Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 268, No. 28, Issue of October 5, pp. 20737–20740, 1993

A GTP-dependent Step in the Activation Mechanism of Capacitative Calcium Influx*

(Received for publication, July 22, 1993, and in revised form, August 9, 1993)

Cristina Fasolato,‡ Markus Hoth, and Reinhold Penner§

From the Department of Membrane Biophysics, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg, 37077 Göttingen, Federal Republic of Germany

Calcium influx in electrically non-excitable cells is regulated by the filling state of intracellular calcium stores. Depletion of stores activates plasma membrane channels that are voltage-independent and highly selective for Ca^{2+} ions. We report here that the activation of plasma membrane Ca^{2+} currents induced by depletion of Ca^{2+} stores requires a diffusible cytosolic factor that washes out with time when dialyzing cells in the whole-cell configuration of the patch-clamp technique. The activation of calcium release-activated calcium current (I_{CRAC}) by ionomycin- or inositol 1,4,5-trisphosphate-induced store depletion is blocked by guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) and guanyl-5′-yl imidodiphosphate, non-hydrolyzable analogs of GTP, suggesting the involvement of a GTP-binding protein. The inhibition by GTPγS occurs at a step prior to the activation of I_{CRAC} and is prevented by the addition of GTP. We conclude that the activation mechanism of depletion-induced Ca^{2+} influx encompasses a GTP-dependent step, possibly involving an as yet unidentified small GTP-binding protein.

Capacitative Ca^{2+} influx is a general feature of many cell types characterized by induction of Ca^{2+} entry upon depletion of intracellular Ca^{2+} pools (1, 2). The mechanism by which the filling state of stores is communicated to the plasma membrane is unknown; current hypotheses have considered both direct protein-protein interactions and indirect gating through messengers generated by the storage organelles (3, 4). In rat peritoneal mast cells, depletion of intracellular Ca^{2+} stores by three different experimental procedures (inositol 1,4,5-trisphosphate ($InsP_3$),[1] ionomycin, Ca^{2+} chelators) activates a calcium current (I_{CRAC} = calcium release-activated calcium current) (5, 6). Similar depletion-activated Ca^{2+} currents have been identified in Jurkat T cells (7, 8) and other cell types (2). In this study, we investigated I_{CRAC} in rat basophilic leukemia cells (RBL-2H3), a mast cell line that is advantageous for several reasons: 1) it is an easily accessible cell line; 2) cell size and Ca^{2+} currents are larger compared with peritoneal mast cells; 3) secretory activity is smaller and much slower than in peritoneal mast cells. Store depletion in RBL cells by $InsP_3$, ionomycin, or Ca^{2+} chelators activates a current that is essentially indistinguishable from I_{CRAC} in peritoneal mast cells (6) in terms of kinetics, current-voltage relationship, Ca^{2+} selectivity, Ca^{2+}-induced inactivation, and block by divalent ions (data not shown). In the experiments of this study, I_{CRAC} was activated by photoreleasing intracellular caged $InsP_3$ or applying ionomycin extracellularly at appropriate times after establishment of the whole-cell configuration of the patch-clamp technique. Pipette solutions were designed to probe the involvement of diffusible messengers, in particular GTP-binding proteins. $[Ca^{2+}]_i$ was buffered to about resting levels with a mixture of 8 mM BAPTA and 2 mM Ca-BAPTA (free $[Ca^{2+}]_i \approx 60$ nM) to prevent spontaneous activation of I_{CRAC}, which occurs when $[Ca^{2+}]_i$ is buffered to very low values (5). Pipette solutions contained Cs^+ instead of K^+ to avoid G protein-mediated activation of outward potassium currents (9).

