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Am J Physiol Cell Physiol 284:497-505, 2003. First published Oct 16, 2002;
doi:10.1152/ajpcell.00183.2002

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Regulation of Ca^{2+} release-activated Ca^{2+} channels by INAD and Ca^{2+} influx factor

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Submitted 22 April 2002; accepted in final form 8 October 2002

Su, Zhengchang, Douglas S. Barker, Peter Csutora, Theresa Chang, Richard L. Shoemaker, Richard B. Marchase, and J. Edwin Blalock. Regulation of Ca^{2+} release-activated Ca^{2+} channels by INAD and Ca^{2+} influx factor. *Am J Physiol Cell Physiol* 284: C497–C505, 2003. First published October 16, 2002; 10.1152/ajpcell.00183.2002.—The coupling mechanism between depletion of Ca^{2+} stores in the endoplasmic reticulum and plasma membrane store-operated ion channels is fundamental to Ca^{2+} signaling in many cell types and has yet to be completely elucidated. Using Ca^{2+} release-activated Ca^{2+} (CRAC) channels in RBL-2H3 cells as a model system, we have shown that CRAC channels are maintained in the closed state by an inhibitory factor rather than being opened by the inositol 1,4,5-trisphosphate receptor. This inhibitory role can be fulfilled by the *Drosophila* protein INAD (inactivation-no after potential D). The action of INAD requires Ca^{2+} and can be reversed by a diffusible Ca^{2+} influx factor. Thus the coupling between the depletion of Ca^{2+} stores and the activation of CRAC channels may involve a mammalian homologue of INAD and a low-molecular-weight, diffusible store-depletion signal.

store-operated ion channels; inositol 1,4,5-trisphosphate receptor

AGONIST-RECEPTOR INTERACTIONS at the plasma membrane often lead to the generation of inositol 1,4,5-trisphosphate (IP_3), which in turn releases Ca^{2+} from internal stores in the endoplasmic reticulum (ER). Such depletion of Ca^{2+} stores gives rise to a signal that activates Ca^{2+}-permeable store-operated channels (SOCs) in the plasma membrane, allowing for the sustained Ca^{2+} influx termed capacitative or stored-operated Ca^{2+} entry (6, 36). Four not necessarily distinct models have been proposed to account for the activation mechanism of SOCs (37). First, a physical link between IP_3 receptors (IP_3R) and SOCs is proposed (15, 17, 22, 35). Changes in the conformation of IP_3R upon IP_3 binding and/or store depletion activates SOCs. In the second, secretory-like transport vesicles are proposed as a source for the insertion of channels previously not present in the plasma membrane (30, 55). Third, a freely diffusible soluble messenger molecule, termed Ca^{2+} influx factor (CIF), is formed upon Ca^{2+} store depletion and activates the SOCs (8, 14, 38, 39, 44, 47), either directly or through interaction with closely associated regulatory proteins. Fourth, SOCs are kept in an inhibited state by an inhibitory mechanism when the Ca^{2+} stores are full, and discharge of Ca^{2+} stores removes such an inhibitory mechanism (37).

The Ca^{2+} release-activated Ca^{2+} (CRAC) channel is the best characterized of the SOCs (29). Its activation mechanism remains largely unknown (7), although several aspects of this regulation have been established. First, it is clear that depletion of intracellular Ca^{2+} stores (12, 28) leads to the activation of I_{CRAC} (current conducted by CRAC channels) within tens of seconds. Second, CRAC channels in RBL cells are not likely activated by a secretion-like coupling mechanism (1). Third, CRAC channels in chicken DT40 cells in which all isoforms of IP_3R are knocked out are activated normally upon store depletion, suggesting that physical coupling between IP_3R and CRAC channels is not a mechanism of CRAC activation (33, 45). Fourth, whole cell patch-clamp experiments have established that achieving this electrophysiological configuration itself leads to I_{CRAC} development, but only after hundreds of seconds and progressively less rapidly as the $[Ca^{2+}]$ in the pipette solution is buffered to levels approaching or exceeding 100 nM (12, 28). Importantly, this activation is seen well before global ER Ca^{2+} store depletion is observed (19). This finding suggests that low Ca^{2+} itself is sufficient to activate I_{CRAC}, at least in the context of the large dilution of cytoplasmic constituents resulting from equilibration with the patch pipette solution. One explanation for this result invokes the dissociation of a regulatory factor from the CRAC channel, a dissociation that is facilitated by low levels of Ca^{2+} and dilution of the cytoplasm. Fifth, we have recently determined (8) that partially purified acid extracts from cells in which Ca^{2+} stores have been depleted, either with thapsigargin or by genetic disruption of sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase, will accelerate the activation of I_{CRAC} in whole cell patch-clamp experiments. This pro-

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vides compelling evidence for the existence of a CIF-like molecule in CRAC channel regulation. However, how CIF might activate CRAC channels is essentially unknown. Here, we begin to reconcile these findings and suggest a unified model for CRAC channel activation.

