In many nonexcitable cells, depletion of the inositol 1,4,5-trisphosphate-sensitive store activates Ca\(^{2+}\) influx, a process termed store-operated Ca\(^{2+}\) entry. In rat basophilic leukemia cells, emptying of the stores activates a highly selective Ca\(^{2+}\)-release-activated Ca\(^{2+}\) current (CRAC), \(I_{\text{CRAC}}\). We have recently found that \(I_{\text{CRAC}}\) activates in an essentially all-or-none manner when the current is evoked by receptor stimulation, dialysis with inositol 1,4,5-trisphosphate via the patch pipette, or through the Ca\(^{2+}\)-ATPase inhibitor thapsigargin (Parekh, A. B., Fleig, A., and Penner, R. (1997) Cell 89, 973-980). Regulatory mechanisms must therefore operate to control the overall amount of Ca\(^{2+}\) that enters through CRAC channels. Such mechanisms include membrane potential and protein kinase C. In the present study, we have investigated additional inhibitory pathways that serve to determine just how much Ca\(^{2+}\) can enter through \(I_{\text{CRAC}}\). We have directly measured the current using the whole cell patch clamp technique. We report the presence of a slow Ca\(^{2+}\)-dependent inactivation mechanism that curtails Ca\(^{2+}\) entry through CRAC channels. This inactivation mechanism is switched on by Ca\(^{2+}\) entering through CRAC channels, and therefore constitutes a slow negative feedback process. Although it requires a rise in intracellular Ca\(^{2+}\) for activation, it maintains CRAC channels inactive even under conditions that lower intracellular Ca\(^{2+}\) levels. The inactivation mechanism does not involve store refilling, protein phosphorylation, G proteins, nor Ca\(^{2+}\)-dependent enzymes. It accounts for up to 70% of the total inactivation of \(I_{\text{CRAC}}\) and therefore appears to be a dominant inhibitory mechanism. It is likely to be an important factor that shapes the profile of the Ca\(^{2+}\) signal in these nonexcitable cells.

In many nonexcitable cells, depletion of the inositol 1,4,5-trisphosphate (InsP\(_3\))-sensitive intracellular Ca\(^{2+}\) stores activates a Ca\(^{2+}\) influx pathway in the plasma membrane (1). This mechanism was originally proposed by Putney (2) and called capacitative Ca\(^{2+}\) influx. Patch-clamp experiments have identified a variety of Ca\(^{2+}\)-permeable channels in the plasma membrane that seem to underlie capacitative Ca\(^{2+}\) influx (reviewed in Ref. 3). These channels differ in their biophysical properties and are generally referred to as store-operated Ca\(^{2+}\) channels (3, 4).

Of the store-operated Ca\(^{2+}\) currents, the best characterized is \(I_{\text{CRAC}}\), which was originally discovered in mast cells (5). \(I_{\text{CRAC}}\) has subsequently been shown to exist in several different nonexcitable cells including basophils, T cells and megakaryocytes (3). CRAC channels are remarkably selective for Ca\(^{2+}\) ions and have a low single-channel conductance (3, 5).

Just how depletion of the stores activates CRAC channels is still unclear. Several potential mechanisms have been proposed but the signal has not been unequivocally identified (reviewed in Refs. 3 and 4). One interesting aspect of \(I_{\text{CRAC}}\) is that the current activates in an essentially all-or-none manner, irrespective of whether activation is evoked by dialysis with inositol 1,4,5-trisphosphate, receptor stimulation, or thapsigargin (6). One consequence of this is that, if \(I_{\text{CRAC}}\) activates in an all-or-none manner, mechanisms must exist that control the amount of Ca\(^{2+}\) entering the cell through CRAC channels. This is required in order to achieve graded activation of Ca\(^{2+}\)-dependent processes like secretion that correlate with the level of cell stimulation by receptors. We have recently reported that Ca\(^{2+}\) entry through CRAC channels in rat basophilic leukemia (RBL) cells can be graded, despite all-or-none activation, because of several regulatory mechanisms that serve to control CRAC channel activity (6, 7). One way is by changing the membrane potential. Hyperpolarization increases the electrical gradient for Ca\(^{2+}\) entry, thus favoring further Ca\(^{2+}\) influx, whereas depolarization decreases the driving force and hence reduces Ca\(^{2+}\) entry (6).

\(I_{\text{CRAC}}\) is also regulated by protein kinase C. Stimulation of this enzyme inactivates \(I_{\text{CRAC}}\). Since protein kinase C will be activated by diacylglycerol, which is produced following stimulation of receptors that engage the phosphoinositide pathway, it constitutes an important negative feedback mechanism on Ca\(^{2+}\) influx (7).

In mast cells and jurkat T lymphocytes, \(I_{\text{CRAC}}\) is subjected to a fast inactivation process operating on a milliseconds time scale. This arises from Ca\(^{2+}\) ions entering the cell through CRAC channels and then binding to sites probably located on the channels themselves (5, 8).

