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Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells

(Ca^{2+} release-activated Ca^{2+} current/protein kinase/fura-2/patch clamp)

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ABSTRACT **Whole-cell patch-clamp recordings and single-cell Ca^{2+} measurements were used to study the control of Ca^{2+} entry through the Ca^{2+} release-activated Ca^{2+} influx pathway (I_{CRAC}) in rat basophilic leukemia cells. When intracellular inositol 1,4,5-trisphosphate ($InsP_3$)-sensitive stores were depleted by dialyzing cells with high concentrations of $InsP_3$, I_{CRAC} inactivated only slightly in the absence of ATP. Inclusion of ATP accelerated inactivation 2-fold. The inactivation was increased further by the ATP analogue adenosine 5′-[γ-thio]triphosphate, which is readily used by protein kinases, but not by 5′-adenylyl imidodiphosphate, another ATP analogue that is not used by kinases. Neither cyclic nucleotides nor inhibition of calmodulin or tyrosine kinase prevented the inactivation. Staurosporine and bisindolylmaleimide, protein kinase C inhibitors, reduced inactivation of I_{CRAC}, whereas phorbol ester accelerated inactivation of the current. These results demonstrate that a protein kinase-mediated phosphorylation, probably through protein kinase C, inactivates I_{CRAC}. Activation of the adenosine receptor (A_3 type) in RBL cells did not evoke much Ca^{2+} influx or systematic activation of I_{CRAC}. After protein kinase C was blocked, however, large I_{CRAC} was observed in all cells and this was accompanied by large Ca^{2+} influx. The ability of a receptor to evoke Ca^{2+} entry is determined, at least in part, by protein kinase C. Antigen stimulation, which triggers secretion through a process that requires Ca^{2+} influx, activated I_{CRAC}. The regulation of I_{CRAC} by protein kinase will therefore have important consequences on cell functioning.**

In nonexcitable cells, hormones and neurotransmitters that couple to the ubiquitous second messenger inositol 1,4,5-trisphosphate ($InsP_3$) produce a biphasic increase in cytosolic free Ca^{2+} (1, 2). The early, transient phase predominantly reflects Ca^{2+} release from internal stores, whereas the secondary, plateau phase reflects Ca^{2+} influx into the cell. This Ca^{2+} influx is important not only for refilling the stores (3) but also modulates the spatiotemporal pattern of Ca^{2+} wave propagation through the cell (4, 5).

Various Ca^{2+} entry pathways have been observed in nonexcitable cells (1). It appears that the most widely distributed Ca^{2+} entry mechanism is provided by the so-called capacitative Ca^{2+} influx (6). In this latter mechanism, the filling state of the internal stores regulates Ca^{2+} entry in that depletion of the stores activates a Ca^{2+} entry pathway. This entry pathway, which is voltage independent, inwardly rectifying, and selective for Ca^{2+}, has been termed I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current; ref. 7). Depletion-activated Ca^{2+} currents have been recorded in several nonexcitable cells (8, 9) including rat basophilic leukemia cells (RBL-2H3; ref. 10). The mechanism whereby I_{CRAC} is activated, as well as its regulation, is largely unknown. Biochemical experiments have led to the suggestion that capacitative Ca^{2+} influx is under control of phosphorylation, but the site of action of the kinase is not clear (11–13).

In the present study, we have found that I_{CRAC} is inactivated by a protein kinase-mediated phosphorylation. Furthermore, the ability of a receptor to evoke Ca^{2+} influx is determined by protein kinase C. Because secretion has an absolute requirement for Ca^{2+} influx in RBL cells (14–16), this kinase-mediated inactivation of I_{CRAC} is likely to be of physiological importance.