MATERIALS AND METHODS

Cell culture. Rat basophilic leukemia (RBL-2H3) cells were purchased from the American Type Culture Collection (Rockville, MD) and were maintained at 37°C in 5% CO_2 in Eagle's minimum essential medium (Sigma, St. Louis, MO) with the following substitutions (Mediatech, Herndon, VA): 10% heat-inactivated fetal calf serum, 10,000 IU/ml penicillin, 10,000 μg/ml streptomycin, 2 mM glutamine, 10 mM sodium pyruvate, 10 mM nonessential amino acid, and 25 mM HEPES. Cells were treated by trypsin, plated on coverslips, and used on *days 3* and *4*.

Patch-clamp recordings. Whole cell recordings were performed with RBL-2H3 cells at room temperature. Standard external solution contained (in mM) 143 NaCl, 10 $CaCl_2$, 1 $MgCl_2$, 5 D-glucose, 4.5 KCl, 0.5 $BaCl_2$, and 10 HEPES, pH 7.3 adjusted with NaOH. Ca^{2+}-free solution (CFS) was made by replacing $CaCl_2$ in this solution with an equimolar concentration of $MgCl_2$ to abolish I_{CRAC} and to obtain a leak current for subtraction to eliminate possible contamination current from Mg^{2+}-inhibited cation (MIC) channels (34). Divalent cation-free solution (DVF) contained (in mM) 160 Na-methane sulfonate, 5 D-glucose, 2 Na-EGTA, and 10 HEPES, pH 7.2 adjusted with NaOH. Standard internal solution for whole cell recordings contained (in mM) 110 Cs-glutamate, 10 CsCl, 2.9 $MgCl_2$, 0.6 $CaCl_2$, 10 Cs-EGTA, and 30 HEPES, pH 7.2 adjusted with CsOH. The calculated free [Ca^{2+}] and [Mg^{2+}] were 10 nM and 2 mM, respectively. To obtain 100 nM free [Ca^{2+}], the $CaCl_2$ and $MgCl_2$ concentrations in the standard internal solution were changed to 3.7 and 2.6 mM (free [Mg^{2+}] = 2 mM), respectively. In some experiments, intracellular solution contained (in mM) 110 Cs-glutamate, 7 $MgCl_2$, 5 Mg·ATP, 0.5 $CaCl_2$, 10 Cs-EGTA, and 30 HEPES, pH 7.2 adjusted with CsOH. The calculated free [Ca^{2+}] and [Mg^{2+}] were 10 nM and 5 mM, respectively. This solution was used to block MIC channels (18, 26, 34, 40). Whole cell I_{CRAC} was detected by applying a 100- or 320-ms voltage ramp from −100 to 100 mV while the cell was held at −40 mV at a frequency of 1 or 0.2 Hz. The currents measured when the cells were perfused by CFS were used as leak current for subtraction to eliminate possible MIC current contamination (34).

INAD expression and purification. Truncated *inad* gene produced by *Nco*I-*Pst*I restriction endonuclease digestion of pCMV4/InaD was ligated into pBAD*mychis*A (Invitrogen) to generate pBADInaDTr. An 890-bp PCR product generated from pCMV4/InaD with the primers Pr.INAD3′END (5′-TCG TCT CTA AAG CTT GGG TGC CTC-3′) and Pr.INAD3 (5′-ATG GTC ATC TAT GGC AAG C-3′) was digested with *Hin*dIII and ligated to *Pst*I-*Hin*dIII-digested pBADInaDTr. This linear construct was digested with *Pst*I and self-ligated to produce pBADInaD. Nucleotide sequences of both pBADInaD and pBADInaDTr were confirmed at the Iowa State University DNA Sequencing Facility (Ames, Iowa).

INAD expression from pBADInaD was induced in logarithmically growing *Escherichia coli* TOP10 cells with 0.2% L-arabinose in Luria-Bertani broth with 100 μg/ml ampicillin for 4 h at 37°C. Cells from 6 liters of induction medium were collected and resuspended in 10 ml of lysis buffer (6 M guanidine·HCl, 20 mM Na·phosphate, pH 7.4, 0.5 M NaCl, 0.1 mM PMSF, 0.1 μg/ml leupeptin, and 0.1 μg/ml Pefabloc) with gentle shaking on ice for 30 min and then disrupted by sonication. Cell debris was pelleted, and soluble proteins were renatured at 4°C by dialysis, first against dialysis buffer (1 M guanidine·HCl, 0.1 mM PMSF, 0.1 μg/ml leupeptin, and 0.1 μg/ml Pefabloc) and twice against water and protease inhibitors (0.1 mM PMSF, 0.1 μg/ml leupeptin, and 0.1 μg/ml Pefabloc). Precipitated proteins were removed by centrifugation, and the supernatant was brought to 30% saturation with ammonium sulfate at 4°C. Precipitated proteins were pelleted, and the supernatant was brought to 40% ammonium sulfate saturation at 4°C. Precipitated protein was again pelleted and then resuspended in loading buffer (20 mM Na·phosphate, pH 5.0, 0.5 M NaCl). Insoluble protein was removed by centrifugation, and the supernatant was applied to a HiTrap chelating column (Pharmacia Biotech) preequilibrated with loading buffer. The column was washed with 10 bed volumes of loading buffer, and INAD was eluted with 5 bed volumes of elution buffer (20 mM Na·phosphate, pH 4.0, 0.5 M NaCl). INAD was then dialyzed twice at 4°C against 1 liter of standard internal solutions for whole cell recordings, with precipitated proteins removed by centrifugation. Final protein concentrations were determined by Bradford assay using a BSA standard curve. The supernatant typically contained a protein concentration of 50–100 μg/ml and was used in the electrophysiological experiments without further dilution.

