+} binding to the channel allosterically regulates the channel's affinity for the depletion signal, in which case the kinetics of CDP arise from the slow interaction of the depletion signal with the channels ("modulated affinity" model). Finally, in a third class of model, extracellular Ca^{2+} binds to the open state of the channel to prolong openings, and the slow kinetics of CDP derive from slow transitions between closed states and the first open state ("modulated opening" model). In each of these models, the voltage dependence of CDP can be explained by assuming that the binding affinity of Ca^{2+} is exponentially dependent on voltage, a consequence of its binding to a site within the membrane electric field. The inability to record currents through single CRAC channels because of their extremely small size (<10 fA; Zweifach and Lewis, 1993) and the lack of information about the depletion signal itself severely limit attempts to distinguish among such models.

Implications of CDP

CDP is likely to influence the behavior of CRAC channels in several significant ways. First, CDP acts to increase the apparent selectivity of I_{CRAC} for Ca^{2+} over other divalent cations and monovalent cations when measured under steady-state conditions. Because Ba^{2+} appears not to support potentiation of CRAC channels (Fig. 8), replacement of extracellular Ca^{2+} with Ba^{2+} results in channel closure. Therefore, the decreased steady-state current commonly seen in the presence of

Ba²⁺ (Hoth and Penner, 1992; Zweifach and Lewis, 1993; Premack et al., 1994; Hoth, 1995) reflects not only the ionic selectivity of CRAC channels but also the effects of Ba²⁺ on channel gating. A recent reexamination of Ba²⁺ permeation using rapid solution changes has shown that CRAC channels are in fact more permeant to Ba²⁺ than was previously concluded based on steady-state measurements (Hoth, 1995). CDP also contributes to the steady-state Ca²⁺ dependence of I_{CRAC} ; as $[\text{Ca}^{2+}]_o$ is lowered, I_{CRAC} can decline because of channel depotentiation as well as a reduction in unitary current. As shown in Fig. 5 C, this effect will be more pronounced at depolarized potentials. The extent to which CDP has influenced the previously reported permeation properties of CRAC channels is unclear. To investigate CRAC channel permeation separately from the confounding effects of CDP, it will be necessary to make non-steady-state measurements with rapid solution changes or to use a negative holding potential at which CDP remains maximal for the course of the experiment.

CDP also confers a complex voltage and time dependence on CRAC channel activity that may shape the dynamics of I_{CRAC} in two ways. If CRAC channels can only become potentiated after the generation of the store depletion signal, then CDP would limit the rate of CRAC channel activation. This type of mechanism is described by the modulating opening model presented above, in which CDP occurs through binding of Ca²⁺ to an open state of the channel. Even though CRAC channels appear to be fully potentiated under physiological conditions (~ -60 mV, 2 mM Ca_o²⁺; see Fig. 5), our experiments were conducted using cells with predepleted stores. Thus, we cannot ascertain from these studies whether CRAC channels can become potentiated before store depletion. If CDP occurs only after store depletion, it will impose a lower limit of several seconds on the time constant of CRAC channel activation. In-

terestingly, the fastest published time constant for I_{CRAC} activation is ~ 3 s, in mast cells dialyzed with a supra-maximal level of intracellular IP₃ to empty stores quickly (Hoth and Penner, 1993).

CDP may also limit the reopening rate of CRAC channels after transient depolarizations. For example, depolarization to 0 mV would be expected to reduce channel activity by $\sim 50\%$ because of depotentiation (Fig. 5 A). As this depolarization would also reduce the open-channel current by $\sim 85\%$ (Fig. 1 C), the effect of CDP on Ca²⁺ influx would be relatively minor. However, by limiting the rate at which CRAC channels repotentiate upon repolarization, CDP could exert a substantial effect on the time course and magnitude of I_{CRAC} during voltage fluctuations. Thus, even relatively brief depolarizations might exert long-lived effects on Ca²⁺ entry after repolarization.

In T cells, $[\text{Ca}^{2+}]_i$ oscillations induced through stimulation of the T cell receptor or through partial depletion of Ca²⁺ stores appear to be generated by periodic fluctuations in I_{CRAC} (Lewis and Cahalan, 1989; Dolmetsch and Lewis, 1994). To explain these oscillations, we have proposed a mechanism which depends critically upon lags between changes in store content and I_{CRAC} activity (Dolmetsch and Lewis, 1994). Two types of slow Ca²⁺-dependent inactivation may generate the delay between the rise in $[\text{Ca}^{2+}]_i$ and the decline of I_{CRAC} required by the model (Lewis et al., 1996). As discussed above, CDP may contribute to the requisite lags in channel activation by slowing the initial activation of CRAC channels by the depletion signal and by slowing channel reopening after periodic hyperpolarizations.

Note added in proof: Since submission of the revised manuscript, we received notice of a paper in press describing the calcium-dependent enhancement of I_{CRAC} in Jurkat cells (Christian, E.P., K.T. Spence, J.A. Togo, P.G. Dargis, and J. Patel. 1996. *J. Membr. Biol.* In press).

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