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Fast Calcium-Dependent Inactivation of Calcium Release-Activated Calcium Current (CRAC) in RBL-1 Cells

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Abstract. Fast inactivation of the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) was studied using whole cell patch-clamp recordings in rat basophilic leukemia (RBL-1) cells. Application of hyperpolarizing voltage steps from the holding potential of 0 mV revealed that I_{CRAC} declined in amplitude over tens of milliseconds during steps more negative than −40 mV. This fast inactivation was predominantly Ca^{2+}-dependent because first, it could be more effectively suppressed when BAPTA was included in the recording pipette instead of EGTA and second, replacing external Ca^{2+} with Sr^{2+} resulted in less inactivation. Recovery from inactivation was faster in the presence of BAPTA than EGTA. The extent of fast inactivation was independent of the whole cell I_{CRAC} amplitude, compatible with the notion that the inactivation arose from a local feedback inhibition by permeating Ca^{2+} ions only on the channel it permeated. Ca^{2+} release from stores did not affect fast inactivation, nor did FCεRI receptor stimulation. Current clamp recordings showed that the majority of RBL cells had a membrane potential close to −90 mV following stimulation of FCεRI receptors. Hence fast inactivation is likely to impact on the extent of Ca^{2+} influx through CRAC channels under physiological conditions and appears to be an important negative feedback process that limits Ca^{2+} increases.

Introduction

It has now been established that the major Ca^{2+} entry pathway in nonexcitable cells is activated by the emptying of the intracellular Ca^{2+} store, a process that has been dubbed capacitative calcium entry (Putney, 1986). In mast cells, jurkat T-lymphocytes and rat basophilic leukemia cells, capacitative Ca^{2+} entry is manifested through a highly selective Ca^{2+} current called I_{CRAC} (reviewed in Parekh & Penner, 1997). Hallmarks of I_{CRAC} include its' voltage-independence, high selectivity for Ca^{2+} over monovalent cations and very low single-channel conductance (<1 pS).

In order to fine-tune Ca^{2+} entry, cells are endowed with mechanisms that regulate the activity of CRAC channels. In rat basophilic leukemia (RBL) cells, which are a model system for studying store-operated Ca^{2+} influx mainly because large I_{CRAC} can be measured, several processes exist that limit the duration of the current. These include changes in electrical driving force through fluctuations in membrane potential (Parekh, Fleig & Penner, 1997), feedback inactivation by protein kinase C (Parekh & Penner, 1995), store refilling (Parekh, *in preparation*) and an intracellular Ca^{2+}-dependent but store-independent mechanism (Parekh, 1998). These pathways all operate over a time-scale of tens of seconds to minutes.

In mast cells and jurkat T-cells, a rapid inactivation mechanism has been reported that operates on a time-scale of tens of milliseconds (Hoth & Penner, 1993; Zweifach & Lewis, 1995). This has been characterized in detail in T-cells, and arises through a negative feedback mechanism triggered by permeating Ca^{2+} ions. The Ca^{2+} ions that permeate each channel induce rapid inactivation only in that particular channel, and this is accomplished by binding to a site estimated to be within 4 nm of the intracellular mouth of the pore (Zweifach & Lewis, 1995).

In RBL cells, I_{CRAC} has been found to decline in amplitude during a hyperpolarizing voltage pulse (Hoth, 1995). However, a detailed analysis of rapid inactivation has not been carried out for any other cell-type that expresses I_{CRAC} other than jurkat T-cells. This may be particularly relevant because it has been suggested that there may be specialized channel subtypes within a

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CRAC superfamily (Hoth, 1995). Hence extrapolation of basic features of I_{CRAC} from one cell-type to another may not be valid.

Continuing our examination of feedback processes that operate on I_{CRAC}, we have now investigated the properties of fast inactivation in RBL cells. We find that this inactivation mechanism shares some striking similarities to that already characterized in jurkat T-cells, but there are some notable differences. By directly measuring the resting membrane potential, we find that the majority of the cells are sufficiently hyperpolarized to support fast inactivation. We have also examined the effects of redox potential on I_{CRAC}, since inactivation of certain types of K^+ channel is affected by cysteine oxidation. We find that neither the activation mechanism nor the fast inactivation process are affected by redox potential. Finally we present the first report of the effects of different divalent cations on the activation mechanism of I_{CRAC}. The rate of activation of the current depends on the charge carrying species.