CIF preparation. We used a previously described procedure to prepare CIF from human platelets (8).

Data analysis. Patch-clamp data were analyzed using pCLAMP 8 (Axon, Foster City, CA). The activation time course of CRAC channels was fitted to equations

$$I = I_0 + I_1 \exp[-(t - \tau_{delay})/\tau)]$$

where I is current, t is time, I_0 and I_1 are constants, τ is the activation time constant, and τ_{delay} is the time when the current develops to 10% of the maximum level after whole cell break-in. Data are shown as means ± SE, and the t-test was used for statistical analysis. The inhibitory effect of 2-aminoethoxydiphenyl borate (2-APB) on CRAC channels in Fig. 2*D* is defined by

$$\text{Inhibition} = (I_{before} - I_{after})/I_{before}$$

where I_{before} is steady-state I_{CRAC} before 2-APB application and I_{after} is steady-state I_{CRAC} after 2-APB application. The data are fitted to the Hill equation

$$\text{Inhibition} = \text{Inhibition}_{max}/[1 + (IC_{50}/[2 - \text{APB}]^{n_H})]$$

where Inhibition_{max} is the maximum inhibition of I_{CRAC} by 2-APB, IC_{50} is the 2-APB concentration that causes half-maximum inhibition, and n_H is the Hill coefficient.

RESULTS

Whole cell I_{CRAC} can be isolated by a leak current subtraction method. Several groups have recently demonstrated that omission of Mg^{2+} or Mg·ATP in the whole cell pipette can activate a cation current termed Mg^{2+}-nucleotide-regulated metal ion (MagNum) current (11) or Mg^{2+}-inhibited cation (MIC) current (34) in both Jurkat and RBL cells that is presumably encoded by LTRPC7/TRP-PLIK/TRPM7 gene (11, 18, 26, 34). Even though MIC channels can be inhibited intracellularly by Mg^{2+} alone with an IC_{50} of 0.5 mM, they still can be sporadically activated during whole cell record-

ings when intracellular free [Mg^{2+}] is <3 mM (34). Prakriya and Lewis (34) have suggested that pure I_{CRAC} can be isolated from possible MIC-contaminated current by subtracting a leak current taken when cells are exposed to a Ca^{2+}-free but Mg^{2+}-containing solution. To validate this method, we compared the divalent and monovalent currents recorded with two different intracellular solutions after the aforementioned leak current subtraction was performed. As shown in Fig. 1*A*, when the pipette contained 7 mM $MgCl_2$ and 5 mM Mg·ATP, a typical I_{CRAC} carried by Ca^{2+} with an inwardly rectifying current-voltage (*I-V*) relationship and a reversal potential >50 mV (Fig. 1*B*) was spontaneously activated after whole cell break-in. The channels activated were not permeable to Mg^{2+} (Fig. 1*A*), which is a hallmark property of CRAC channels (18, 20, 34). Switching the bath solution from the standard external solution to DVF invoked a larger Na^+-carried current with an inwardly rectifying *I-V* relationship and a reversal potential around 50 mV, which rapidly inactivated in ~20 s to a steady state, another hallmark of I_{CRAC} (2, 18, 20, 34), further suggesting that the current is solely conducted by CRAC channels. These results thus confirmed that high levels of Mg^{2+}/ATP (~5 mM) in the pipette solution could completely block spontaneous activation of MIC channels (18, 20, 34). As shown in Fig. 1, *C* and *D*, when the free [Mg^{2+}] level in the pipette was buffered to 2 mM and the aforementioned leak current subtraction method was used, Ca^{2+} and Na^+ currents were recorded that were indistinguishable from those when the pipette contained 7 mM $MgCl_2$ and 5 mM Mg·ATP in terms of Mg^{2+} impermeability, inwardly rectified *I-V* relationships for both Ca^{2+} and Na^+ currents, reversal potentials, and inactivation of the monovalent current. These results suggest that the current isolated was solely conducted by CRAC channels and confirms that this leak current subtraction method is sufficient to eliminate possible MIC current contamination (34). We thus used this procedure of leak current subtraction in all the following experiments.