Here we report an additional mechanism that serves to regulate CRAC channels. We find that Ca\(^{2+}\) influx through CRAC channels exerts a slow feedback inhibition that curtails further Ca\(^{2+}\) entry and which is dependent on a rise in intracellular Ca\(^{2+}\) levels. Slow inactivation accounts for up to 70% of the inhibition of \(I_{\text{CRAC}}\). Once activated, this slow inactivation mechanism can maintain CRAC channels in an inactivated state for several minutes, even after intracellular Ca\(^{2+}\) levels have been reduced. Hence slow inactivation appears to be a dominant inhibitory mechanism that determines the time span of Ca\(^{2+}\) entry through CRAC channels.
Calcium-dependent Inactivation of Calcium Entry

**RESULTS**

**Slow Inactivation of I_{CRAC} in the Presence of Moderate Intracellular Calcium Buffering**—Previous work has demonstrated that $I_{CRAC}$ inactivates partially when RBL cells are dialyzed with a patch pipette solution containing a high concentration of the slow Ca$^{2+}$ chelator EGTA (10 mM), and this is due to a kinase-mediated phosphorylation (7). In the present study, we have examined the effects of more moderate calcium buffering on the properties of $I_{CRAC}$ by including 1.4 mM EGTA in the recording pipette. Fig. 1A(i) shows an experiment in which the internal solution contained 30 $\mu$M InsP$_3$ (a supramaximal concentration) and 1.4 mM EGTA. Cells were voltage clamped at 0 mV and $I_{CRAC}$ was monitored using voltage ramps applied every two seconds (shown in Fig. 1A(ii)). The current was measured at −80 mV. Fig. 1A(ii) depicts the time course of the current during the experiment. Following the onset of whole-cell recording, $I_{CRAC}$ activated as InsP$_3$ diffused into the cell from the recording pipette and depleted the stores. The time constant for activation ($\tau$) was 15.2 ± 1.6 s, similar to our previous measurements (19.4 ± 1.2 s) (9). The current peaked after 50–80 s and then inactivated substantially with time. For the cell shown in Fig. 1A(i), the current had fallen by 70% (relative to the peak amplitude) after 300 s. Fig. 1A(ii) shows after breaking in and then subtracting this from all subsequent traces. Several parameters (capacitance, series resistance, holding current) were displayed simultaneously on a second monitor at a slower rate (2 Hz) using the X-Chart display (HEKA Electronics). Data are presented as mean ± S.E., and statistical evaluation was carried out using Student's unpaired t test.

**EXPERIMENTAL PROCEDURES**

Rat basophilic leukemia cells (RBL-2H3) cells were kindly supplied by Michael Pilot, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, and were cultured essentially as described previously (6, 7). Patch-clamp experiments were conducted in the tight seal whole cell configuration at room temperature (18–25 °C) as described previously (6, 7). Patch pipettes were pulled from borosilicate glass (Hilgenberg), sylgard-coated, and fire-polished. Pipettes had d.c. resistances of 2.5–4 megohms when filled with standard internal solution that contained (in mM): cesium glutamate 145, NaCl 8, MgCl$_2$ 1, MgATP 1, InsP$_3$ 0.03, EGTA 1.4, HEPES 10, pH 7.2, with CsOH. A correction of +10 mV was applied for the subsequent liquid junction potential. In some experiments, the EGTA concentration was raised to 14 mM (indicated in text) or substituted with BAPTA. Drugs were added to this internal solution as described in the text. Extracellular solution contained (in mM): NaCl 145, KCl 2.8, CaCl$_2$ 10, MgCl$_2$ 2, CsCl 10, glucose 10, HEPES 10, pH 7.2 (NaOH). CsCl was present to block the activity of the inwardly rectifying potassium channel. High resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA Electronics, Germany). Capacitative currents were canceled before each voltage ramp using the automatic compensation of the EPC-9. Series resistance was between 5 and 15 megohms. Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitized at 100 $\mu$s. $I_{CRAC}$ was measured using either voltage ramps (−100 to +100 mV in 50 ms) or voltage steps (pulses to −80 mV for 200 ms) applied every 2 s using PULSE software (HEKA Electronics) on a 9500 PowerMac. Cells were held at 0 mV between pulses. All currents were leak subtracted by averaging the first two to four ramps/steps after breaking in and then subtracting this from all subsequent traces.
Calcium-dependent Inactivation of Calcium Entry