MATERIALS AND METHODS

Rat basophilic leukemia cells (RBL-2H3) were cultured as described (10). Patch-clamp experiments were conducted in the tight-seal whole-cell configuration (17) at room temperature (20–25°C) in standard saline solution containing 140 mM NaCl, 2.8 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM CsCl, 11 mM glucose and 10 mM Hepes·NaOH (pH 7.2). Sylgard-coated, fire-polished patch pipettes had resistances of 2–3 MΩ after filling with the standard intracellular solution, which contained 145 mM potassium glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 2 mM MgATP, 10 mM EGTA, 0.1 mM fura-2, 40 μM $InsP_3$ (Amersham), and 10 mM Hepes·KOH (pH 7.2). In some experiments, Ca^{2+} was clamped to 60 nM by varying the EGTA/CaEGTA ratio. When the adenosine receptor was stimulated, cesium glutamate replaced potassium glutamate in the internal solution in order to block the outwardly rectifying K^+ current activated by receptor stimulation (18) and 100 μM GTP was also included in the pipette solution, since the receptor couples to phospholipase C through a heterotrimeric G protein. High-resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9). Capacitative currents were canceled before each voltage ramp using the automatic capacitance compensation of the EPC-9. Series resistance was between 5 and 12 MΩ and inactivation of the Ca^{2+} current did not correlate with series resistance. RBL cells possess an inward rectifying K^+ current (19), whose I–V relationship could overlap with I_{CRAC} under the present conditions. When cells were dialyzed without $InsP_3$ (Ca^{2+} buffered to 60 nM to prevent passive activation of I_{CRAC}), very small currents were observed (<0.5 pA/pF at −80 mV; $n = 10$) that were constant over a 10-min recording period. However, to eliminate any contribution from the inward rectifying K^+ current in our experiments, 10 mM Cs^+ was always present in the external solution. The Ca^{2+} current was analyzed at −80 mV. Currents were filtered at 2.3 kHz and digitized at 100 μs. Ramps were given every second (−100 to +100 mV in 50 ms), and cells were held at 0 mV between ramps. All currents were leak subtracted by averaging the first three to six ramps after breaking in and then subtracting this from all subsequent

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Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; ATP[γS], adenosine 5′-[γ-thio]triphosphate; NECA, 5′-(*N*-ethylcarboxamido)adenosine; PMA, phorbol 12-myristate 13-acetate; I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current; BAPTA, bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate.

traces. Extracellular solution changes were made by local pressure application from a wide-tipped micropipette placed within 20 μm of the cell. Intracellular [Ca^{2+}] was monitored with a photomultiplier-based system as described (20). The fluorescent indicator dye fura-2 was loaded by diffusion from the patch pipette. Intracellular [Ca^{2+}] was calculated from the fluorescence ratio (360/390) as described (20). For single-cell Ca^{2+} measurements, coverslips were incubated in normal saline solution (140 mM NaCl/2.8 mM KCl/1 mM $MgCl_2$/2 mM $CaCl_2$/11 mM glucose/10 mM Hepes·NaOH, pH 7.2) to which 5 μM fura-2 acetoxymethyl ester (AM) had been added. After 30 min, the coverslips were washed five times in normal saline solution and then incubated at 37°C for 15 min. Single cell Ca^{2+} levels were measured by using a photomultiplier-based system as described above. Cells were sensitized to antigen [2,4-dinitrophenyl-conjugated bovine serum albumin (100 ng/ml)] by incubating the cells overnight with IgE (2 μg/ml). Both IgE and antigen were kindly provided by I. Pecht (Weizmann Institute of Science, Rehovot, Israel). Three different preparations of RBL cells were sensitized in this way, since responses in mast cells are variable from batch to batch (21).

Drugs were from Sigma with the exception of adenosine 5′-[γ-thio]triphosphate (ATP[γS]) and 5′-adenylyl imidodiphosphate (Boehringer Mannheim) and staurosporine and bisindolylmaleimide (Calbiochem).

RESULTS AND DISCUSSION

Depletion of internal Ca^{2+} stores in rat basophilic leukemia cells (RBL-2H3) by three independent procedures (inclusion of InsP_3 in the pipette, applying the Ca^{2+} ionophore ionomycin, or dialyzing cells with high concentrations of Ca^{2+} chelators to passively deplete stores) activates the voltage-independent, highly selective Ca^{2+} current termed I_{CRAC} (10). Fig. 1 shows that when single RBL-2H3 cells were dialyzed via a patch pipette with solutions lacking ATP, I_{CRAC} (activated by inclusion of InsP_3 in the pipette) decreased only 18% ± 4% (mean ± SEM) after a 250-s whole-cell recording (Fig. 1 *a* and *d*; n = 20 cells). However, when 2 mM ATP was included in the pipette solution, I_{CRAC} inactivated by 42% ± 4% (Fig. 1 *a* and *d*; n = 15).

The present results differ from a previous report, which failed to find any effect of ATP on either activation or inactivation of I_{CRAC} in RBL cells (10). The difference seems to lie in the choice of Ca^{2+} chelator. We have used EGTA, whereas the previous study used bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate (BAPTA). Using 10 mM BAPTA instead of EGTA under paired experiments, we failed to see an effect of ATP on inactivation of I_{CRAC}, whereas I_{CRAC} was inactivated in cells from the same coverslip when EGTA was used (n = 3). BAPTA has numerous pharmacological effects in addition to chelating Ca^{2+} (22, 23), including blocking protein kinase C (24). Since inactivation of I_{CRAC} involves a kinase, likely protein kinase C (see below), this pharmacological action of BAPTA might account for the lack of inactivation to ATP previously reported (10). Clearly, caution is required when interpreting results with BAPTA.