MATERIALS AND METHODS

Rat basophilic leukemia cells (RBL-2H3) were cultured on glass coverslips with Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), $NaHCO_3$ (45 mM), glucose (5 mM), streptomycin (0.12 mg/ml), and penicillin (0.60 mg/ml). Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 23–27 °C in a Ringer's solution containing (in mM): NaCl, 140; KCl, 2.8; $CaCl_2$, 10; $MgCl_2$, 2; glucose, 11; Hepes·NaOH, 10, pH 7.2. Sylgard-coated patch pipettes had resistances between 2 and 4 megaohms after filling with the standard intracellular solution, which contained (in mM): cesium glutamate, 145; NaCl, 8; $MgCl_2$, 1; Fura-2, 0.1; Mg-ATP, 0.5; BAPTA (8 mM); Ca-BAPTA (2 mM); Hepes·CsOH, 10, pH 7.2. This solution was supplemented with GTPγS, GDPβS, GDP, GTP, GMP-PNP, ATPγS, ATP, NaF, and $AlCl_3$, or caged $InsP_3$ (Calbiochem) when needed. For nucleotide concentrations above 0.5 mM, equimolar amounts of $MgCl_2$ were also included. Extracellular solution changes were made by short application (5–10 s) from a wide-tipped micropipette that contained standard Ringer's supplemented with ionomycin (14 μM, Calbiochem). The concentration of $[Ca^{2+}]_i$ was monitored with a photomultiplier-based system as described (10) in order to control the efficacy of Ca^{2+} buffers in the cell. All cells included in the analysis had no significant changes in $[Ca^{2+}]_i$. For experiments with caged $InsP_3$, the filter wheel (360/390 nm) was stopped in the dark position before starting an experiment, and a short illumination at 360 nm was delivered when needed. A 10-s flash was enough to fully activate I_{CRAC} when 400 μM caged $InsP_3$ was included in the patch pipette. Low resolution currents were sampled at 2 Hz and filtered at 500 Hz. High resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9; HEKA, Lambrecht, Federal Republic of Germany). Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. Holding potential was usually 0 mV. Most of the current amplitudes were determined at a potential of −40 mV, taken from high resolution currents in response to voltage ramps. Currents were filtered at 2.3 kHz and digitized at 100-μs intervals. For analysis and presentation, currents were filtered digitally to 1 kHz.

RESULTS AND DISCUSSION

To test for washout of diffusible cytosolic constituents required for signaling the filling state of Ca^{2+} stores to plasma membrane Ca^{2+} channels, we dialyzed RBL cells for variable

* We acknowledge support by the following institutions: Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 236, Hermann- und Lilly-Schilling-Stiftung (to R. P.), and the European Molecular Biology Organization (EMBO) (to C. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dip. di Scienze Biomediche, Università di Padova, Via Trieste 75, 35121 Padova, Italy.

§ To whom correspondence should be addressed. Tel.: 49-551-201-640; Fax: 49-551-201-688; E-mail: RPENNER@GWDU03.GWDG.DE.

[1] The abbreviations used are: $InsP_3$, inositol 1,4,5-trisphosphate; BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GDPβS, guanyl-5′-yl thiophosphate; GMP-PNP, guanyl-5′-yl imidodiphosphate; ATPγS, adenosine 5′-*O*-(thiotriphosphate); F, farad; S, siemens.

periods of time prior to emptying the stores by the Ca^{2+} ionophore ionomycin. As shown in Fig. 1*A*, at the instance of break-in, there was an initial outward current, likely due to a small K^+ conductance at 0 mV, which rapidly decayed to zero as the cesium-containing pipette solution equilibrated in the cytosol. Thereafter, depletion of Ca^{2+} stores by ionomycin activated a small inward current at 0 mV. Voltage dependence as assessed by voltage ramps (Fig. 1*A*, *right panel*), and kinetic behavior of the inward current identified it as the previously characterized I_{CRAC} (5, 6). Depletion-activated Ca^{2+} currents were larger when depleting stores early in the experiment as compared with currents activated at later times. To compare the amplitude among different cells we corrected the traces for cell capacitance (which is a measure for cell size) and for series conductance (which determines the rates of diffusional exchange between cell and pipette) (11). The details are given in the figure legend. Fig. 1*B* (*left panel*) shows the normalized traces of Fig. 1*A* (*left panel*). The relationship between the time of store depletion after break-in (normalized for series conductance) and the resulting amplitude of I_{CRAC} (normalized for cell capacitance) is depicted in Fig. 1*B* (*right panel*) for all tested cells. The relationship follows an exponential time course with a time constant of about 250 s (considering the average series conductance of 174 ± 74 nS, mean ± S.D., n = 91). The gradual decrease in the maximum amplitude of I_{CRAC} with prolonged whole-cell dialysis suggests that activation of I_{CRAC} requires a cytosolic component that diffuses out of the cell during the time of dialysis.