Materials and Methods

Rat basophilic leukemia cells (RBL-1) cells, which were kindly provided by Michael Pilot from the Max Planck Institute for Biophysical Chemistry in Goettingen, Germany, were cultured essentially as previously described (Parekh, Fleig & Penner, 1997). Briefly, cells were maintained in medium containing DMEM, 10% heat-inactivated fetal bovine serum and 3% streptomycin-penicillin (all from Life Technologies), and kept in a 5% CO_2-humidified atmosphere at 37°C. Cells were trypsinized and then plated on glass coverslips around 24 hr before use. Patch-clamp experiments were conducted in the tight-seal whole-cell configuration at room temperature (18–25°C) as previously described (Hamill et al., 1981; Parekh, 1998). Patch pipettes were pulled from borosilicate glass (Hilgenberg), sylgard coated and fire-polished. Pipettes had d.c. resistances of 2.5–4 MΩ when filled with standard internal solution that contained (in mM): cesium glutamate 145, NaCl 8, $MgCl_2$ 1, HEPES 10, pH 7.2 with CsOH. Depending on the experiment (described in the text), the Ca^{2+} chelators EGTA or BAPTA were added to this solution, as was $InsP_3$ (0.03 mM). In some experiments, RBL cells were sensitized to antigen (DNP-BSA) by incubation in IgE (500 ng/ml) for >3 hr. Antigen and IgE were kindly supplied by Professor Israel Pecht (Weizman Institute, Rehovot, Israel). A correction of +10 mV was applied for the subsequent liquid junction potential that arose from this glutamate-based internal solution. 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). In divalent substitution experiments, 10 mM Ca^{2+} was replaced by 10 mM Ba^{2+} or Sr^{2+}. External CsCl was present to block the activity of the inwardly rectifying potassium channel (McCloskey & Cahalan, 1990). In some current clamp experiments, CsCl was not present in the external solution (pH was adjusted with NaOH), and potassium glutamate was used instead of caesium glutamate in the internal solution (pH with KOH). 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 or step using the automatic compensation of the EPC 9. Series resistance was between 4 and 14 MΩ. Because of the small size of the currents (usually <50 pA at −80 mV), series resistance compensation was not applied. Voltage errors were therefore <1 mV. Currents were filtered using an 8-pole Bessel filter at 2.5 kHz and digitized at 100 μsec. I_{CRAC} was measured using both voltage ramps (−100 to +100 mV in 50 msec) and voltage steps (described in text) applied every 2 sec using PULSE software (HEKA Electronics) on a 9500 PowerMac, as described in Parekh, 1998. Peak currents were measured 2 msec after the start of the pulse to minimize potential contributions from uncompensated capacitative current (time constant <0.5 msec). Steady-state currents were measured as a 5 msec average at the end of the pulse. Cells were held at 0 mV between pulses. All currents were leak subtracted by averaging the first two to four ramps or steps after breaking in and then subtracting this from all subsequent traces. Local application was achieved by means of a second pipette, placed within 30 μm of the cell. Several parameters (capacitance, series resistance, holding current) were displayed simulataneously on a second monitor at a slower rate (2 Hz) using the X-Chart display (HEKA Electronics). Data are presented as mean ± SEM, and statistical evaluation was carried out using Students unpaired *t*-test.

Results

I_{CRAC} Inactivates Within Milliseconds during Hyperpolarizing Voltage Steps

Application of hyperpolarizing pulses in mast cells and jurkat T-lymphocytes have demonstrated that I_{CRAC} exhibits a rapid, Ca^{2+}-dependent inactivation (Hoth & Penner, 1993; Zweifach & Lewis, 1995). To examine whether I_{CRAC} inactivated during a sustained hyperpolarizing step in RBL cells, we applied voltage pulses from 0 to −100 mV. Figure 1*A* shows a recording that was taken with 10 mM EGTA in the pipette after the whole cell CRAC current had reached a stable value. High EGTA passively depletes the stores. The leak current, obtained before I_{CRAC} had developed, has been subtracted. On stepping to −100 mV, there was an instantaneous increase in the current which then declined to reach a steady-state amplitude that was around 50% of the peak. The speed of inactivation could, in most cases, be fitted with fast and slow exponential components at −100 mV (τs in the range of 10 and 120 msec, respectively).

To examine the voltage-dependence of this inactivation, we applied voltage steps up to −120 mV from the holding potential of 0 mV. Pooled data from 5 cells are summarized in Fig. 1*B*. As the hyperpolarizing step increased in size, the extent of inactivation (steady-state current divided by the initial peak value) became greater. At potentials positive to −40 mV however, inactivation became weak, at best.

Fast Inactivation is Dependent on Intracellular Ca^{2+}: Effects of Bapta

To examine whether fast inactivation in RBL cells was dependent on intracellular Ca^{2+}, we replaced EGTA in the recording pipette with the faster Ca^{2+} chelator

BAPTA. BAPTA reduced the extent of fast inactivation on stepping to −100 mV when compared with EGTA (Fig. 1*A*). In fact, BAPTA was substantially more effective than EGTA at reducing the extent of inactivation at all those voltages (<−40 mV) where fast inactivation was apparent (Fig. 1*B*, each point reflects data from 5 cells).

Further support of the notion that fast inactivation is Ca^{2+}-dependent is provided from experiments in which Ca^{2+} was replaced by Sr^{2+}, and is described later.

CRAC Channels Inactivate Independently of Each Other

To see whether Ca^{2+} influx through one CRAC channel could induce fast inactivation in another CRAC channel, we examined how the extent of fast inactivation changed as the number of conducting CRAC channels increased (monitored through the increased whole cell CRAC current). The extent of inactivation stayed reasonably constant as the whole cell Ca^{2+} current increased (8 cells, *data not shown*). Hence, fast inactivation would appear to arise from the local buildup of Ca^{2+} in the vicinity of each specific channel, and not from overlapping microdomains of elevated Ca^{2+} from several independent channels. This is in good agreement with results from T-cells (Zweifach & Lewis, 1995).

Ca^{2+} Release from $InsP_3$-Sensitive Stores Does Not Trigger Fast Inactivation

Because the $InsP_3$-sensitive Ca^{2+} stores that activate I_{CRAC} are thought to be close to the plasma membrane in RBL cells (Parekh & Penner, 1995), we entertained the possibility that Ca^{2+} released from these stores might enhance the fast inactivation process. To address this issue, we dialyzed cells with 30 μM $InsP_3$ and 10 mM EGTA, and applied voltage steps to −80 mV from the holding potential of 0 mV. Ca^{2+} release evoked by $InsP_3$ concentrations >1 μM occurs within seconds of obtaining the whole-cell configuration (Parekh, Fleig & Penner, 1997), and would occur before appreciable amounts of EGTA have diffused into the cell. A typical record is shown in Fig. 1*C*. The extent of fast inactivation was relatively constant throughout the recording. If Ca^{2+} release had contributed to fast inactivation, one would have predicted a high extent of inactivation initially and this would subsequently decline as the Ca^{2+} released by $InsP_3$ was buffered by the high levels of EGTA diffusing from the recording pipette. This was clearly not the case. Similar results were obtained in 11 other cells. Figure 1*C* also shows that the extent of inactivation was constant despite a fivefold increase in whole cell CRAC current amplitude.