**Fig. 2. Slow inactivation of $I_{CRAC}$ is dependent on Ca$^{2+}$ influx.** A, effects of changing the electrical gradient for Ca$^{2+}$ entry on slow inactivation. In (i), reducing the driving force from $-80$ to $-40$ mV reduces slow inactivation, whereas increasing the driving force to $-120$ mV (ii) has no effect. The current recording at $-40$ mV in (i) has been scaled to match the response at $-80$ mV in peak amplitude, as has been the recording at $-80$ mV in (ii) so that it matches the response at $-120$ mV. The absolute values for the currents in (i) are $-1.6 \text{ pA/pF}$ ($-80$ mV) and $-1.1 \text{ pA/pF}$ ($-40$ mV). For (ii) they are $-1.4 \text{ pA/pF}$ ($-80$ mV) and $-3.2 \text{ pA/pF}$ ($-120$ mV). All cells in Fig. 2A were from the same coverslip. $B$, reducing the concentration gradient for Ca$^{2+}$ influx from 10 mM to 4 mM at $-80$ mV results in less inactivation. C, the fast Ca$^{2+}$ chelator BAPTA (1.4 mM) does not prevent slow inactivation from occurring (six cells). See Table I for further details.

The current measured in the ramps exhibited the classic features of $I_{CRAC}$: voltage-independent activation, inward rectification, and a reversal potential close to $+60$ mV.

We also monitored activity of $I_{CRAC}$ by applying voltage steps to $-80$ mV for 200 ms every 2 s from a holding potential of 0 mV. In Fig. 1B(i), the time course of $I_{CRAC}$ is plotted using this protocol, and Fig. 1B(ii) shows the inward current measured during these hyperpolarizing pulses. During the step, $I_{CRAC}$ inactivated by up to 40% with a time constant in the range of 50 ms (Fig. 1B(ii)). This fast inactivation has been described in detail in T cells and arises from local feedback inactivation by Ca$^{2+}$ ions in the vicinity of the channel pore (8). We measured the steady-state current during the step (indicated by the arrow in 1B(ii)), and this amplitude is plotted against time in Fig. 1B(i). As with the ramp protocol employed in Fig. 1A, $I_{CRAC}$ inactivated substantially during the experiment. For the cell shown in Fig. 1B, the current amplitude at 300 s had fallen by 68% relative to the peak value.

Fig. 1C plots the time course of $I_{CRAC}$ measured using either the ramp or step methods (six cells each). These experiments were carried out such that ramps or steps were applied to alternate cells on the same coverslip (henceforth referred to as paired experiments). There was no significant difference between ramp or step protocols with respect to $\tau_{activation}$ (15.2 ± 1.6 versus 17.7 ± 2.0 s, respectively) peak amplitude ($-2.98 ± 0.24$ versus $-2.58 ± 0.19 \text{ pA/pF}$) or in the extent of steady-state inactivation (fallen by 53.4 ± 8.9% versus 56.1 ± 9.3%, respectively, Fig. 1C).

This inactivation in the presence of moderate Ca$^{2+}$ buffering will henceforth be referred to as slow inactivation to distinguish it from the fast inactivation seen during the hyperpolarizing step.

**Variability in the Overall Extent of Slow Inactivation between Cell Preparations**—In some cells, slow inactivation was very prominent and contributed to an almost complete inactivation of $I_{CRAC}$. Typical examples of this type of response are shown in Figs. 3B and 5A. In some other cells, slow inactivation contributed to around a 50% decrease in the amplitude of $I_{CRAC}$ (e.g. Fig. 5C). Overall, there was little difference between preparations, with the average decrease being 64.8 ± 7.9% (n = 35, measured using the step protocol after 300 s).

In experiments where drugs were used to interfere with slow inactivation, control responses were always obtained from the same preparation and the data were compared with these controls.

**Slow Inactivation Is Reduced by a Higher EGTA Concentration in the Recording Pipette**—To confirm that slow inactivation is dependent on a rise in intracellular Ca$^{2+}$ levels, we dialyzed cells with a pipette solution in which the EGTA concentration had been increased 10-fold (to 14 mM). Pooled data from 10 cells in 14 mM EGTA and eight cells in 1.4 mM EGTA are summarized in Fig. 1D (paired recordings). Slow inactivation was reduced by almost 2-fold in the presence of the higher EGTA concentration and appears therefore to require a rise in intracellular free Ca$^{2+}$ because it can be suppressed by increasing the concentration of a slow mobile Ca$^{2+}$ buffer in the cytosol. The inset of Fig. 1D shows current traces taken from the voltage steps from two cells dialysed with 1.4 and 14 mM EGTA. Fast inactivation was the same in both cells, yet slow inactivation was less pronounced in the presence of higher EGTA. Fast and slow inactivation therefore reflect distinct processes.