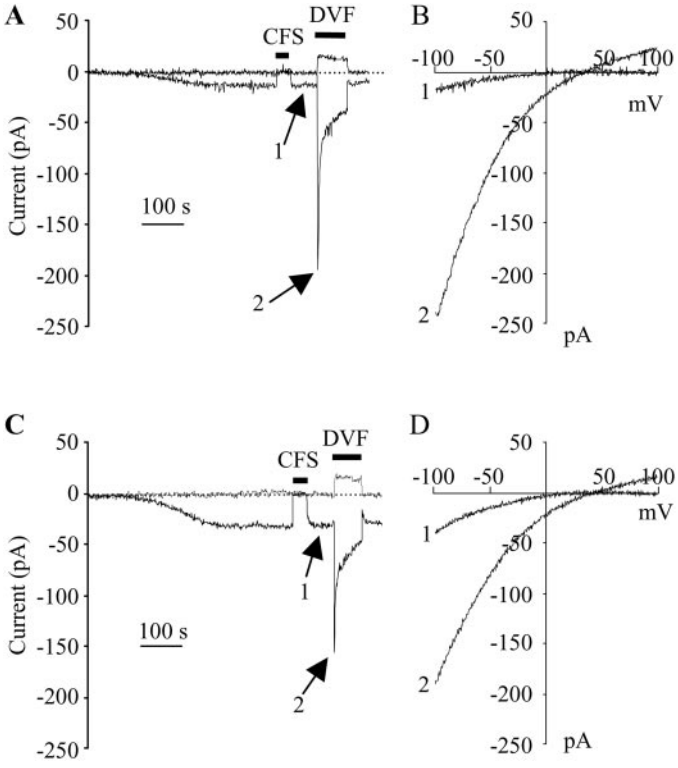

Fig. 1. Current conducted by Ca^{2+} release-activated Ca^{2+} (CRAC) channels (I_{CRAC}) can be isolated by leak current subtraction. *A*: pipette solution contained 7 mM $MgCl_2$ and 5 mM Mg·ATP. *Top trace* was measured at 80 mV; *bottom trace* was measured at −80 mV. Number-labeled arrows indicate time points for display of the current-voltage (*I-V*) relationship in *B*. CFS, Ca^{2+}-free solution; DVF, Divalent cation-free solution. *B*: *I-V* relationships of current taken at the corresponding time points in *A*. *C*: pipette solution contained 2.9 mM $MgCl_2$ (free [Mg^{2+}] = 2 mM). The labels have the same meaning as in *A*. *D*: *I-V* relationships of current taken at the corresponding time points in *C*.

CRAC channels are not activated by physical interaction between IP_3R and CRAC channels. Recent results with an IP_3R blocker 2-APB have been used to argue that SOC activation is due to an IP_3- and/or depletion-mediated conformational change in the IP_3R (22). The relative unimportance of the IP_3R as an activator for CRAC channels is suggested by the observation that I_{CRAC} spontaneously and normally developed during whole cell dialysis even in the presence of the IP_3 receptor antagonist heparin (0.5 mM) in the pipette solution (Fig. 2*A*; τ_{delay} = 95 ± 6.1 s for heparin and 100 ± 8.4 s for control, $P > 0.1$; τ = 149.4 ± 20.9 s for heparin and 149.5 ± 34.1 s for control, $P > 0.25$). These results are also consistent with previous reports (1, 5). Similar results were observed with another IP_3R inhibitor, xestospongin C (17) (20 μM in pipette solution, data not shown). Thus, if the IP_3R is involved in CRAC activation, a conformational change due to IP_3 seems not to be required.

To further explore the possible regulatory role of the IP_3R on the CRAC channel, we have also used 2-APB. As previously reported (24), this compound, as shown in Fig. 2*B*, when included in the pipette solution at 75 μM, blocked IP_3 action as evidenced by its delaying the onset of I_{CRAC} development (τ_{delay} = 15.7 ± 0.9 s, n = 7; 41.4 ± 1.4 s, n = 7; and 85.2 ± 3.3 s, n = 8 for IP_3 alone, IP_3 plus 2-APB, and passive dialysis, respectively; $P < 0.001$ for both IP_3 alone vs. IP_3 plus 2-APB and IP_3 vs. passive dialysis) as well as by its inhibition of the IP_3-mediated acceleration of the development of I_{CRAC} (τ = 30.0 ± 1.1 s, n = 7; 168.6 ± 8.6 s, n = 7; and 85.2 ± 3.3 s, n = 8 for IP_3 alone, IP_3 plus 2-APB, and passive dialysis, respectively; $P < 0.001$ for IP_3 alone vs. IP_3 plus 2-APB; $P < 0.005$ for IP_3 vs. passive dialysis). However, in the presence of 2-APB in the pipette solution, I_{CRAC} spontaneously developed, although with a smaller amplitude (Fig. 2*B*; 0.82 ± 0.22 pA/pF for IP_3 and 0.52 ± 0.18 pA/pF for IP_3 plus 2-APB, $P < 0.01$). The concentration required to delay I_{CRAC} activation to the time seen with passive dialysis alone was found to be approximately that previously reported for its effects on the IP_3R, an IC_{50} of 42 μM (24). Interestingly, with 75 μM 2-APB in the pipette, I_{CRAC} was completely and reversibly blocked by the