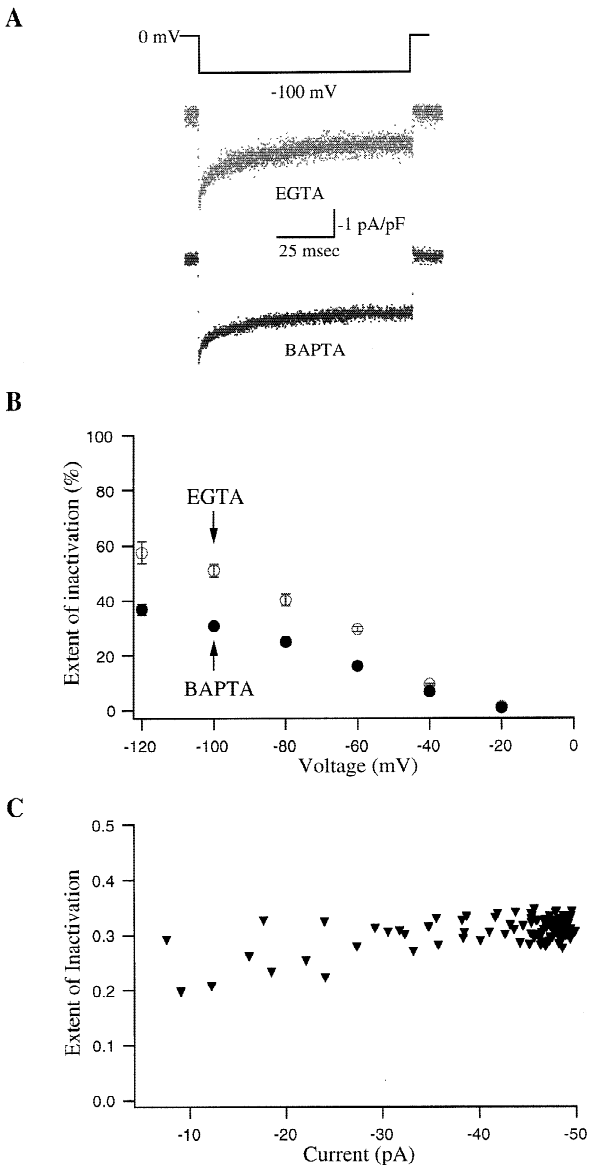

Fig. 1. Fast Ca-dependent inactivation of I_{CRAC}. (*A*) I_{CRAC} inactivates more during a voltage step in the presence of 10 mM EGTA than BAPTA. *B* plots the voltage dependence of the extent of inactivation. BAPTA significantly reduced the extent of inactivation at all voltages where it occurred compared with EGTA. In *A* and *B*, I_{CRAC} was activated passively. *C* plots the extent of inactivation *vs.* whole cell I_{CRAC} amplitude for a cell in which I_{CRAC} was evoked by dialysis with $InsP_3$.

Kinetics of Recovery from Fast Inactivation

To assess how quickly CRAC channels recovered from fast inactivation, we employed a paired-pulse protocol which is illustrated at the top of Fig. 2*A*. Cells were initially clamped at 0 mV and then stepped to −100 mV for 250 msec (first pulse). The potential was then returned to 0 mV for a variable period of time (initially for 10 msec which then increased by a factor of two) before

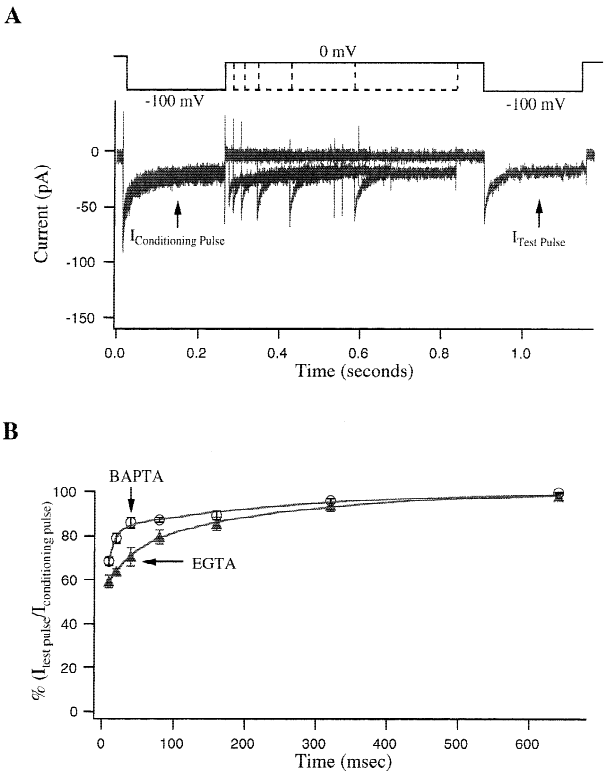

Fig. 2. Kinetics of recovery from fast inactivation. The rate of recovery from fast inactivation was determined using a two-pulse protocol (depicted at the top of the upper panel), as described in the text. I_{CRAC} was evoked by passive depletion using EGTA. *A* shows the time course of recovery from fast inactivation. The currents were recorded once I_{CRAC} had reached its peak amplitude, and the background currents have been subtracted. *B* plots the ratio of test current to conditioning current *vs.* time between pulses. Recovery was faster in BAPTA than EGTA. Recovery in BAPTA and EGTA could be fit best with bi-exponential functions.

stepping back to −100 mV once more (second pulse). In these experiments, we triggered I_{CRAC} using passive store depletion. Because I_{CRAC} activates slowly when stores are depleted passively, we were able to obtain the entire set of background currents using the paired-pulse protocol and then subtract these from the currents obtained once I_{CRAC} had fully developed. Fig. 2*A* shows a typical leak-subtracted record, and Fig. 2*B* summarizes the pooled data from 5 cells. Recovery from fast inactivation could be best fitted with a double-exponential function yielding time-constants $\tau_{recovery}$ of 34 and 233 msec (Fig. 2). Hence, recovery from fast inactivation appears to be a biphasic process.