We considered that inactivation of I_{CRAC} in the presence of ATP might reflect a kinase-mediated phosphorylation. If this is true then ATP[γS], an ATP analogue that is readily used by kinases but is relatively resistant to dephosphorylation by phosphatases resulting in irreversible phosphorylation (25, 26) should evoke greater inactivation of I_{CRAC}. Dialyzing cells with 2 mM ATP[γS] (instead of ATP) resulted in almost complete inactivation of the Ca^{2+} current (Fig. 1 *a* and *d*; n = 11). This inhibitory effect of ATP[γS] was not mimicked by 5′-adenylyl imidodiphosphate, another nonhydrolyzable analogue of ATP but one that is not utilized by kinases (27), where inactivation was 23% ± 4% after a 250-s recording (n = 11). This indicates

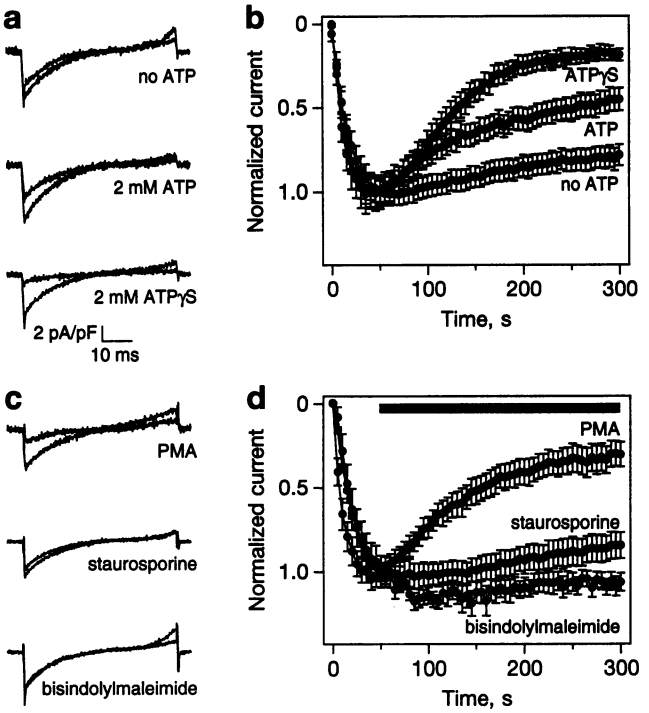

FIG. 1. Protein kinase inactivates I_{CRAC}. The Ca^{2+} current was activated by depleting stores with 40 μM InsP_3. EGTA (10 mM) was included in the pipette solution to clamp the cytosolic Ca^{2+} to very low values. The Ca^{2+} current was monitored by voltage ramps going from −100 to +100 mV in 50 ms, as described (7). (*a*) Raw data of ramps measured at 50 s (when I_{CRAC} had peaked) and 250 s in the presence of the indicated adenine nucleotides. (*b*) Pooled data from all the cells analyzed (20 cells for 0 mM ATP, 15 cells for 2 mM ATP, and 11 cells for 2 mM ATP[γS]). (*c*) Ramps (50 and 250 s, as in *a*) obtained in the presence of pharmacological agents that interact with protein kinase C. (*d*) Pooled data of these agents [6 cells for 100 nM phorbol 12-myristate 13-acetate (PMA), 7 cells for 2 μM staurosporine, and 5 cells for 500 nM bisindolylmaleimide]. Drugs were applied at 50 s, when the Ca^{2+} current had peaked, and were maintained throughout.

that the inhibitory effects of ATP and ATP[γS] are due to kinase-mediated phosphorylation. Although it is difficult to rule out refilling of stores, especially if the stores are very close to the plasma membrane, the inactivation of I_{CRAC} by ATP is unlikely to reflect refilling of InsP_3 stores for several reasons. First, the high concentrations of InsP_3 used (30–50 μM) would maintain depleted stores. Maximal activation of I_{CRAC} occurs with 3 μM under our conditions, which differs from a previous report that found 10–20 μM InsP_3 was necessary to activate I_{CRAC} (10). Again, the difference lies in the chelator, in that the previous report used BAPTA, a competitive inhibitor of the InsP_3 receptor (22). When Ca^{2+} was clamped to 60 nM, no difference in the extent of inactivation was observed compared with 10 mM EGTA (both had 2 mM ATP and 50 μM InsP_3), demonstrating little, if any, store refilling in the continuous presence of high InsP_3 levels. Second, the extent of inactivation of I_{CRAC} by ATP was increased further when ATP[γS] was used. ATP[γS] is not used by ATPases, so refilling of stores by Ca^{2+}-ATPases would not occur.