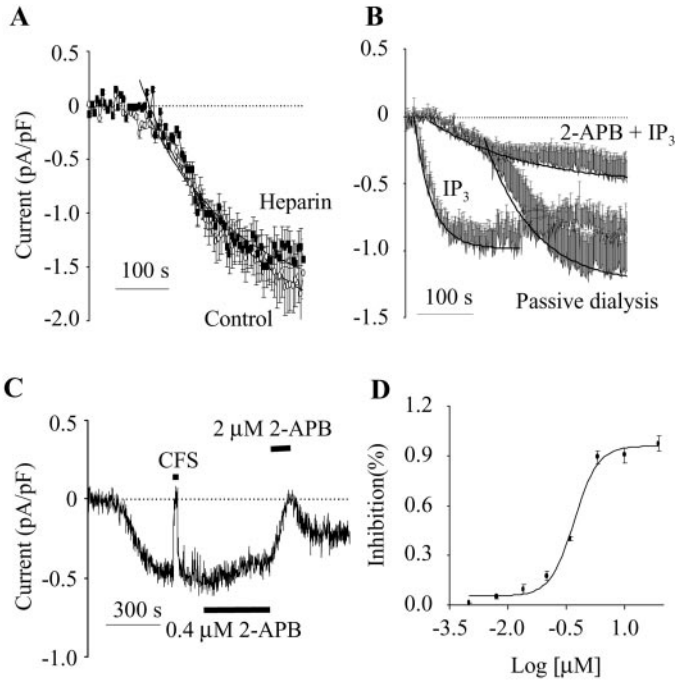

Fig. 2. CRAC channels are not activated by inositol 1,4,5-trisphosphate receptor (IP_3R)/channel interaction. *A*: 0.5 mM heparin in the pipette solution fails to inhibit the activation of I_{CRAC}. τ_{delay} = 95 ± 6.1 and 100 ± 8.4 s for heparin and blank control, respectively ($P > 0.1$). τ = 149.4 ± 20.9 and 149.5 ± 34.1 s for heparin and blank control, respectively ($P > 0.25$; n = 6 for both groups). Data were collected at 0.2 Hz and are presented as means ± SE. *B*: 75 μM 2-aminoethoxydiphenyl borate (2-APB) in the pipette solution prevents IP_3 (10 μM)-induced acceleration of the CRAC channel activation by whole cell dialysis. τ = 30.0 ± 1.1 s, n = 7; 168.6 ± 8.6 s, n = 7; and 85.2 ± 3.3 s, n = 8, for dialysis with IP_3 alone or IP_3 plus 2-APB in the pipette solution and for passive dialysis, respectively ($P < 0.001$ for both IP_3 vs. IP_3 + 2-APB and IP_3 vs. passive dialysis). τ_{delay} = 15.7 ± 0.9 s, n = 7; 41.4 ± 1.4 s, n = 7; and 85.2 ± 3.3 s, n = 8, for dialysis with IP_3 alone or IP_3 + 2-APB in the pipette solution and for passive dialysis, respectively ($P < 0.001$ for IP_3 vs. IP_3 plus 2-APB; $P < 0.005$ for IP_3 vs. passive dialysis). Data were collected at 1 Hz and are presented as means + SE for clarity. *C*: 75 μM 2-APB in the pipette solution fails to inhibit the activation of I_{CRAC} by whole cell dialysis of the cytosol. However, 0.4 or 2 μM 2-APB from the extracellular side of the patch can partially or completely block I_{CRAC}, respectively. *D*: dose-response relation of 2-APB inhibition of I_{CRAC} from extracellular side (n = 5). Fitting the data to the Hill equation yielded an IC_{50} of 0.5 μM and a Hill coefficient of 1.4. Data are presented as means ± SE.

application of 2 μM 2-APB to the extracellular side of the patched cell (Fig. 2*C*). The IC_{50} for this effect was ~0.5 μM (Fig. 2*D*). This finding suggests that 2-APB was effective in blocking I_{CRAC} not because of an effect on the IP_3R but, rather, by directly affecting the extracellular face of the CRAC channel. The reduced I_{CRAC} in the presence of 75 μM 2-APB in the pipette solution (Fig. 2, *B* and *C*) may solely result from its diffusion across the plasma membrane (24) so as to affect the CRAC channel from the extracellular side. Thus, while 2-APB is clearly an IP_3R/channel blocker, it appears to be a far more potent CRAC channel blocker. These results cause us to question the possibility that the IP_3R is a direct activator of the CRAC channel (22) and strengthen our previous hypothesis that CRAC channels are activated by a CIF-like molecule directly or through a regulatory intermediate (8, 44). The extracellular effects of 2-APB on CRAC channels have also been found by others (1, 4, 33). However, we did not observe a potentiation effect of 2-APB on I_{CRAC} at low concentrations (Fig. 2*C*) as reported previously (33). This discrepancy might be due to different cells used in the current vs. the previous (Jurkat and DT40 cells) studies.

CRAC channels do not require the continued presence of a diffusible factor once they are activated in whole cell configuration. Although our previous results suggested that CRAC channels are activated by CIF (8, 44), how CIF activates CRAC channels is unknown. If CRAC channels are activated by direct binding of CIF, then I_{CRAC} might be predicted to inactivate after a period of time in whole cell recordings. This might occur because the concentration of CIF would decrease if its rate of synthesis fell behind its rate of removal due to degradation and/or the exchange between the cytosol and pipette solution. In contrast to this prediction, as shown in Fig. 3, after I_{CRAC} was activated by dialysis of the cytosol by pipette solution, it could be recorded more than 120 min after whole cell break-in (n = 4), even though the channels underwent slow inactivation process as previously reported (27, 57). As a result of diffusion (23), this period of time should have washed out a small molecule such as CIF with a molecular weight <1,000 Dalton (38). This might suggest that, once activated, maintaining the CRAC channels in an open state does not require the continued presence of a diffusible molecule such as CIF under this recording condition. In other words, CRAC channels are unlikely activated by direct binding of CIF. Our hypothesis to explain this is that the CRAC channel might be activated by CIF through removal of an inhibitory molecule that otherwise keeps it in a closed state. Krause et al. (19) have recently found that I_{CRAC} could be activated in the absence of global ER store