**Slow Inactivation Is Dependent on Ca$^{2+}$ Entry into the Cell**—To assess the contribution of Ca$^{2+}$ influx through CRAC channels to the slow Ca$^{2+}$-dependent inactivation, we carried out a series of experiments in which the electrochemical gradient for Ca$^{2+}$ influx was altered. First, we modified the electrical gradient by applying voltage pulses to potentials that altered Ca$^{2+}$ entry ($-40$ mV) or enhanced it ($-120$ mV), relative to the responses obtained on pulsing to $-80$ mV. Typical results are shown in Fig. 2A, and the data are summarized in Table I. Stepping the voltage to $-40$ mV reduced the rate and extent of inactivation almost 2-fold (Fig. 2A(i) and Table I). The current at $-40$ mV in Fig. 2A(i) has been scaled so that it has the same amplitude as that at $-80$ mV in order to clearly show the slower inactivation. Voltage pulses to $-120$ mV, however, did not increase the level of steady-state inactivation compared with that seen on stepping to $-80$ mV (Fig. 2A(ii)), where the current at $-80$ mV has been scaled, see also Table I,
which might suggest that sufficient Ca\(^{2+}\) enters at -80 mV to maximally activate the inhibitory process.

In a second set of experiments, we reduced the concentration gradient for Ca\(^{2+}\) influx, while leaving the electrical driving force unchanged. This was accomplished by lowering the external Ca\(^{2+}\) concentration in the bath to 4 mM (from 10 mM). A typical example is shown in Fig. 2B, in which the cell was hyperpolarized to -80 mV at 2-s intervals. Steady-state inactivation was less pronounced (22% for this cell, see also Table I). The fact that slow inactivation was reduced following a decrease only in the concentration gradient for Ca\(^{2+}\) entry while the electrical gradient was unchanged suggests that it is predominantly a Ca\(^{2+}\)-dependent rather than a voltage-dependent phenomenon.

### Ca\(^{2+}\)-dependent Slow Inactivation Is Not Affected by a Fast Ca\(^{2+}\) Chelator—The preceding results demonstrate that \(I_{\text{CRAC}}\) is subject to a negative feedback mechanism in which Ca\(^{2+}\) influx through CRAC channels induces a slow Ca\(^{2+}\)-dependent inactivation. Fast negative feedback inactivation of CRAC channels operates on a milliseconds time-scale and can be substantially reduced by inclusion of the fast Ca\(^{2+}\) chelator BAPTA in the recording pipette, whereas it is not altered by increasing the concentration of the slower Ca\(^{2+}\) chelator EGTA (8). Replacing EGTA with the fast chelator BAPTA (both at 1.4 mM) did not significantly affect the rate or extent of inactivation (six cells, Fig. 2C and Table I). However, rapid inactivation of \(I_{\text{CRAC}}\) was slightly slowed by this concentration of BAPTA (1.2–1.4-fold, data not shown), reinforcing the notion that the inactivation we observe is distinct from the fast inactivation process.

### Once Initiated, Slow Inactivation Is Not Strongly Dependent on Ca\(^{2+}\) Entry—Although Ca\(^{2+}\) entry is important for initiating slow inactivation, we set out to determine whether slow inactivation still required Ca\(^{2+}\) entry even after the inactivation process had started to develop. Fig. 3 describes two types of experiment which were designed to address this. In Fig. 3A, hyperpolarizing steps to -80 mV were repetitively applied every 2 s from a holding potential of 0 mV. Once inactivation had clearly developed (at 200 s), the cell was held continuously at +20 mV for 30 s. At this positive potential, very little Ca\(^{2+}\) entered through CRAC channels (6). After 30 s, the cell was held again at 0 mV and hyperpolarizing steps were resumed. The amplitude of \(I_{\text{CRAC}}\) increased only slightly relative to the level it had reached prior to clamping the cell at +20 mV (four of nine cells). In the other five cells, no recovery was observed at all.

Fig. 3B shows an experiment in which \(I_{\text{CRAC}}\) had inactivated fully by 300 s. Hyperpolarizing pulses were stopped and the cell was held at 0 mV for 120 s. Hyperpolarizing pulses were then resumed. No recovery of \(I_{\text{CRAC}}\) occurred (three of four cells). In one cell, the amplitude of \(I_{\text{CRAC}}\) recovered by 9%. Taken together, these results suggest that, once initiated, Ca\(^{2+}\)-depend-

### Table I

| Treatment          | Normalized \(I_{\text{max}}\) \(\mu A/\mu F\) | Steady-state current \% peak | \(t_{\text{activation}}\) s | No. of cells |
|--------------------|---------------------------------------------|-----------------------------|-----------------------------|--------------|
| 1.4 BAPTA, -80 mV  | -1.90 ± 0.17                               | 48.9 ± 8.6                  | 17.6 ± 1.2                  | 6            |
| 1.4 EGTA, -40 mV   | -1.20 ± 0.07                                | 65.3 ± 3.8                  | 18.6 ± 4.4                  | 4            |
| 1.4 EGTA, -80 mV   | -1.60 ± 0.05                                | 35.7 ± 6.7                  | 16.8 ± 1.8                  | 4            |
| 1.4 EGTA, -120 mV  | -2.81 ± 0.30                                | 42.0 ± 5.8                  | 13.9 ± 1.7                  | 4            |
| 1.4 EGTA, 4 Ca\(^{2+}\), -80 mV | -1.20 ± 0.17 | 62.0 ± 6.5 | 18.6 ± 4.1 | 3            |