The rather slow time course of washout indicates that the diffusible factor is a fairly large molecule rather than a nucleotide, inositol phosphate, or other small second messenger; these would washout in less than 1 min (11). Nevertheless, we tested a number of second messengers and metabolites (cAMP, 50 μM; cGMP, 50 μM; ATP, 0.5–5 mM; GTP, 0–8 mM; GDP, 0–5 mM; inositol 1,3,4,5-tetrakisphosphate, 100 μM) and found none to significantly activate or inhibit I_{CRAC} when applied intracellularly. However, although there was no apparent requirement for GTP in the pipette solution, we did find an inhibitory effect of non-hydrolyzable analogs of GTP on the activation of I_{CRAC}. Fig. 2*A* illustrates that intracellular application of GTPγS (100 μM) prevents the ionomycin-induced activation of I_{CRAC} normally seen in control cells. The relationship between the time of store depletion (after establishment of the whole-cell configuration) and the resulting amplitude of I_{CRAC} in the presence of GTPγS is shown in Fig. 2*B* (*left panel*). The relationship follows an exponential time course with a time constant of about 80 s (considering the average series conductance of 201 ± 67 nS, mean ± S.D., n = 42). The effect of GTPγS was dose-dependent over the concentration range tested (10–500 μM, data not shown), saturating at about 100 μM.

The inhibitory effect of GTPγS (100 μM) could be antagonized by adding GTP (5 mM) to GTPγS-containing pipette solutions (Fig. 2*A*), which restored the extent to which store depletion could activate I_{CRAC} to control levels (Fig. 2*B*, *left panel, open squares*). Another non-hydrolyzable analog of GTP, GMP-PNP, was also effective in preventing ionomycin-induced activation of I_{CRAC} but required somewhat higher concentrations (500 μM) to produce equivalent effects. GTPγS effects do not seem to result from γ-thiophosphorylation since ATPγS (100 μM) had no inhibitory effect (Fig. 2*A* and 2*B*, *right panel, open diamonds*) and ATP (up to 5 mM) did not antagonize the GTPγS effect (data