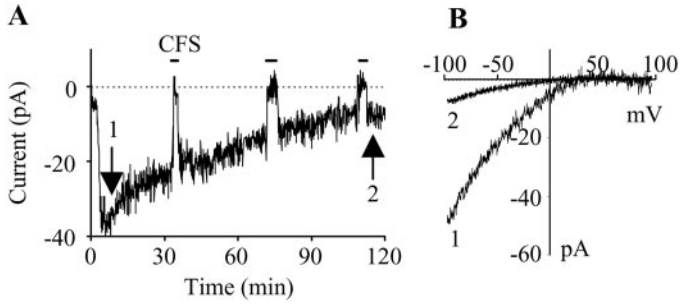

Fig. 3. Activation of CRAC channels does not require the continued presence of a diffusible factor once they are activated. *A*: I_{CRAC} can be recorded as long as the whole cell configuration is maintained. CRAC channels are activated by whole cell dialysis of the cytosol. I_{CRAC} was detected by a −100- to 100-mV voltage ramp in 320 ms at a 5-s interval, and the current at −80 mV was plotted as a function of time. Horizontal bars indicate the replacement of extracellular Ca^{2+} by equimolar Mg^{2+} (CFS; see MATERIALS AND METHODS). *B*: *I-V* relationship of current taken at the corresponding number-labeled time points in *A*. Note the positive reversal potential (>50 mV) and inwardly rectifying *I-V* relationships of I_{CRAC}.

depletion by low cytoplasmic [Ca^{2+}]. One explanation of their results is that a low cytoplasmic [Ca^{2+}] environment promotes the dissociation of the putative inhibitor from the CRAC channel, which then activates. In other words, the inhibitory factor associates with the CRAC channel in a Ca^{2+}-dependent manner. Thus what is the putative inhibitory factor for CRAC channels?

INAD inhibits CRAC channels in a Ca^{2+}-dependent manner. Detection of light by photoreceptors in the eye of *Drosophila* requires activation of a cation channel that includes transient receptor potential (TRP) channel subunits (54). Mammalian cells express homologues of TRP, and certain of these can function as SOCs (3). Indeed, there is evidence that mammalian TRPs may be components of CRAC channels (32, 51, 56). In *Drosophila*, inactivation-no after potential D (INAD), a multivalent PDZ domain-containing protein, functions as a scaffold protein as well as a regulator of the TRP/TRPL channel (21, 41, 43, 48, 49, 53). In particular, it associates with TRP/TRPL through one or two of its PDZ domains with a peptide motif, -S/T-X-V/I, in the carboxyl (COOH) terminal cytoplasmic domain of TRP/TRPL (25, 42), as well as the COOH terminus itself (21). This leads to deactivation of TRP and/or TRPL Ca^{2+} channel activity (21, 42). A recent study further demonstrated that overexpression of INAD inhibits TRP channels (10). Interestingly, we have found a number of S/T-X-V/I motifs as well as another PDZ domain-binding motif, ϕ-x-ϕ, where ϕ is a hydrophobic amino acid, in the COOH-terminal domain of TRP1–6 of the mammalian TRP family (data not shown). Indeed, TRP4 and TRP5 can bind to a PDZ-containing scaffold protein, Na^{2+}/H^{+} exchanger regulatory factor (NHERF) (46). In addition, we also found a putative EF-hand Ca^{2+}-binding motif from G279-I291 in the second PDZ domain of INAD (NH_2-GVDPNGALGSVDI-COOH). Consequently, we tested INAD as a surrogate inhibitor of I_{CRAC}.