**Calcium-dependent Inactivation of Calcium Entry**

**Effect of changing the electrochemical gradient for calcium entry on slow inactivation**

In all experiments, 30 \(\mu\)M InsP\(_3\) was included in the recording pipette. 1.4 mM BAPTA/EGTA refers to 1.4 mM of total chelator. The voltage represents the potential to which the cells were stepped. All experiments were carried out in 10 mM external Ca\(^{2+}\), except the last row in which the bath solution contained 4 mM instead. The difference in steady-state current between 1.4 EGTA, -40 mV and 1.4 EGTA, -80 mV was significant (\(p < 0.02\)), whereas the difference between 1.4 EGTA, -80 mV and 1.4 EGTA, -120 mV was not (\(p > 0.4\)). The difference between 1.4 EGTA, -80 mV and 1.4 BAPTA, -80 mV was also not significant (\(p > 0.2\)). There were no significant differences between the \(t_{\text{activation}}\) values.

**Investigation into the Mechanism of Ca\(^{2+}\)-dependent Slow Inactivation**—The following set of experiments were aimed at elucidating the molecular mechanism that gave rise to the slow inactivation of \(I_{\text{CRAC}}\).

**Refilling of Intracellular Ca\(^{2+}\) Stores**—In the presence of moderate concentrations of EGTA, it is conceivable that Ca\(^{2+}\) entry through CRAC channels might enable the intracellular Ca\(^{2+}\) stores to refill, a process that would turn off \(I_{\text{CRAC}}\). If such a mechanism were responsible for the inactivation of \(I_{\text{CRAC}}\), then one would predict that maneuvers directed toward reducing Ca\(^{2+}\) uptake into the stores should prevent slow inactivation from occurring. Thapsigargin is a specific inhibitor of the Ca\(^{2+}\)-ATPase on the endoplasmic reticulum and prevents store refilling (10). We therefore carried out paired recordings in which six control cells were dialyzed with internal solution containing InsP\(_3\) and 1.4 mM EGTA, whereas six other cells from the same preparations were dialyzed with this solution supplemented with 2 \(\mu\)M thapsigargin. Fig. 4A shows the averaged results. Slow inactivation was only slightly reduced by thapsigargin, but this was not statistically significant. Thapsigargin is lipophilic, so it is possible that the drug diffuses out of the cell into the bath solution. Because we included a high concentration of thapsigargin in the pipette (2 \(\mu\)M), one would be surprised if the steady-state cytoplasmic concentration was less than a few hundred nanomolar, a concentration that is sufficient to reduce Ca\(^{2+}\)-ATPase activity in a variety of cell types (10). In two cells, we applied thapsigargin (1 \(\mu\)M) from the outside just after the onset of \(I_{\text{CRAC}}\) (thapsigargin was also included in the recording pipette). The current still inactivated (by 53 and 61%). Nevertheless, we sought additional ways to probe the effects of compromised store refilling on slow inactivation. One method would be to employ the Ca\(^{2+}\) ionophore ionomycin. Ionomycin increases the permeability of the store membrane to Ca\(^{2+}\), and this would enable any Ca\(^{2+}\) that had been pumped into the stores to diffuse back into the cytosol thereby preventing stores from refilling. We included 2 \(\mu\)M ionomycin in the recording pipette together with InsP\(_3\) and 1.4 mM EGTA. Fig. 4B shows the effects of ionomycin. \(I_{\text{CRAC}}\) activated slightly faster in ionomycin-treated cells (7 of 16.1 ± 1.7 s versus 22.7 ± 3.0 s in control paired cells), which might indicate that ionomycin is diffusing into the cells rather quickly and accelerating store depletion in combination with InsP\(_3\). Slow inactivation of \(I_{\text{CRAC}}\) was still apparent in the presence of ionomycin, and the current declined to a value similar to that seen in the absence of ionomycin in paired recordings (six cells for ionomycin, five for controls).

**Protein Kinase-mediated Phosphorylation**—We tested the possible involvement of a kinase-mediated phosphorylation reaction in the Ca\(^{2+}\)-dependent slow inactivation in RBL cells in two independent ways. First, we examined the effects of remov-
ing ATP from the pipette solution and then replacing it with ATPγS, and second, we tested the effects of broad kinase and phosphatase inhibitors on the inactivation process.

Slow inactivation was still present when ATP was omitted from the pipette solution (three cells, not shown), suggesting that global ATP levels are probably not important for the inactivation process. We then replaced ATP with ATPγS in the pipette solution. ATPγS is a nonhydrolyzable analogue of ATP that is readily used by a variety of protein kinases resulting in “irreversible phosphorylation” of the target (11). Slow inactivation was slightly increased by ATPγS (Table II). Importantly, it was not reduced, which one might have expected if recovery from inactivation required a phosphorylation (12).