To identify the protein kinase(s), various activators and inhibitors of known kinases were tested. When either cAMP or cGMP (each at 100 μM) was included in the pipette solution (in addition to 2 mM ATP), no difference in the rate or extent of inactivation of I_{CRAC} was observed compared with experiments in which the cyclic nucleotides were omitted (n = 6 in both cases). Hence, neither cAMP- nor cGMP-dependent protein kinases inactivate I_{CRAC} under the present conditions. Neither the calmodulin inhibitor calmidazolium (50 μM; n =

4) nor the tyrosine kinase inhibitor genistein (100 μM; $n = 4$) prevented inactivation of I_{CRAC}, suggesting that neither calmodulin-dependent nor tyrosine kinases contribute significantly to inactivation of I_{CRAC}.

However, the protein kinase C inhibitor staurosporine (2 μM) reduced inactivation of I_{CRAC} in the presence of ATP and InsP_3 (Fig. 1*d*; $n = 7$). Staurosporine alone failed to activate I_{CRAC} ($n = 3$). The more selective kinase C inhibitor bisindolylmaleimide (500 nM; ref. 28) also reduced inactivation of I_{CRAC} (Fig. 1*d*). Since staurosporine and bisindolylmaleimide both reduced inactivation of I_{CRAC}, whereas conventional inhibitors of other kinases did not, it seems likely that protein kinase C inactivates I_{CRAC}. Consistent with this notion was the finding that direct activation of the kinase by the phorbol ester PMA (100 nM; applied 50 s after obtaining whole-cell mode when I_{CRAC} had peaked) increased inactivation of I_{CRAC} in the presence of 2 mM ATP (Fig. 1*d*; $n = 6$ cells). In two additional cells, however, PMA did not clearly increase inactivation compared with ATP alone.

Taken together, these results demonstrate that a protein kinase inactivates I_{CRAC} and, although one must always bear in mind that the current specific inhibitors will inevitably affect other kinases, the pharmacological profile of the kinase would be compatible with protein kinase C. These results are in agreement with previous studies that used Ca^{2+} measurements in cell populations and that suggested that protein kinase C can reduce capacitative Ca^{2+} influx (11–13). They extend those studies by demonstrating a direct current suppression independent of other factors such as receptor desensitization, membrane potential, and pump activity. Using the Ca^{2+}-activated Cl^- current to follow capacitative Ca^{2+} influx in oocytes, two studies found a role for a kinase in regulating Ca^{2+} entry. Blocking protein phosphatases potentiated Ca^{2+} influx (9) and the phorbol ester PMA increased Ca^{2+} entry, implicating kinase C (29). Sustained exposure to PMA subsequently inhibited Ca^{2+} influx, demonstrating a dual role for kinase C on Ca^{2+} influx in oocytes (30). In RBL cells, we did not observe an initial potentiation of I_{CRAC} with phorbol ester but instead only the inhibition. On the other hand, capacitative Ca^{2+} influx appears not to be inhibited by protein kinase C in some cell types (31). Which one of the two effects is being observed may be dependent on the cell type or the recording situation.

One interesting observation is that kinase-mediated inactivation begins after a delay of ≈50 s (Fig. 1*d*). If kinase activity were constant, one might have expected a gradual increase in I_{CRAC} until it reached a steady-state value rather than a large transient activation followed by some inactivation. This could be explained if depletion of stores activated either a phosphatase or the inhibitory kinase. Because kinase C can be activated by an increase in Ca^{2+} (32), and kinases can associate tightly with channels in the plasma membrane (27), an increase in subplasmalemmal Ca^{2+} arising from activation of I_{CRAC} might stimulate the kinase, resulting in inactivation of I_{CRAC}. This would provide a means whereby activation and inactivation are tightly coupled and might also contribute to the Ca^{2+}-dependent inactivation of I_{CRAC} (7).