Figure 4*A* shows an average whole cell activation profile of I_{CRAC} when the pipette solution is buffered to 10 nM Ca^{2+} ($n = 8$). After a 140.0 ± 10.4-s delay, I_{CRAC} begins to activate spontaneously with a time constant τ of 85.6 ± 3.3 s. In the presence of 100 nM Ca^{2+} in the pipette solution (Fig. 4*B*, $n = 8$), the onset was delayed (τ_{delay} = 320.3 ± 20.1 s, $P < 0.05$) compared with that when pipette solution contains 10 nM Ca^{2+}, and the activation course was also slower (τ = 499.3 ± 53.9 s, $P < 0.05$) compared with that when pipette solution contains 10 nM Ca^{2+}. When electrophoretically pure INAD (73 μg/ml, Fig. 4*I*) was included in the pipette solution with Ca^{2+} buffered to 10 nM (Fig. 4*C*, $n = 6$), the onset was somewhat delayed (τ_{delay} = 281.1 ± 17.6 s) and more gradual (τ =160.3 ± 6.6 s) compared with that when INAD was not included (τ_{delay} = 140.0 ± 10.4 s, $P < 0.001$ and τ = 85.6 ± 3.3 s, $P < 0.001$), but full activation was routinely achieved. In contrast, when INAD was included at 100 nM Ca^{2+}, no activation was observed (Fig. 4*D*, $n = 7$). This inhibition persisted even after store-depletion with 10 μM ionomycin, suggesting that endogenous CIF, hypothesized to be produced in response to ionomycin-mediated depletion of stores, was not sufficient to activate CRAC in the presence of INAD. No such inhibition was seen in the presence of other proteins, including an extract of *E. coli* proteins from strains not expressing INAD (Fig. 4*E*, $n = 6$, τ_{delay} = 327.7 ± 23.5 s, $P > 0.5$; τ = 498.6 ± 67.9 s, $P > 0.5$). The inability of endogenous CIF to reverse INAD was likely due to the relatively large amount of recombinant INAD and the relatively low concentration of CIF such that only a fraction of INAD is bound to CIF and, hence, CRAC channels stays inactive with INAD bound. To test this hypothesis, we used exogenous CIF at a presumably higher concentration. As shown in Fig. 4*F*, inclusion of both INAD and platelet CIF (1:50 dilution) in the patch pipette resulted in full activation of I_{CRAC} (τ_{delay} = 128.8 ± 4.6 s, τ = 44.1 ± 1.4 s, $n = 8$). In contrast, a small dose of CIF (1:500 dilution) could not reverse the inhibitory effect of INAD on I_{CRAC} activation (Fig. 4*G*, $n = 5$), even though this dose of CIF could tremendously accelerate the activation of I_{CRAC} when intracellular [Ca^{2+}] was buffered at 100 nM (Fig. 4*H*, τ_{delay} = 20.2 ± 2.4 s, τ = 97.96 ± 3.9 s, $n = 5$, $P < 0.001$ compared with data in Fig. 4*B*).

Because of CIF's property of overcoming INAD's inhibition of I_{CRAC}, we asked whether a direct association between the two could be established. Affinity-purified INAD was covalently linked to a Sepharose matrix, and a partially purified extract from activated platelets containing CIF was passed over the column. In contrast to a column lacking INAD, the column run-through, although containing nearly all the optical density at 262 nm, was found to be devoid of the CIF activity of the original extract, as assessed by activity in *Xenopus* oocytes (Fig. 5) (8). Fractions eluting several bed volumes later contained >80% of the activity originally applied to the column. These results suggest that CIF can directly bind to INAD.

DISCUSSION

Several groups have recently demonstrated that low levels of free [Mg^{2+}] (<3 mM) in the pipette solution may activate MIC and I_{CRAC} spontaneously and simultaneously (11, 18, 34) in both Jurkat and RBL cells during whole cell recordings. MIC may be responsible for the previously claimed single-channel current of CRAC channels when divalent cations are taken from both sides of the plasma membrane (4, 9, 13). Single-channel conductance of CRAC channels for Na^{+} in the absence of extracellular Ca^{2+} was estimated at 0.2 pS by noise analysis (34), which is beyond the current resolution of patch-clamp recording techniques. Although I_{CRAC} and MIC can be simultaneously and spontaneously activated during whole cell recordings when intracellular free [Mg^{2+}] is low, there are still ways to separate I_{CRAC} and MIC by using the different properties of these currents. In this study we isolated I_{CRAC} from possible MIC-contaminated current by subtracting the leak current recorded while cells were exposed to Ca^{2+}-free but Mg^{2+}-containing solutions

after I_{CRAC} was fully activated, as suggested previously by Prakriya and Lewis (34) on the basis that MIC channels are equally permeable to Ca^{2+} and Mg^{2+} whereas CRAC channels are only permeable to Ca^{2+}. The success of this method is manifested by the fact that the isolated currents were indistinguishable from currents recorded when MIC was presumably completely blocked by high concentrations of Mg/ATP, in terms of current size, inwardly rectifying *I-V* relationship, and inactivation of monovalent cation currents (Fig. 1).

It has recently been suggested that the IP_3R is involved in the activation of some SOCs (15, 17, 35). However, this appears not to be the case for CRAC channels on the following basis. First, inclusion of the IP_3R inhibitor heparin in the whole cell pipette failed to affect the spontaneous activation of I_{CRAC} (Fig. 2*A*), which is consistent with previous reports (1, 5). Another IP_3R inhibitor, xestospongin C, also could not inhibit I_{CRAC} during whole cell recordings when applied from either the intracellular or extracellular side of the plasma membrane (unpublished observation). Second, knockout of all isoforms of IP_3R in DT40 cells fails to affect I_{CRAC} activation in these cells (33, 45) (but see Ref. 16).

Although 2-APB can efficiently inhibit CRAC channels, it apparently exerts the effect by directly blocking the CRAC channel extracellularly, instead of disrupting the physical interaction between CRAC channels and IP_3R. Some of the previous evidence for SOC/IP_3R coupling (22) can now be explained by the finding that 2-APB is a potent and direct CRAC channel blocker. Thus caution needs to be taken when interpreting data with 2-APB. This, of course, does not negate the possibility that the IP_3R directly couples to and regulates certain SOCs or mammalian TRP channels such as TRP3 that do not show the precise features of I_{CRAC} (17, 37, 50).