Dialysis of cells with H-7 (200 μM) did not affect slow inactivation compared with control cells in which the inhibitor was not present. A control response and one in which H-7 was present are shown in Fig. 5A. Both cells were from the same coverslip. Although H-7 seemed to slow the rate of inactivation in these paired recordings, in other cells control responses decayed at a slower rate than H-7 treated ones. The data from several cells are summarized in Table II. There was no significant difference in inactivation between H-7-treated and control cells. In three cells that were dialyzed with H-7, we stopped the application of hyperpolarizing steps after 300 s and waited

**FIG. 3.** Once initiated, slow inactivation does not require continuous Ca²⁺ entry. A, the development of slow inactivation was monitored using hyperpolarizing steps to −80 mV applied every 2 s. Once inactivation had clearly developed, the cell was held at +20 mV for 30 s to reduce further Ca²⁺ influx into the cytoplasm. No hyperpolarizing steps were given during this time. After 30 s, the pulses were resumed. Little recovery of ICrAc occurred relative to the levels it had shown prior to reducing Ca²⁺ influx. In B, slow inactivation completely turned off ICrAc. Hyperpolarizing steps were stopped and the cell was held at 0 mV for 2 min. Resumption of the steps did not result in any recovery of the current.

**FIG. 4.** Slow inactivation occurs even when store refilling is compromised. Dialysis with InsP₃ and 2 μM thapsigargin (A) or InsP₃ and 2 μM ionomycin (B) did not prevent slow inactivation from occurring relative to control responses taken from the same coverslips in the absence of these drugs (p > 0.1 and 0.3, respectively).
Ca2+-dependent Inactivation of Calcium Entry

TABLE II
Effects of various treatments on slow inactivation

| Treatment          | Normalized Imax | Steady-state current | τactivation | N of cells |
|--------------------|-----------------|----------------------|-------------|------------|
| ATPβS              | -1.80 ± 0.40    | 29.5 ± 4.2           | 13.5 ± 2.4  | 4          |
| H-7                | 1.77 ± 0.15     | 28.4 ± 8.0           | 14.7 ± 1.4  | 6          |
| Bisindolylmaleimide| -5.0 ± 0.13     | 54.1 ± 6.0           | 17.8 ± 1.9  | 6          |
| Okadaic acid       | -1.13 ± 0.30    | 38.3 ± 12.5          | 19.2 ± 1.2  | 4          |
| GDPβS              | -1.86 ± 2.01    | 39.7 ± 10.2          | 22.5 ± 2.5  | 4          |
| Calpeptin          | -1.34 ± 0.19    | 34.0 ± 9.3           | 16.0 ± 1.0  | 5          |

FIG. 5. Slow inactivation is not affected by a variety of intracellular signaling pathways. A, dialysis with the broad kinase inhibitor H-7 (200 μM) did not affect slow inactivation relative to a control cell from the same coverslip. See also Table II. B, preincubation for 30 min with the broad phosphatase inhibitor okadaic acid (1 μM) was ineffective on slow inactivation. Okadaic acid was also included in the recording pipette. Okadaic acid enhanced Ca2+ influx in Xenopus oocytes after application of lysophosphatidic acid (n = 2), as described in Petersen and Berridge (19). Hence okadaic acid was active. C, dialysis with the nonhydrolyzable GDP analogue GDPβS (300 μM) did not interfere with slow inactivation relative to control cells. GDPβS prevented activation of ICRACh by the adenosine receptor agonist NECA in two cells, indicating it was active (7). D, pretreatment with the Ca2+-activated protease inhibitor calpeptin (2 μM), as well as its inclusion in the recording pipette, failed to alter slow inactivation.

2–4 min at a holding potential of 0 mV. On resumption of the hyperpolarizing pulses, no recovery of ICRACh was found.

The protein phosphatase inhibitor okadaic acid has been reported to inhibit slow inactivation of ICRACh in Jurkat T cells (13). We therefore pretreated RBL cells for at least 20 min with 1 μM okadaic acid and then included the same concentration of the inhibitor in the pipette solution. Slow inactivation still occurred, demonstrating that it was not sensitive to okadaic acid (Fig. 5B and Table II).

GTP-dependent Proteins and Ca2+-activated Proteases—To assess the role of G proteins, we included 300 μM GDPβS in the recording pipette together with InsP3. Slow inactivation still occurred (Fig. 5C and Table II) and it was not significantly different from control cells. Dialysis with 250 μM GTPβS did not affect slow inactivation either (inactivation of 77 ± 9%). Later on in these same cells (after a 5–10-min recording), the GTPβS-dependent Na+ current activated (14), demonstrating that the GTPβS was active under these conditions.