We asked whether protein kinase inactivation of I_{CRAC} had a physiological significance. RBL cells possess native A_3 adenosine receptors, which increase InsP_3 by coupling to phospholipase C via a G protein (33). Surprisingly, however, receptor stimulation evokes only transient Ca^{2+} influx despite a robust increase in InsP_3 levels (33). In whole-cell recordings, 5′-(*N*-ethylcarboxamido)adenosine (NECA), a nonmetabolizable agonist of the A_3 receptor, failed to activate I_{CRAC} in a significant fraction of the cells (50%; $n = 8$), and in those that responded a Ca^{2+} current occurred that inactivated almost completely. Responses of three different cells are shown in Fig. 2*a* and averaged data from eight cells are provided in Fig. 2*b*. Consistent with this was that NECA evoked only a small Ca^{2+} plateau, indicative of weak Ca^{2+} entry, in fura-2 AM-loaded single cells (Fig. 3*b* shows the averaged Ca^{2+} response of 20 single cells, and Fig. 3*a* depicts responses of three individual cells). However, when staurosporine was coapplied with NECA, then not only did all cells evoke prominent I_{CRAC} but the Ca^{2+} current was larger and inactivation was modest (Fig. 2 *c* and *d*). I_{CRAC} evoked by NECA was identical to that activated by ionomycin or InsP_3 in terms of inward rectification, voltage-independent gating, and positive reversal potential (>+30 mV). Consistent with the current measurements was the finding that sustained Ca^{2+} influx was evoked when staurosporine, at the same concentration at which it prevented inactivation of I_{CRAC} in whole-cell recordings, was coapplied with NECA in single fura-2 AM-loaded cells (Fig. 3 *c* and *d*). Now a prominent Ca^{2+} plateau was observed. This effect of staurosporine was mimicked by bisindolylmaleimide at a concentration of 500 nM (Fig. 3*c*).

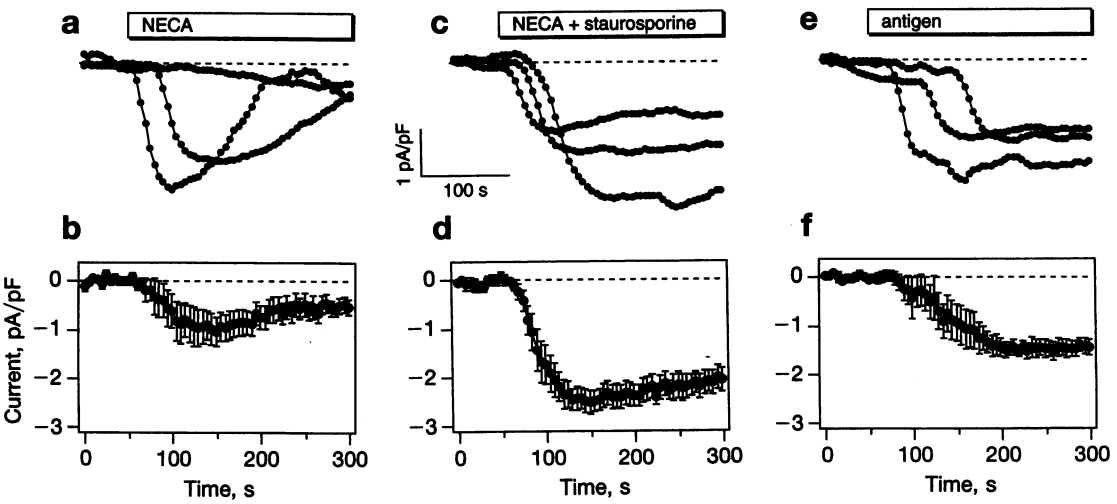

FIG. 2. Receptor activation of the Ca^{2+} current is regulated by protein kinase C. (*a*) Responses of three different cells to the adenosine receptor agonist NECA (20 μM). (*b*) Averaged data from eight cells. Note the rapid inactivation and that many cells evoke only a weak response. (*c* and *d*) Coapplication of NECA and staurosporine (2 μM) evokes a prominent Ca^{2+} current in all cells, which inactivates only slightly. *c* shows three representative cells and *d* shows pooled data from all cells. (*e* and *f*) Antigen stimulation (2,4-dinitrophenyl-modified bovine serum albumin at 200 ng/ml) evokes a prominent Ca^{2+} current in all cells tested. The longer latency after antigen application reflects the numerous processes in its signal transduction pathway leading to depletion of InsP_3 stores. In all cases, receptor agonists were applied at 50 s and maintained.