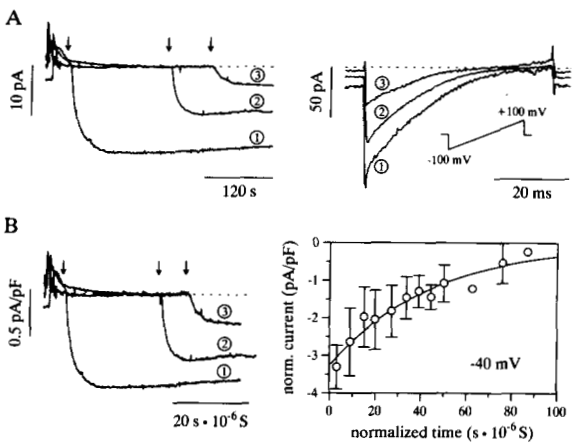

FIG. 1. **Current amplitudes of ionomycin-induced I_{CRAC} become smaller with prolonged dialysis.** *A*, superimposed current records of cells perfused with the standard pipette solution for variable times before applying ionomycin (14 μM, indicated by the *arrows*) to induce store depletion and triggering of I_{CRAC}. Temporal pattern of current activation at 0 mV holding potential is shown in the *left panel*. The *right panel* depicts the corresponding high resolution net currents across voltage in response to the ramp protocol shown (*inset*). *B*, the *left panel* shows the same currents as in *A* normalized for capacitance (C_m) and series conductance (G_s). The washout of diffusible substances from the cytosol is dependent on series resistance (diffusion rates increase proportionally with series conductance), and the amplitudes of currents are dependent on both the concentration of diffusible messengers and on cell capacitance. The parameters for the cells were: *1*, C_m = 16.9 pF, G_s = 130 nS; *2*, C_m = 11.6 pF, G_s = 130 nS; *3*, C_m = 13.5 pF, G_s = 141 nS. In the *right panel*, the normalized current amplitude taken from voltage ramps at −40 mV is plotted as a function of the time delay of ionomycin application. The data of 91 cells were pooled at intervals of 6 s·10^{-6} S. The standard deviation of the current is given by the *error bars*; the standard deviation of the normalized time is in the range of 0.2–2 s·10^{-6} S for all points. The data could be fitted by a single exponential function of the form A exp ($-t/T$), where the amplitude A = −3.3 pA/pF and effective time constant T = 44.9 s·10^{-6} S. Considering the mean series conductance of 174 nS of all these experiments, the time constant for the current decrease is ~250 s.

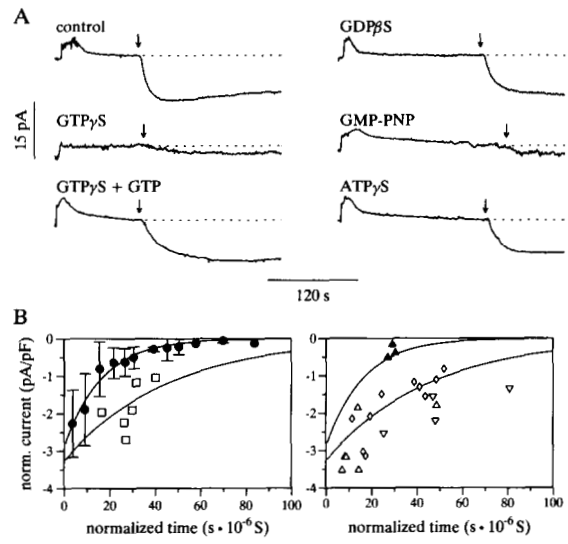

FIG. 2. **GTPγS inhibits ionomycin-induced I_{CRAC}.** *A*, ionomycin-induced I_{CRAC} under different experimental conditions (holding potential, 0 mV). As indicated in the graphs, the standard pipette solution was supplemented with GTPγS (100 μM), GTPγS (100 μM) plus GTP (5 mM), GDPβS (500 μM), GMP-PNP (500 μM), or ATPγS (100 μM). The *arrows* indicate the start of the ionomycin application (5–10 s). *B*, current amplitudes of I_{CRAC} at −40 mV as a function of time delay of ionomycin application (normalized as described in Fig. 1). In the *left panel* the data for GTPγS (n = 42) were pooled (●) and fitted with a single exponential function A exp ($-t/T$), where A = -2.9 pA/pF and T = 16.3 s·10^{-6} S, yielding a time constant of about 80 s for the current decrease (considering the mean series conductance of 201 nS, 42 experiments). The fitted curve under control conditions (taken from Fig. 1) and the points for GTPγS plus GTP (□) are also shown in the *left panel*. The *right panel* depicts the two fitted curves (control and GTPγS) and the points for GDPβS (△, = 100 μM, ▽ = 500 μM), ATPγS (◇), and GMP-PNP (▲).

not shown). GDPβS (up to 500 μM) had no inhibitory effect on I_{CRAC} (Fig. 2*A*). Indeed, in the presence of GDPβS current amplitudes were slightly larger than control currents (Fig. 2*B*, *right panel, open triangles*). One possible reason for the increased amplitudes in the presence of GDPβS may be that I_{CRAC} is tonically inhibited by basal trimeric G protein activity, possibly through kinase phosphorylation (see AlF_4^- effects below). The inhibition of trimeric G proteins by GDPβS may relieve this tonic modulation, resulting in an enhanced response following store depletion.