To examine whether a Ca2+-dependent protease was responsible for slow inactivation, as is the case for voltage-gated calcium channels (15), we preincubated cells for 30 min with 2 μM calpeptin, a potent Ca2+-dependent protease inhibitor. In some experiments, calpeptin was also added to the internal solution. Slow inactivation still occurred (Fig. 5D and Table II), and this was indistinguishable from control cells taken from different coverslips from the same preparations.

Because of the variability in the kinetics of slow inactivation, small effects by the agents used would not be detected. Although the findings therefore do not rule out a contribution from these signaling pathways, they demonstrate that these mechanisms are not dominant.

DISCUSSION

Negative Feedback by Ca2+ Ions Entering through CRAC Channels Gives Rise to Slow Inactivation—Our observation that slow inactivation could be significantly reduced by increasing the concentration of the Ca2+ chelator EGTA in the patch pipette solution (Fig. 1D) suggests that the inactivation mechanism requires a rise in cytoplasmic Ca2+ concentration. This rise in Ca2+ is accomplished by Ca2+ entry through CRAC channels because maneuvers that reduce the electrochemical gradient for Ca2+ influx result in less inactivation (Fig. 2).

It is likely that Ca2+ entry is the main source of Ca2+ for activating the slow inhibitory pathway with little contribution from Ca2+ release from the stores (e.g. Ca2+ entry evoked Ca2+ release) for the following reasons. First, dialysis with InsP3 and either ionomycin or thapsigargin, agents that would reduce the Ca2+ content of the stores, did not alter slow inactivation. Second, cells were dialyzed with a supramaximal concentration of InsP3 which is likely to maintain stores in a Ca2+-depleted state (6). Although the InsP3 receptor might desensitize, thereby enabling stores to refill, we feel this process is not particularly active under our conditions. The InsP3 receptor that is involved in the activation of ICRACh in RBL cells does not seem to desensitize much because Ca2+ influx can still be activated more than 10 min after dialyzing with high InsP3 levels (6). Finally, slow inactivation still occurs in the presence
of 2 mM ATPγS and no exogenous ATP (Table II). Ca2+ pumps cannot use ATPγS as a substrate and therefore will be much less effective in taking up Ca2+ to replenish the stores.

Because an increase in the concentration of a slow Ca2+ chelator like EGTA can reduce inactivation (Fig. 1D), and that there is little difference between BAPTA (a fast chelator) and EGTA (Fig. 2C), the Ca2+-dependent inactivation step seems to have slow kinetics. Furthermore, Ca2+ buffering, rather than the speed of binding, is important in determining the rate and extent of the slow inactivation.

Mechanism of Slow Inactivation—Just how a rise in intracellular Ca2+ switches on the slow inactivation process is not clear. It is unlikely to reflect refilling of the stores because inclusion of thapsigargin or ionomycin in the pipette solution did not prevent the inactivation from occurring. Direct involvement of the Ca2+-binding protein calmodulin in the inactivation process is also unlikely because inclusion of the specific calmodulin inhibitory peptide fragment from Calbiochem failed to prevent slow inactivation (three cells, not shown).

One important clue to the mechanism of slow inactivation was the finding that, once activated, it did not require the continuous presence of elevated Ca2+ (Fig. 3). Such long-lasting effects are often mediated by protein phosphorylation reactions. However, several treatments designed to interfere with phosphorylation failed to affect slow inactivation of ICRACh. Involvement of Ca2+-activated proteases was also unlikely since a specific and potent inhibitor of this class of enzyme did not alter inactivation. It also seems unlikely that CRAC channels are being endocytosed slowly from the membrane in a Ca2+-dependent manner, because membrane capacitance did not change despite substantial inactivation of the Ca2+ current (data not shown). Finally, during whole cell recording small soluble components diffuse out of the cell and this washout may cause a general loss of CRAC channel activity. The fact that increasing the concentration of EGTA in the recording pipette reduced slow inactivation suggests that loss of cytoplasmic factors might not explain the inactivation. However, we cannot rule out the possibility that a rise in intracellular Ca2+ accelerates washout of an important component, perhaps by promoting its Ca2+-dependent dissociation from a bound to a diffusible form.

A slow Ca2+-dependent inactivation of store-operated Ca2+ influx has been described in jurkat T cells (13) and NIH-3T3 fibroblasts (16). In T cells, it was reported that slow inactivation was inhibited by okadaic acid and by the kinase inhibitor H-7 (11, 13). It was concluded that recovery from slow inactivation occurred through the actions of a protein kinase. In NIH-3T3 cells, Ca2+ influx was measured using the fluorescent dye fura-2. Unlike T cells, slow inactivation was not affected by kinase or phosphatase inhibitors nor by Ca2+ chelators (loaded via the membrane-permeable acetoxymethyl ester form). Slow inactivation was suggested to arise from an action of Ca2+ not subject to Ca2+ chelation, perhaps by binding to an external part of the channel. The slow inactivation pathway described in this report exhibits some notable differences from these other inactivation ones. Unlike T cells, it was not sensitive to H-7 or okadaic acid, nor to conditions designed to facilitate protein phosphorylation. Slow inactivation was reduced by increasing the concentration of Ca2+ chelator in the recording pipette, demonstrating an intracellular action of Ca2+ rather than an extracellular site as proposed for the NIH-3T3 cells (16). Ca2+-dependent slow inactivation of ICRACh might therefore arise through different mechanisms in different nonexcitable cells.