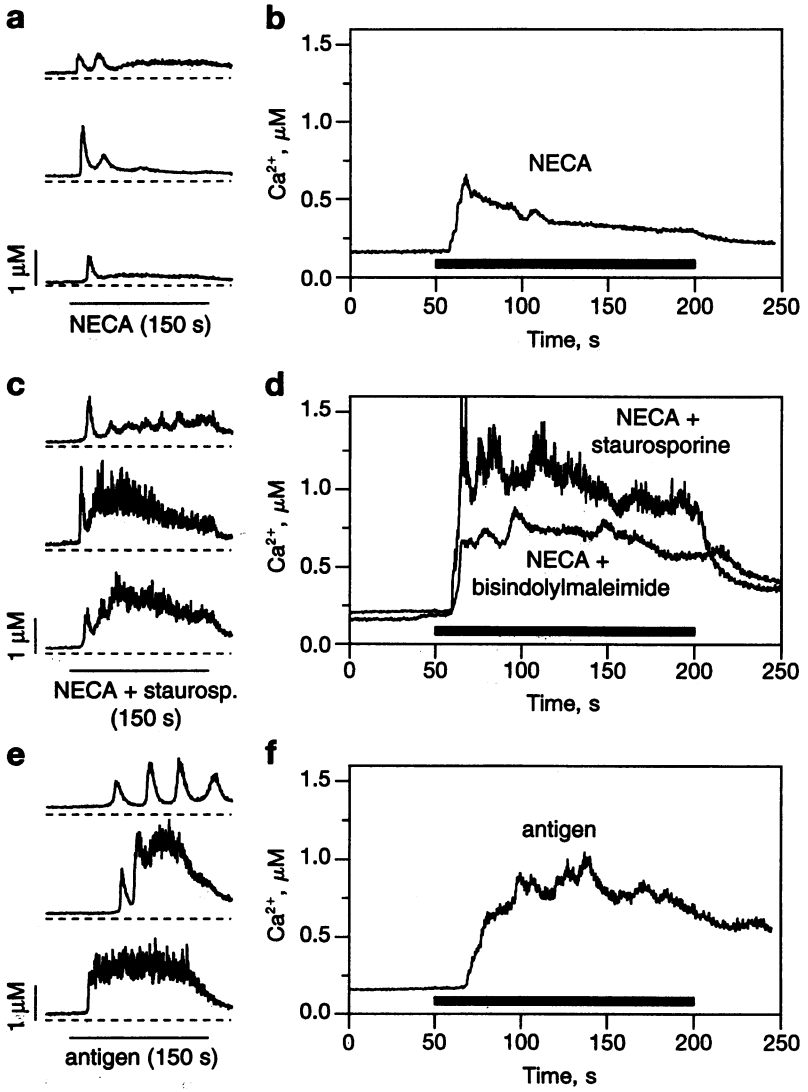

FIG. 3. Protein kinase C regulates receptor-operated Ca^{2+} influx. (*b*) Averaged response of 20 fura-2 AM-loaded single cells to 20 μM NECA. (*a*) Three single cell responses. (*c* and *d*) Responses to coapplication of NECA and 2 μM staurosporine. *d* is the averaged response of 20 cells and *c* shows responses of three individual cells. Also included in *d* is the averaged response of 20 cells that were exposed to NECA and GX-109230X (500 nM), a more selective inhibitor of protein kinase C. A higher concentration of GX-109230X (2 μM) evoked a larger response. (*e* and *f*) Responses to antigen. *f* is the averaged response of 21 single cells, and *e* depicts responses of three individual cells.

The preceding results have demonstrated that activation of protein kinase C by PMA inhibits I_{CRAC}, while inhibition of protein kinase C by staurosporine or bisindolylmaleimide can substantially increase receptor-operated Ca^{2+} influx induced by NECA. In principle, protein kinase-mediated inhibition of I_{CRAC} could at least partially account for the relatively small size of the influx phase observed with NECA stimulation. However, there are probably additional sites in the signal transduction pathway where protein kinase C can interfere. It is known that many receptors that couple to phospholipase C desensitize, and protein kinase C has been implicated in this fast desensitization process through phosphorylation of the receptor, G protein, or phospholipase C (34). Inhibition in this early chain of events would decrease the amount of $InsP_3$ produced and therefore the extent of store depletion, resulting in less capacitative Ca^{2+} influx. Protein kinase C might also decrease the amount of Ca^{2+} released by $InsP_3$, since it has been reported to phosphorylate, and thereby regulate, the $InsP_3$ receptor (30). This could also reduce the amount of store depletion and thus Ca^{2+} entry. NECA-evoked Ca^{2+} transients do desensitize when the agonist is applied at an interval of 100 s (33), although it is not clear whether this reflects poor refilling of the stores, inhibition of Ca^{2+} release, or receptor desensitization. Our findings on $InsP_3$-evoked I_{CRAC} described above reveal a direct site of kinase action on capacitative Ca^{2+} influx that is independent of receptor signaling. In these experiments, we dialyzed cells with supramaximal concentrations of $InsP_3$ and interfered with kinase C activity after I_{CRAC} had peaked. Thus, receptor desensitization and $InsP_3$ receptor inhibition were bypassed. Under these conditions, protein kinase C inhibits either I_{CRAC} or the message coming from depleted stores. It is not clear, and it is beyond the scope of this study, to what extent each of the above-mentioned mechanisms contributes to the negative feedback regulation of receptor-mediated Ca^{2+} influx. Nevertheless, since I_{CRAC} is the limiting factor for Ca^{2+} influx, its inhibition is likely to be of paramount importance. If this inhibition were a general feature of capacitative influx, then one would expect this mechanism to operate even with receptor stimulation with nondesensitizing agonists.