To exclude that GTPγS interferes with ionomycin-induced store depletion we used photohydrolyzable caged $InsP_3$ to activate I_{CRAC} at later times. As depicted in Fig. 3*A* the inhibitory effect of GTPγS was also observed in these experiments. The data obtained with caged $InsP_3$ well fitted to the relationship

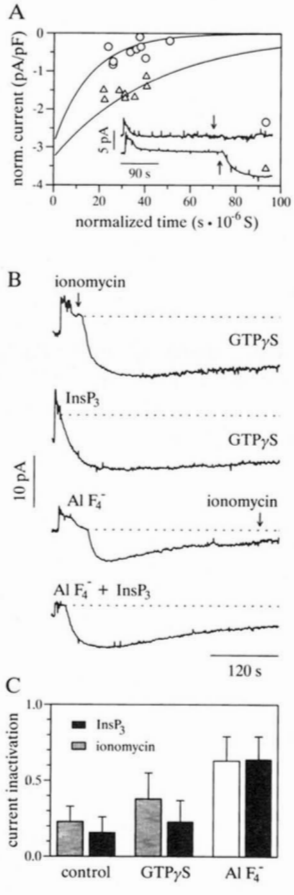

FIG. 3. **GTPγS and AlF_4^- act in different ways.** *A*, GTPγS inhibits $InsP_3$-induced activation of I_{CRAC}. Data points represent current amplitudes of I_{CRAC} at −40 mV in the absence (△) and presence of 100 μM GTPγS (○) as a function of time delay of photoreleased $InsP_3$ (400 μM). Currents were normalized as described in Fig. 1. For comparison, the two curves for ionomycin-induced current amplitudes under control conditions and in the presence of GTPγS are also shown (taken from Fig. 2*B*). The *insets* illustrate two examples of current responses (△ = control, ○ = 100 μM GTPγS) recorded at 0 mV upon release of caged $InsP_3$ (time of photolysis is indicated by *arrows*). *B*, GTPγS does not significantly inhibit I_{CRAC} when it is activated by applying ionomycin quickly after break-in or by co-perfusion with $InsP_3$ (*upper traces*). In contrast, AlF_4^- (5 mM NaF, 50 μM $AlCl_3$) induces a fast inactivation of the current activated by AlF_4^- alone, or by co-perfusion of $InsP_3$ (*lower traces*). *C*, the relative amount of current inactivation at a holding potential of 0 mV after 390 s of perfusion of the cell is shown for different activation procedures (as labeled in the graph). Current inactivation represents the ratios of current amplitude after 390 s and the maximal current. Data are means ± S.D. of experiments in which I_{CRAC} was activated by ionomycin (n = 9), $InsP_3$ (n = 10), ionomycin + GTPγS (n = 7), $InsP_3$ + GTPγS (n = 12), AlF_4^- (n = 9), AlF_4^- + $InsP_3$ (n = 10). Only experiments in which activation of the current was induced within 100 s following break-in were taken into account.

calculated with ionomycin, both in the absence and presence of GTPγS.

We asked whether the inhibition of I_{CRAC} by GTPγS occurred prior to the activation of I_{CRAC} or whether it was due to a modulatory effect, possibly resulting from activation of G protein-mediated negative feedback mechanisms, which might be anticipated to regulate a process as paramount as Ca^{2+} influx. Interestingly, however, GTPγS does not significantly inhibit I_{CRAC} once it is activated as evidenced by Fig. 3*B*. In this experiment GTPγS was applied through the patch pipette, and ionomycin was applied shortly after break-in, resulting in a fast and sustained activation of I_{CRAC}. Likewise, GTPγS was unable to reduce I_{CRAC} when current activation was induced even earlier by $InsP_3$ co-applied through the patch pipette (Fig. 3, *B* and *C*). Together, these results suggest that a GTP-dependent step is involved in activating I_{CRAC} and that non-hydrolyzable analogs interfere with the activation at some crucial early step in the signal transduction pathway. A channel block by GTPγS itself, a G protein, or some other second messenger is highly unlikely, as one would have to postulate that channels can only be blocked in their closed conformation and, once activated, they prevail in a state of high open probability, rendering them no longer susceptible to GTPγS inhibition. In fact, however, open probability of activated channels seems to be low, as suggested by noise analysis of I_{CRAC} in Jurkat cells (7).