Comparison of Slow Inactivation with Other Inhibitory Mechanisms That Regulate ICRACh—Several inhibitory pathways have been found to regulate CRAC channels in RBL cells, and it might be instructive to ascertain their relative contributions to controlling Ca2+ entry. ICRACh is subject to a fast inactivation process (8). The peak current declines by up to 40% following a hyperpolarization to −80 mV (Fig. 1B). Because we have measured ICRACh at steady state (i.e. once the rapid inactivation component was over, Fig. 1B), fast inactivation is not contributing to the inactivation that we have measured and is therefore not relevant to the discussion that follows.

In the presence of high EGTA in the recording pipette, ICRACh is also subject to a slow inactivation pathway that involves protein phosphorylation, probably through protein kinase C (7). Because this protein kinase-mediated inactivation is dependent on ATP in the recording pipette and is sensitive to protein kinase C blockers, it occurs through a mechanism distinct to that involved in the Ca2+-dependent slow inactivation described in this report. The kinase-mediated action accounts for up to 30% (Fig. 1D) and 45% of the inactivation (7).

Since depletion of stores activates ICRACh, one might expect that refilling of stores turns the current off. Using fluorescent dyes, it has been reported that the duration of Ca2+ influx correlates with the Ca2+ uptake into the stores, the latter being assessed through the extent of Ca2+ release by an agonist in Ca2+-free solution (17). To date, only one study has suggested that store refilling turns off ICRACh. In T cells, Zweifach and Lewis (13) reported that ICRACh fully inactivated within 100 s in the presence of a moderate EGTA concentration (1.2 mM) and the inactivation could be partially reduced by thapsigargin. In our experiments, we have found no evidence that store refilling contributes to inactivation of ICRACh (Fig. 4), despite dialyzing cells with a similar concentration of EGTA to that used in T cells (1.2 versus 1.4 mM). The reason why store refilling contributes significantly to slow inactivation in T cells whereas it seems to play little, if any, role in RBL cells under similar conditions is unclear. Differences in current density are unlikely to explain this because increasing the RBL cell ICRACh by stepping to −120 mV, Fig. 2B) did not enhance slow inactivation, despite the current now exhibiting a similar current density to that in the T cells. It is conceivable that local saturation of the Ca2+ buffers might contribute to these differences in the role of store refilling, especially if stores were situated close to such areas of buffer depletion. It is interesting to note that InsP3-sensitive stores are physically adjacent to the plasma membrane in T cells (18). Ca2+ buffers just below the plasma membrane are likely to be saturated following activation of ICRACh, thereby facilitating the refilling process. We have suggested previously that the InsP3-sensitive stores involved in activation of ICRACh in RBL cells are close to the plasma membrane (9), but no morphological data is available to indicate just how close they are. Finally, Ca2+ATPase activity might differ between jurkat T and RBL cells in whole cell recording, such that Ca2+ uptake is less effective in the RBL cells. Clearly, further work is needed to assess the contribution of refilling to the deactivation of ICRACh in RBL cells.

In this report, we have found a new Ca2+-dependent negative feedback inactivation pathway that turns off ICRACh. In the presence of moderate Ca2+ buffering (1.4 mM EGTA), ICRACh inactivates by between 50 and 100%. With high buffering (14 mM EGTA), inactivation is reduced to 30% (Fig. 1D). Hence the slow Ca2+-dependent inactivation accounts for between 20 and 70% of the inactivation of ICRACh and operates on a time scale of tens of seconds. Although it is activated by Ca2+ entry through CRAC channels, it does not require the continuous presence of elevated Ca2+ (Fig. 3). Slow inactivation is therefore endowed with the important property of maintaining CRAC channels inactive, even when the signal that switches on the inactivation pathway (a rise in Ca2+) has been removed. Variable levels of
activation of this slow inhibitory pathway will lead to different amounts of Ca\(^{2+}\) entry through CRAC channels, thereby providing a further mechanism for generating graded Ca\(^{2+}\) influx despite all-or-none activation of \(I_{\text{CRAC}}\). This feature, as well as its slow time course, suggests that slow inactivation plays an important role in shaping the profile of the Ca\(^{2+}\) signal, and hence it will be an important factor that regulates slow Ca\(^{2+}\)-dependent processes like exocytosis and gene transcription in nonexcitable cells.

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