When we used BAPTA instead of EGTA in the recording pipette, recovery from inactivation was accelerated at shorter time intervals between pulses (Fig. 2*B*, 5 cells). Recovery from inactivation was best fit with a double exponential, yielding time-constants of 9.0 and 250 msec.

Effects of Group II Divalent Cations on Fast Inactivation

In the first set of experiments, we simply replaced Ca^{2+} from the extracellular solution with either Ba^{2+} or Sr^{2+} prior to recording. This protocol enabled us to examine the effects of different divalent cations on the rate of activation of I_{CRAC} as well as on its final extent, in addition to actions on fast inactivation. To see whether I_{CRAC} could activate in the presence of Ba^{2+} and Sr^{2+}, we first measured the current by applying voltage ramps after dialysing cells with $InsP_3$ and 10 mM EGTA. Figure 3*A* depicts the current-voltage relationship for I_{CRAC} in the presence of 10 mM Ca^{2+}, Ba^{2+} and Sr^{2+} from three different cells from the same cell preparation. Irrespective of the charge carrier, a voltage-independent inwardly rectifying current was observed with a reversal potential >+40 mV. However, the amplitude of the current was smallest with Ba^{2+} at all negative voltages. I_{CRAC} was slightly smaller in Sr^{2+} than Ca^{2+}. In Fig. 3*B*, the time-course of I_{CRAC} (measured at −80 mV) is plotted against time of whole cell recording (6 cells for each divalent cation). Steady-state I_{CRAC} in the presence of Ba^{2+} was 4-fold smaller than in the presence of Ca^{2+} (−0.78 ± 15 pA/pF and −3.15 ± 0.50 pA/pF respectively, $P < 0.01$), whereas, with Sr^{2+}, it was only 1.4 times smaller (−2.30 ± 0.15 pA/pF, $P < 0.05$). Strikingly, the time-constant for activation of I_{CRAC} ($\tau_{activation}$, measured for each cell using an exponential fit and then pooled together) was significantly slower in the presence of Sr^{2+} than Ca^{2+} (30.8 ± 3.7 sec *vs.* 15.6 ± 2.1 sec, respectively. $P < 0.01$). With Ba^{2+}, $\tau_{activation}$ was 24.5 ± 4.8 sec. Because of the small size of the currents however, only 3 cells could be fitted well.

Using passive store depletion to activate I_{CRAC}, we then examined the effects of replacing Ca^{2+} with Sr^{2+} on fast inactivation (because of the small currents, we did not study Ba^{2+}). Fast inactivation was less prominent when Sr^{2+} was the charge carrier. Figure 3*C* shows a recording in the presence of Sr^{2+} when the membrane potential was stepped from 0 to −100 mV. The current inactivated by around 20% only. In Fig. 3*D*, we have plotted the extent of fast inactivation at different voltages in the presence of Sr^{2+} and Ca^{2+}. Fast inactivation was significantly less at all voltages when Sr^{2+} was the charge carrier. Hence Sr^{2+} is not able to fully mimic the intracellular actions of Ca^{2+} in inducing fast inactivation, reinforcing the notion that fast inactivation is predominantly a Ca^{2+}- and not voltage-dependent phenomenon.

In the second set of experiments, we replaced external Ca^{2+} with Ba^{2+} or Sr^{2+} by local application after I_{CRAC} had developed. To do this properly, one needs to obtain the leak currents in the presence of each divalent cation before the current starts to activate. This is essential in order to record the true Ba^{2+} and Sr^{2+} currents through CRAC channels. However, we noticed that the