Primary targets of non-hydrolyzable GTP analogs are heterotrimeric (40–45 kDa) and monomeric GTP-binding proteins of small molecular mass (19–21 kDa) (12, 13). The former are activated irreversibly by non-hydrolyzable GTP analogs whereas the latter require GTP hydrolysis for functioning and are inhibited by GTPγS. Our results show that the main effect of GTPγS is to increase the rate of washout of the I_{CRAC} response to ionomycin, which we interpret to be the result of interference with the activation cycle of small G proteins. An alternative interpretation would be that GTPγS effects are mediated by a trimeric G protein whose washout is accelerated by dissociation of α and βγ-subunits. Three arguments make this possibility seem unlikely. 1) Washout occurs under non-stimulatory conditions and GDPβS, which should prevent dissociation of trimeric G proteins, does not alter the rate of run-down. 2) GTPγS does not reduce the current once it has been activated, which would have been expected assuming a trimeric G protein mechanism. 3) Similarly, GTP (like GTPγS) is expected to activate and dissociate trimeric G proteins, which in turn would enhance the washout and reduce I_{CRAC}. However, we find that GTP antagonizes GTPγS effects, a key feature of small G protein mechanism.

Pharmacological tools to discern between trimeric and small GTP-binding proteins are sparse. AlF_4^- ions are thought to be specific activators of trimeric G proteins and ineffective at stimulating small GTP-binding proteins (14), prompting us to test AlF_4^- for its effects on I_{CRAC}. Fig. 3 illustrates that AlF_4^- indeed exhibited a different response pattern than GTPγS: perfusing cells with AlF_4^- consistently activated I_{CRAC}. However, the AlF_4^--induced current underwent a fast inactivation that could not be recovered by ionomycin addition (n = 8, Fig. 3*B*). In contrast to GTPγS, the AlF_4^--induced fast inactivation was still evident with early activation of I_{CRAC} by $InsP_3$-induced store depletion (Fig. *B* and *C*). Furthermore, the effects of AlF_4^- were not antagonized by 5 mM GTP (data not shown).

The activation of I_{CRAC} by AlF_4^- could be the result of store depletion by a massive stimulation of $InsP_3$ production via activation of phospholipase C through a trimeric G protein. The inability of GTPγS to induce store depletion through trimeric G proteins could be accounted for by insufficient build-up of $InsP_3$ to induce store depletion. We noted that in RBL cells, the minimal $InsP_3$ concentration required to activate I_{CRAC} (10 μM) is

about 1 order of magnitude higher than in peritoneal mast cells under identical conditions (K^+-based pipette solutions, EGTA as Ca^{2+} chelator). In addition, the presence of Cs^+ and BAPTA in the pipette further reduce the efficacy of $InsP_3$, such that at least 25 μM $InsP_3$ are required to activate I_{CRAC}. Possible explanations for a reduced efficacy of GTPγS as compared to AlF_4^- could be as follows. 1) the GDP/GTP exchange rate of the G protein that couples to phospholipase C, which constitutes the rate-limiting step in GTPγS action, may be too slow to produce levels of $InsP_3$ that overwhelm removal processes; 2) GTPγS effects might be inhibited by BAPTA; in peritoneal mast cells Br_2-BAPTA causes inhibition of Ca^{2+}-independent GTPγS-induced secretion (15).

The AlF_4^--induced inactivation of I_{CRAC} is clearly different from the GTPγS-induced block of activation, as AlF_4^- was still able to inhibit I_{CRAC} after it had been activated. For the reasons outlined above, GTPγS does not seem to effectively stimulate trimeric G proteins and therefore would not induce this inhibitory modulation. The inhibitory effects of AlF_4^- may be due to a modulation of the current itself, possibly through activation of negative feedback mechanisms secondary to trimeric G protein stimulation (*e.g.* phosphorylation through protein kinases). In addition, NaF is known to inhibit phosphatases (16) that might act synergistically to promote phosphorylation processes. Hence, while it is apparent that GTPγS and AlF_4^- exhibit obvious differences in their effects on cellular responses, they seem to do so with respect to both trimeric and monomeric G proteins.