Mast cells and the related RBL cells secrete histamine and serotonin after antigen stimulation. The secretion critically requires Ca^{2+} influx through a non-voltage-gated Ca^{2+} entry pathway (14–16). However, the source of Ca^{2+} for secretion in

RBL cells is unclear. Using perforated patch recordings, it has been shown that antigen activates I_{CRAC} in RBL cells, and it was concluded that I_{CRAC} is the predominant source of Ca^{2+} for secretion (35). In whole-cell dialysis experiments, we also observed that antigen activates I_{CRAC} (Fig. 2 *e* and *f*) as well as a large Ca^{2+} plateau (Fig. 3 *e* and *f*). Since antigen activates phosphotidylinositol breakdown, one might expect a concomitant activation of protein kinase C and hence inhibition of I_{CRAC}. The finding that antigen-evoked I_{CRAC} was on average -1.6 pA/pF compared with -2.8 and -4 pA/pF for NECA and InsP_3, respectively, would indeed point to such inhibition. The large Ca^{2+} influx evoked by antigen (similar to that evoked by NECA and staurosporine) despite the smaller I_{CRAC} likely arises from the ability of antigen to activate the inward rectifier K^+ channel in RBL cells (36). This would clamp the cells at negative potentials and thereby maintain a large driving force for Ca^{2+} influx through I_{CRAC}. This result stresses the caution required when interpreting Ca^{2+} signals from unclamped cells. The fact that antigen could routinely evoke I_{CRAC} without needing to block protein kinase C, unlike the case with NECA, would suggest that the kinase block of I_{CRAC} is not strong in the case of antigen, since both stimulants produce similar increases in InsP_3 (11). The lack of strong inhibition of I_{CRAC} might be due to several possibilities. The stimulation by antigen might result in a mild or slow stimulation of the dual signaling pathway, producing levels of InsP_3 sufficient to deplete stores but not enough kinase activation to substantially inhibit I_{CRAC}. This would be consistent with the delay in the Ca^{2+} signal to antigen compared with NECA. Alternatively, the stimulation might mainly be targeted at a kinase C isozyme (of which five have been identified in RBL cells) that does not effectively suppress I_{CRAC}.

Because antigen evokes secretion through a process dependent on Ca^{2+} influx, Ca^{2+} entry through I_{CRAC} should be an important source of Ca^{2+} for secretion. I_{CRAC} therefore does more than merely refill depleted stores; it regulates key physiological processes like secretion. Clearly, any factor that regulates I_{CRAC}, like protein kinase C, will have important consequences on cell functions such as secretion.

CONCLUSIONS

Our results have directly shown that I_{CRAC} is inhibited by a protein kinase, probably kinase C. Since protein kinase C will be activated by receptors that increase InsP_3 levels, this demonstrates an important negative feedback mechanism on Ca^{2+} homeostasis and supports the dual signaling hypothesis where InsP_3 and protein kinase C interact to regulate the same process (37). The inability of some receptors that couple to InsP_3 to evoke Ca^{2+} influx, like adenosine A_3 receptors, may be due in part to protein kinase C inhibition of I_{CRAC}. Finally, although the mechanism whereby depletion of stores activates Ca^{2+} influx is unclear, our results indicate that any activating signal may need to overcome the protein kinase inhibition. This could be achieved if the activating mechanism involved either stimulation of a phosphatase (to dephosphorylate the kinase-mediated phosphorylations) or if a signal also inhibited the kinase. The efficacy of the activating signal from depleted stores may be governed by the prevalent kinase C activity.