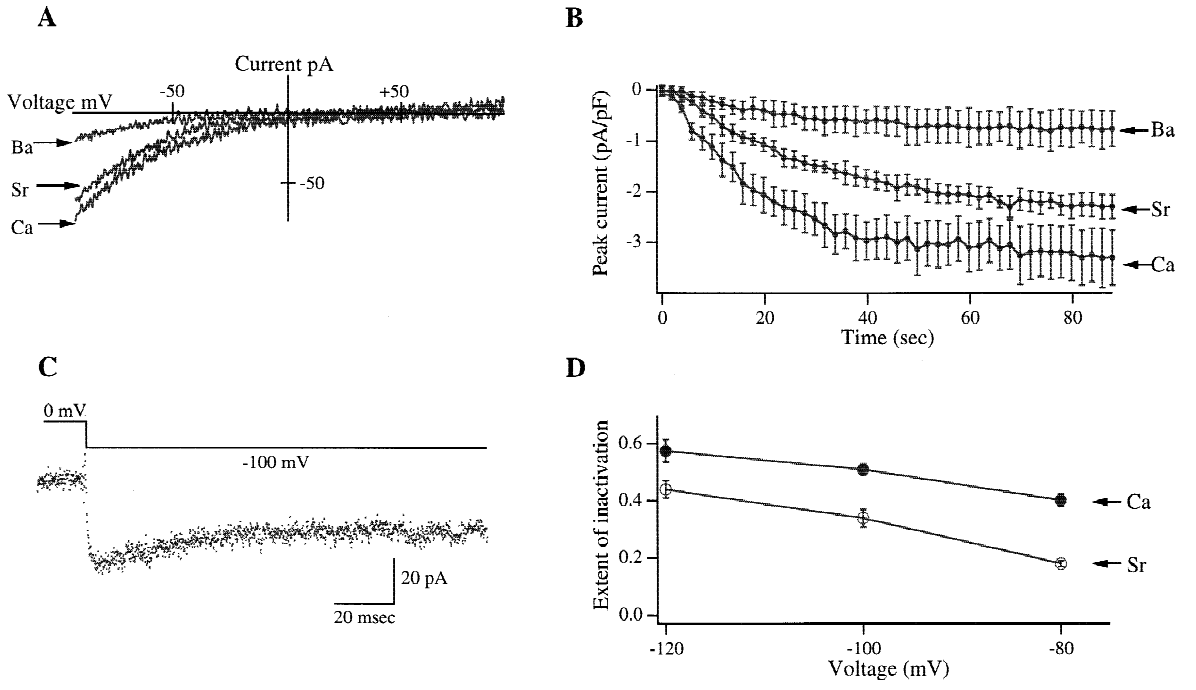

Fig. 3. Effects of different group II divalent cations on I_{CRAC}. (*A*) Current-voltage relationship of steady-state I_{CRAC} in the presence of Ba^{2+}, Sr^{2+} and Ca^{2+}. Each trace represents a different cell. *B* plots the development of I_{CRAC} (measured at −80 mV) *vs.* time. Note that the current developed significantly more slowly with Sr^{2+} as the charge carrier. In these experiments, I_{CRAC} was activated by dialysis with $InsP_3$ and 10 mM EGTA. *C* shows that the extent of fast inactivation was reduced on stepping to -100 mV with Sr^{2+} as the charge carrier. *D* compares the extent of fast inactivation in the presence of Sr^{2+} *vs.* Ca^{2+} at different negative voltages. Fast inactivation was significantly less at all voltages in the presence of Sr^{2+} than Ca^{2+}.

leak currents changed, suggesting that Ba^{2+} and Sr^{2+} were affecting the resting cell conductance. We obtained very variable effects with Ba^{2+}. In some cells, there was a rapid fall in I_{CRAC} amplitude whereas in others a small increase initially occurred. In addition, different divalent cations change the surface potential to different degrees, which would alter the current-voltage relationship and complicate interpretation (Hagiwara & Byerly, 1981). We therefore abandoned this approach.

Fast Inactivation is Unaffected by Cysteine Oxidation

A-type K^+ currents exhibit a rapid N-type inactivation that is due to oxidation of cysteine 13 on the amino ball peptide (Ruppersberg et al., 1991). This inactivation is accelerated by the reduced form of glutathione (GSH; the most abundant thiol in living cells) applied to the cytoplasmic side. To see whether a similar mechanism could account for the fast inactivation of I_{CRAC}, we examined the effects of 5 mM GSH on the decay of the current. I_{CRAC} was activated passively by dialysis with 10 mM EGTA. This method was particularly suitable for our purposes because we were able to ensure that a significant amount of GSH had entered the cytoplasm prior to the development of the current. Dialysis with GSH did not affect the peak amplitude of I_{CRAC} measured in voltage ramps at −80 mV (−2.13 ± 0.19 pA/pF compared with control of −2.15 ± 0.29 pA/pF, 5 cells each), nor was there much change in the rate I_{CRAC} developed. Figure 4A shows fast inactivation in the presence of GSG and Fig. 4*B* plots the extent of inactivation over the voltage range −120 to −40 mV. Fast inactivation was largely unaffected by GSH compared with controls. Under physiological conditions, GSH is in a steady-state with oxidized glutathione (GSSG) with a maximal ratio of 10:1. Dialysis with 5 mM GSH together with 0.5 mM GSSG did not alter the extent of inactivation compared with either GSH or control recordings (Fig. 4*A* and *B*). This mixture also did not affect the development nor the peak amplitude of I_{CRAC} (−1.86 ± 0.34 pA/pF, 4 cells; *data not shown*). Hence cysteine oxidation does not seem to play a role in the fast inactivation of I_{CRAC}, nor in the step that links store depletion to activation of the current.

Is Fast Inactivation Regulated by Receptor Stimulation?

To see whether fast inactivation was regulated by cell-surface receptor stimulation, we activated I_{CRAC} by stimulating FCεRI receptors with the antigen dinitrophe-