In summary, our results are consistent with the notion that the signal for capacitative Ca^{2+} entry following depletion of intracellular Ca^{2+} stores involves a GTP-dependent step. It is tempting to speculate that this step encompasses a small G protein that is blocked by non-hydrolyzable GTP analogs. If this interpretation is correct one could imagine at least three, admittedly speculative, models of how capacitative Ca^{2+} influx might be activated by small G proteins. 1) For direct G protein activation of I_{CRAC} there must be a Ca^{2+} sensor of the luminal Ca^{2+} concentration that translates store depletion into activation of the G protein. This is possibly aided by an accessory protein, such as GDS (GDP dissociation stimulator), which catalyzes GDP-GTP exchange (17). Then the G protein acts as a messenger to gate the Ca^{2+} channels in the plasma membrane. Washout of the small G protein itself, or of accessory proteins, may be responsible for the observed current rundown. Termination of the activated state of the G protein in the intact cell may involve other accessory proteins such as GAPs (GTPase-activating proteins), which will catalyze hydrolysis of GTP (13, 17) and render the G protein inactive. This accessory protein may also be subject to washout in whole-cell experiments, which would result in an irreversible activation of I_{CRAC}. 2) For indirect G protein activation of I_{CRAC} the G protein could act as an intermediate signal to produce another as yet unidentified diffusible messenger. 3) G protein-induced fusion activates I_{CRAC}; this could be proposed on the basis of the well documented role of small G proteins in vesicular traffic (13, 18–20). It is conceivable that activation of a small G protein induces fusion of specialized vesicles that carry Ca^{2+} channels. These channels would then insert in the plasma membrane, and Ca^{2+} influx can occur as long as they remain in the membrane. Termination of the current through these new channels could be accomplished by endocytic activity.

Acknowledgment—We thank M. Pilot for technical assistance.

REFERENCES

1. Putney, J. W. (1990) *Cell Calcium* **11,** 611–624
2. Penner, R., Fasolato, C., and Hoth, M. (1993) *Curr. Opin. Neurobiol.* **3,** 368–374
3. Irvine, R. F. (1992) *FASEB J.* **6,** 3085–3091
4. Berridge, M. J. (1993) *Nature* **361,** 315–325
5. Hoth, M., and Penner, R. (1992) *Nature* **355,** 353–356
6. Hoth, M., and Penner, R. (1993) *J. Physiol. (Lond.)* **465,** 359–386
7. Zweifach, A., and Lewis, R. S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6295–6299
8. McDonald, T. V., Premack, B. A., and Gardner, P. (1993) *J. Biol. Chem.* **268,** 3889–3896
9. McCloskey, M. R., and Cahalan, M. D. (1990) *J. Gen. Physiol.* **95,** 205–227
10. Neher, E. (1989) in *Neuromuscular Junction* (Sellin, L. C., Libelius, R., and Thesleff, S., eds) pp. 65–76, Elsevier Science Publishers B.V., Amsterdam
11. Pusch, M., and Neher, E. (1988) *Pflügers Arch. Eur. J. Physiol.* **411,** 204–211
12. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature* **349,** 117–126
13. Hall, A. (1993) *Curr. Opin. Cell Biol.* **5,** 265–268
14. Kahn, R. A. (1991) *J. Biol. Chem.* **266,** 15595–15597
15. Penner, R., and Neher, E. (1988) *FEBS Lett.* **226,** 307–313
16. Brautigan, D. L., and Shriner, C. L. (1988) *Methods Enzymol.* **159,** 339–346
17. Bollag, G., and McCormick, F. (1991) *Annu. Rev. Cell Biol.* **7,** 601–632
18. Goud, B., and McCaffrey, M. (1991) *Curr. Opin. Cell Biol.* **3,** 626–633
19. Rothman, J. E., and Orci, L. (1992) *Nature* **355,** 409–415
20. Balch, W. (1990) *Trends Biochem. Sci.* **15,** 473–477