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1. Tsien, R. W. & Tsien, R. Y. (1990) *Annu. Rev. Cell Biol.* **6,** 715–760.
2. Berridge, M. J. (1993) *Nature (London)* **361,** 315–325.
3. Parekh, A. B., Foguet, M., Lübbert, H. & Stühmer, W. (1993) *J. Physiol. (London)* **469,** 653–671.
4. Girard, S. & Clapham, D. E. (1993) *Science* **260,** 229–232.
5. Yao, Y. & Parker, I. (1993) *J. Physiol. (London)* **468,** 275–296.
6. Putney, J. W. (1986) *Cell Calcium* **7,** 1–12.
7. Hoth, M. & Penner, R. (1992) *Nature (London)* **355,** 353–356.
8. Zweifach, A. & Lewis, R. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 6295–6299.
9. Parekh, A. B., Terlau, H. & Stühmer, W. (1993) *Nature (London)* **364,** 814–818.
10. Fasolato, C., Hoth, M. & Penner, R. (1993) *J. Biol. Chem.* **268,** 20737–20740.
11. Montero, M., Garcia-Sancho, J. & Alvarez, J. (1993) *J. Biol. Chem.* **268,** 13055–13061.
12. Montero, M., Garcia-Sancho, J. & Alvarez, J. (1994) *J. Biol. Chem.* **269,** 3963–3967.
13. Tornquist, K. (1993) *Biochem. J.* **290,** 443–447.
14. Beaven, M. A. & Cunha-Melo, J. R. (1988) *Prog. Allergy* **42,** 123–184.
15. Ali, H., Cuhna-Melo, J. R., Saul, W. F. & Beaven, M. A. (1990) *J. Biol. Chem.* **265,** 745–753.
16. Mohr, F. C. & Fewtrell, C. (1987) *J. Cell Biol.* **104,** 783–792.
17. Hamill, O., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. (1981) *Pflügers Arch.* **391,** 85–100.
18. Qian, Y. X. & McCloskey, M. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7844–7848.
19. McCloskey, M. A. & Cahalan, M. D. (1990) *J. Gen. Phys.* **95,** 205–227.
20. Neher, E. (1989) in *Neuromuscular Junction*, eds. Sellin, L. C., Libelius, R. & Thesleff, S. (Elsevier, Amsterdam), pp. 65–76.
21. Neher, E. (1991) *Int. Arch. Allergy Appl. Immunol.* **94,** 47–50.
22. Richardson, A. & Taylor, C. W. (1993) *J. Biol. Chem.* **268,** 11528–11533.
23. Penner, R. & Neher, E. (1988) *FEBS Lett.* **226,** 307–313.
24. Dieter, P., Fitzke, E. & Duyster, J. (1993) *Biol. Chem. Hoppe Seyler* **374,** 171–174.
25. Eckstein, F. (1985) *Annu. Rev. Biochem.* **54,** 367–402.
26. Chad, J. E. & Eckert, R. (1986) *J. Physiol. (London)* **378,** 31–51.
27. Chung, S. K., Reinhart, P. H., Martin, B. L., Brautigan, D. & Levitan, I. B. (1991) *Science* **253,** 560–562.
28. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. & Kirilovsky, J. (1991) *J. Biol. Chem.* **266,** 15771–15781.
29. Petersen, C. C. H. & Berridge, M. J. (1994) *J. Biol. Chem.* **269,** 32246–32253.
30. Ferris, C. D., Huganir, R. L., Bredt, D. S., Cameron, A. M. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 2232–2235.
31. Bird, G., Rossier, M. F., Obie, J. F. & Putney, J. W. (1993) *J. Biol. Chem.* **268,** 8425–8428.
32. Asaoka, Y., Nakamura, S., Yoshida, K. & Nishizuka, Y. (1992) *Trends Biochem. Sci.* **127,** 414–417.
33. Beaven, M. A., Rogers, J., Moore, J. P., Hesketh, T. R., Smith, G. A. & Metcalfe, J. C. (1984) *J. Biol. Chem.* **259,** 7129–7136.
34. Wojcikiewicz, R. J., Tobin, A. B. & Nahorski, S. R. (1993) *Trends Pharmacol. Sci.* **14,** 279–285.
35. Zhang, L. & McCloskey, M. A. (1995) *J. Physiol. (London)* **483,** 59–66.
36. Gericke, M., Dar, O., Droogmans, G., Pecht, I. & Nilius, B. (1995) *Cell Calcium* **17,** 71–83.
37. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56,** 159–193.