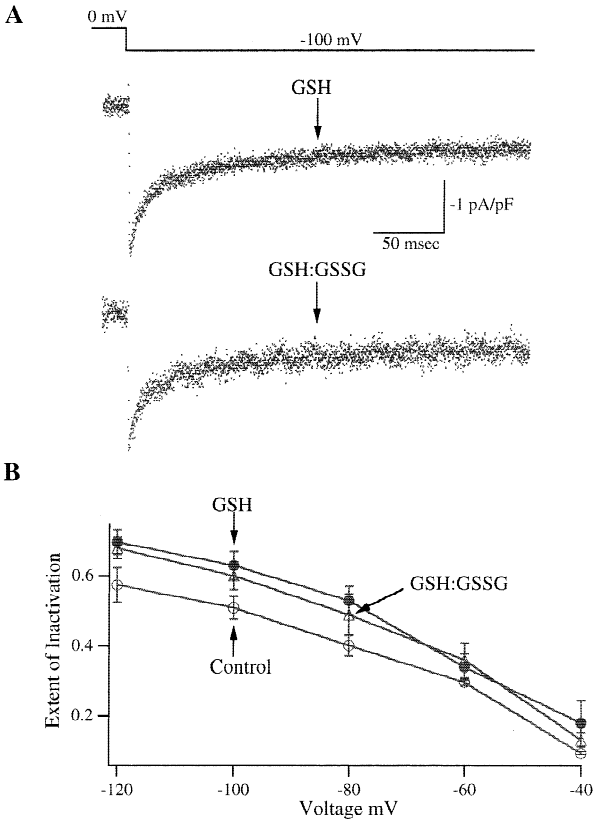

Fig. 4. Fast inactivation is not altered by cysteine oxidation. *A* shows current traces obtained on stepping to −100 mV with a pipette solution containing 5 mM GSH (upper panel) or 5 mM GSH: 0.5 mM GSSG (lower panel). *B* shows that neither 5 mM GSSH nor a mixture containing 0.5 mM GSSG: 5 mM GSH alter the relationship between inactivation and voltage compared with control recordings. I_{CRAC} was activated in all three cases by passive depletion with 10 mM EGTA.

nyl-bovine serum albumin (DNP-BSA) in sensitized RBL cells. FCεRI receptors have endogenous tyrosine kinase activity through which they phosphorylate phospholipase C γ1 to generate $InsP_3$ and diacylglycerol; the latter subsequently stimulates several different protein kinase C isozymes expressed simultaneously in RBL cells (Ozawa et al., 1993). Stimulation of FCεRI receptors with antigen generates I_{CRAC} (Zhang & McCloskey, 1995; Parekh & Penner, 1995; Fig. 5*A*). We applied antigen around 50 sec after the onset of whole cell recording. The cell was dialyzed with a pipette solution in which Ca^{2+} was buffered at 280 nM (6.5 mM Ca-EGTA: 3.5 mM EGTA) in order to prevent spontaneous activation of the current. Once I_{CRAC} had reached a stable amplitude, we applied voltage steps and measured the overall extent of inactivation. Figure 5*B* shows that fast inactivation was unaffected by stimulation of FCεRI receptors when compared with control cells over the voltage range of −120 to −40 mV. Hence, the extent of fast inactivation is not regulated by receptor stimulation.

Changes in Membrane Potential Following Receptor Stimulation

For fast inactivation to be of physiological relevance, the membrane potential following receptor stimulation should become quite hyperpolarized because fast inactivation becomes significant only at these negative potentials. To examine this, we recorded the membrane potential of resting RBL cells and then followed how it changed after stimulation of FCεRI receptors in current clamp mode. In an attempt to mimic physiological conditions, we bathed cells in a solution containing 1.8 mM Ca^{2+} (rather than 10 mM) 1.2 mM Mg^{2+} and we omitted external Cs^+. The pH of the external solution was increased to 7.4. The pipette solution contained K^+ (instead of Cs^+), and Ca^{2+} was weakly buffered with 100 μM EGTA. Under these conditions, we observed two distinct membrane potentials in RBL cells sensitized to antigen. Immediately after break in, 6 of 16 cells had a potential of −26 ± 11 mV (mean ± SD) whereas 11 cells were more hyperpolarized (−89 ± 1 mV). The membrane potential was very stable and did not change with time by more than ±5 mV. Regardless of the initial value, application of antigen failed to alter the membrane potential further. Typical records of a cell with a membrane potential of −33 mV, and one at −88 mV, are shown in Fig. 5*C* and *D*, respectively. Antigen was applied as indicated, and exerted virtually no effect. Hence in the majority of cells, the membrane potential following receptor stimulation is sufficiently negative for fast inactivation to exert a significant on the extent of Ca^{2+} influx through CRAC channels.

Discussion

In this report, we have characterized rapid Ca^{2+}-dependent inactivation of I_{CRAC} in RBL cells. We find that it shares several similarities to that described in jurkat T-cells (Zweifach & Lewis, 1995). These include the fact that fast inactivation accounts for a modest decline in the amplitude of I_{CRAC} unless the membrane potential is hyperpolarized below −60 mV, that the extent of steady-state inactivation reached is similar in RBL and T-cells over the range −120 to −80 mV, that it is predominantly Ca^{2+}-dependent and is largely independent of the macroscopic current. It is more effectively suppressed by the fast chelator BAPTA than by EGTA, suggesting that the intracellular Ca^{2+} binding site(s) are in close proximity to the channel pore.

Two striking differences between the RBL cells and the jurkat T-cells are the kinetics of recovery from inactivation and the Ca^{2+}-dependence of this process. In T-cells, recovery from inactivation in the presence of EGTA is a biphasic process with time-constants of 9 msec and 75 msec (Zweifach & Lewis, 1995). In RBL

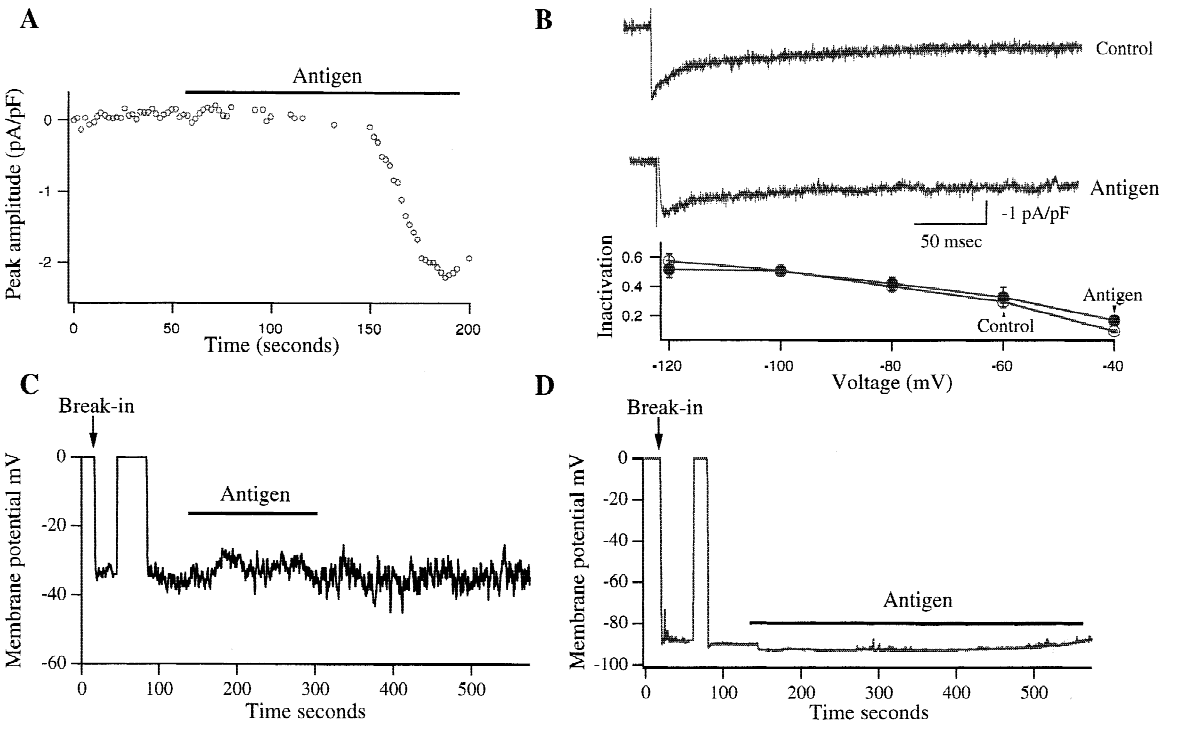

Fig. 5. Effect of receptor stimulation on fast inactivation and membrane potential. *A* shows the development of I_{CRAC} following application of 1 μg/ml antigen. The current was measured from the voltage ramps (−100 to +100 mV in 50 msec) at −80 mV. In B, current records from a control cell and one after exposure to antigen are shown. The potential was stepped to −100 mV from a holding potential of 0 mV. The lower panel in *B* summarizes the pooled data from 5 cells each. *C* and *D* depict current clamp recordings from a cell with a resting potential of −33 mV (*C*) and −90 mV (*D*). Application of antigen did not change the membrane potential for either cell. The break in the recordings after around 50 sec (when membrane potential became 0 mV) reflects our switch from current to voltage clamp, so that we could apply a few voltage ramps in order to assess the state of the cell. Because fast inactivation is not affected by the cytoplasmic EGTA concentration nor global levels of cytoplasmic Ca^{2+}, the buffered Ca^{2+} used in these experiments will not alter the kinetics of the inactivation process (Zweifach & Lewis, 1995; Parekh, 1998).

cells, recovery is significantly slower for both phases (34 msec and 233 msec). A more unexpected difference is that recovery from inactivation appears to be brought about by distinct mechanisms in the two cell-types. In RBL cells, BAPTA accelerated recovery from inactivation (by decreasing the time constant of the faster component) whereas in T-cells recovery was apparently not affected upon replacement of EGTA with BAPTA. Recovery in RBL cells therefore appears to exhibit one Ca^{2+}-dependent component whereas in T cells it does not. One possible explanation for this is that the rate at which Ca^{2+} dissociates from the inactivation site is slower in RBL cells than T-cells, so that BAPTA would compete with the site for Ca^{2+}. Interestingly, in T-cells recovery from inactivation was faster at positive potentials, where less Ca^{2+} would enter the cell due to the reduced electrical gradient, and slower at negative ones, which would favor Ca^{2+} entry (Zweifach & Lewis, 1995). This would fit with a Ca^{2+}-dependent recovery. Alternatively, recovery from inactivation in T-cells might be a purely voltage-dependent process.

To probe the Ca^{2+} selectivity of the inactivation site, divalent cation substitution experiments have been carried out by replacing Ca^{2+} with Ba^{2+}, once I_{CRAC} has developed (Zweifach & Lewis, 1995; Hoth, 1995). However, the effects of different divalent cations on the activation of I_{CRAC} have not been described. Such experiments might provide valuable information on the activation properties of the current. Using this approach, we have found that the activation of I_{CRAC} was significantly slower when Sr^{2+} was the charge carrier than with Ca^{2+}. One attractive explanation for this is that Ca^{2+} entry feeds back positively to accelerate further activation of CRAC channels, either through an action on the channels themselves or directed at the activating signal. Alternatively, with Sr^{2+} as the charge carrier, it is conceivable that the affinity of CRAC channels for the activation signal is reduced, resulting in a slower rate of channel activation as well as a smaller steady-state amplitude.

The group II alkali earth cations also differed in their ability to carry current through CRAC channels at steady state. Ca^{2+} was the best charge carrier, whereas Ba^{2+} carried only around 25%. Sr^{2+} was an effective charge carrier, carrying almost 80% that of Ca^{2+}. This conductivity profile is different from I_{CRAC} in T-cells, where

Ba^{2+} and Sr^{2+} were rather similar in that they carried around 40% of the current compared with Ca^{2+} (Zweifach & Lewis, 1993). The permeability profile of CRAC channels in RBL cells would therefore appear to be distinct from that in T-cells, and would support the notion that they may reflect distinct channel subtypes (Hoth, 1995).

Fast inactivation of voltage-gated Ca^{2+} channels has been well-described (Ashcroft & Stanfield 1981; Chad & Eckert, 1986) and the inactivation site is rather selective in that Ba^{2+} cannot substitute for Ca^{2+}. Ba^{2+} permeability through CRAC channels seems to be complex and confusing. Hoth (1995) observed an initial large increase in current amplitude on replacing external Ca^{2+} with Ba^{2+}, once I_{CRAC} had reached a steady state. However, this increase was not observed by Schofield and Mason (1996). In our hands, we observed effects of Ba^{2+} on the background conductance. In addition, we observed very variable effects when we locally applied it to cells that had generated I_{CRAC}. In T-cells, Ba^{2+} induced less inactivation than Ca^{2+} (Zweifach & Lewis, 1995). In RBL cells, we found that the internal binding site was rather selective for Ca^{2+} in that Sr^{2+} was not able to fully substitute for Ca^{2+} in inducing inactivation. These results are consistent with those described in T-cells with Ba^{2+} in that they point to a highly selective Ca^{2+}-binding site for fast inactivation.

Recently, a Ca^{2+} binding site has been described for CRAC channels in T-cells, which needs to be occupied by Ca^{2+} in order to obtain the maximal channel conductance (Christian et al., 1996; Zweifach & Lewis, 1996). This site is thought to be located on either the extracellular part of the channel or within the pore. Ion substitution experiments may therefore affect the number of available channels in addition to conductivity. It is not clear whether such an external Ca^{2+}-binding site is present on CRAC channels in RBL cells. If one were to exist, then it would appear that Sr^{2+} is better able to occupy the site than Ba^{2+}. In T-cells however, it has been reported that Sr^{2+} cannot support channel activity (Christian et al., 1996).

One key question is whether Ca^{2+} induces fast inactivation by directly binding to a site on the channel itself (or an associated subunit) or whether it recruits an intermediate signal. This latter mechanism is thought to account for the Ca^{2+}-dependent inactivation of voltage-operated, nifedipine-sensitive, Ca^{2+} channels in helix neurons (Chad & Eckert, 1986), where Ca^{2+} influx stimulates Ca^{2+}-dependent phosphatase calcineurin to dephosphorylate the channel, thereby inducing inactivation. Recovery from inactivation occurs through a protein kinase-mediated phosphorylation. Recent evidence has demonstrated that protein kinases colocalize with Ca^{2+} channels, linked by adaptor proteins of the AKAP family (Gao et al., 1997; Gray et al., 1997). Because fast inactivation was remarkably constant throughout our whole cell recordings (>10 minutes in some experiments) and could occur without any ATP in the pipette, we do not think a phosphorylation reaction is fundamental to the fast inactivation process.

Ca^{2+} release from $InsP_3$-sensitive stores was not able to affect the extent of fast inactivation that was induced by Ca^{2+} entry. Ca^{2+} release starts almost immediately after breaking into the cell and Ca^{2+} stays elevated for up to 30 sec. Because I_{CRAC} activates after a delay of around 3 sec, cytosolic Ca^{2+} will still be high as the current starts to develop (appreciable amounts of Ca^{2+} buffer would not have diffused into the cell from the recording pipette at these short times). Since we find that fast inactivation is unaffected, the subplasmalemmal Ca^{2+} elevation in the vicinity of CRAC channels following Ca^{2+} release is not of sufficient amplitude to alter inactivation, and hence Ca^{2+} release is of little consequence to this process. This finding may have broad implications for the activation mechanism of I_{CRAC}. In both avian nasal gland cells and mouse pancreatic acini, a sizeable delay of several seconds between Ca^{2+} release and subsequent Ca^{2+} influx has been observed. Both Shuttleworth (1994) and Toescu and Petersen (1995) have argued that this delay is hard to reconcile with the conformational-coupling model in which $InsP_3$ receptors on the stores are supposed to physically attach to Ca^{2+} channels in the plasma membrane, but are instead compatible with a slow biochemical step such as the generation of a retrograde messenger. Berridge (1995) has argued however, that the delay between Ca^{2+} release and entry reflects recovery from Ca^{2+}-dependent fast inactivation of CRAC channels, the inactivation arising from Ca^{2+} release. Our finding that Ca^{2+} release does not affect fast inactivation would suggest that the delay between Ca^{2+} release and subsequent influx does not reflect recovery from inactivation, but instead would indicate a slow activation mechanism reminiscent of a biochemical process, at least in RBL cells.

For fast inactivation to be of physiological relevance, two criteria need to be fulfilled. First, the membrane potential must be more negative than around −60 mV and second, sufficient Ca^{2+} must enter in order to induce the inactivation process. In our current clamp recordings, we found that the majority of cells had a resting potential of around −90 mV. This would be sufficient for significant fast inactivation of I_{CRAC}. With physiological external Ca^{2+} concentration, I_{CRAC} can be observed in T-cells although the current is small (Zweifach & Lewis, 1995). In agreement with this, we have measured I_{CRAC} in RBL cells in 1.8 mM Ca^{2+} and fast inactivation can be discerned (*data not shown*). Hence fast inactivation is likely to impact on the extent of Ca^{2+} influx through CRAC channels under physiological con-

ditions and appears to be an important negative feedback process that limits Ca^{2+} increases.

The extent of fast inactivation was not modified by stimulation of FCεRI receptors. Because these receptors have endogenous tyrosine kinase activity, stimulate phospholipase Cγ1 (resulting in protein kinase C activation), and activate a variety of SH2 domain-containing proteins (like Grb2 leading to Ras activation), it would appear that fast inactivation is not subject to regulation from a variety of intracellular signaling pathways. Instead its impact is determined solely by the prevailing membrane potential.

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