On the basis of the present results with INAD, it is tempting to speculate that mammalian homologues of this protein are key elements in CRAC channel regulation. These data lead to a model of the signaling mechanism that couples ER Ca^{2+} store depletion in mammalian cells with plasma membrane CRAC channels (Fig. 6). Specifically, there is Ca^{2+}-dependent conformational coupling between a putative INAD homologue (which we termed MINAD, for mammalian INAD) and one or more mammalian TRP subunits comprising the CRAC channel. This occurs through interactions between the TRP and certain PDZ domains on MINAD, which also may serve to anchor the

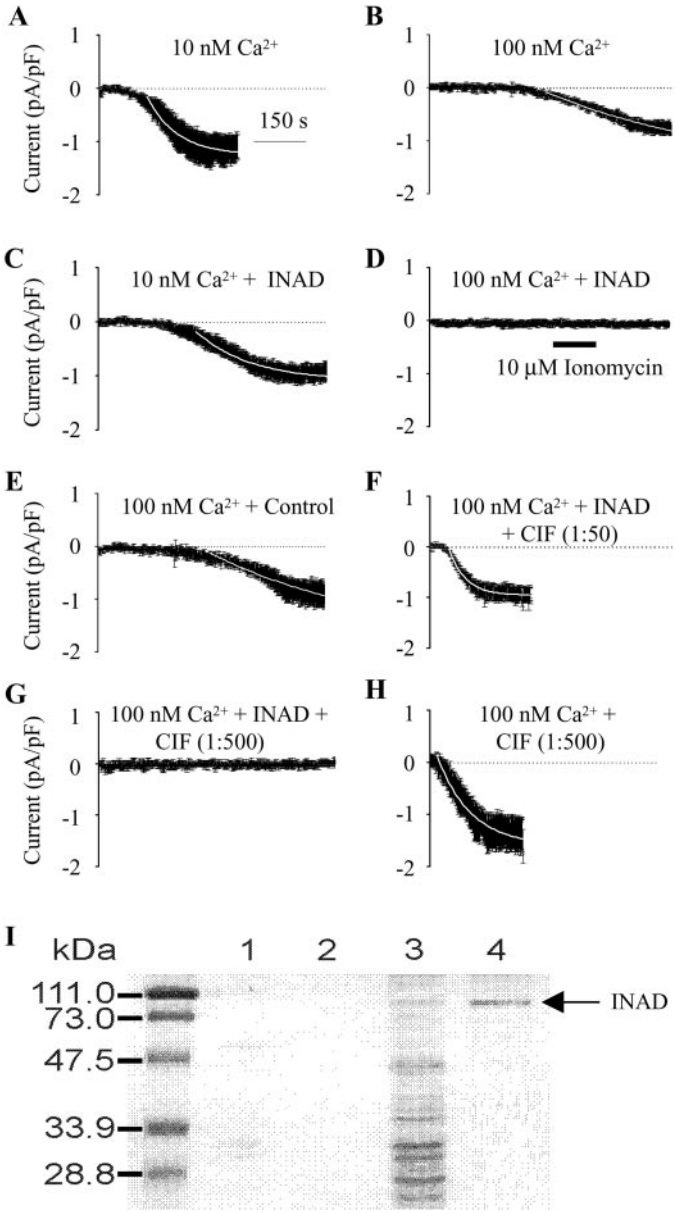

Fig. 4. CRAC channels are inhibited by INAD (inactivation-no after potential D) in a Ca^{2+}-dependent manner, and the inhibition is reversed by Ca^{2+} influx factor (CIF). *A*: whole cell recording with a pipette solution containing 10 nM free Ca^{2+} rapidly activates I_{CRAC}: $\tau_{delay} = 140 \pm 10.4$ s, $\tau = 85.4 \pm 3.3$ s, $n = 8$. *B*: 100 nM free Ca^{2+} in the pipette solution delays the activation of I_{CRAC}: $\tau_{delay} = 320.3 \pm 20.1$ s, $n = 8$ ($P < 0.05$ compared with that in *A*); $\tau = 499.3 \pm 53.9$ s, $n = 8$ ($P < 0.05$ compared with that in *A*). *C*: INAD (73 μg/ml) only delays the activation of I_{CRAC} when the free Ca^{2+} level is 10 nM: $\tau_{delay} = 281.1 \pm 17.6$ s, $\tau = 160.3 \pm 6.6$ s, $n = 6$ ($P < 0.001$ compared with those in *A*). *D*: INAD (73 μg/ ml) completely prevents the activation of I_{CRAC} when the free Ca^{2+} level is 100 nM; even application of 10 μM ionomycin cannot rescue this inhibition ($n = 7$). *E*: *Escherichia coli* extract that does not contain INAD fails to prevent the activation of I_{CRAC} by dialysis in presence of 100 nM free Ca^{2+}: $\tau_{delay} = 327.7 \pm 23.5$ s, $\tau = 498.6 \pm 67.9$ s, $n = 6$ ($P > 0.5$ compared with those in *B*). *F*: CIF (1:50 dilution) in the pipette solution reverses the inhibition of INAD on I_{CRAC}: $\tau_{delay} = 128.8 \pm 4.6$ s, $\tau = 44.1 \pm 1.4$ s, $n = 8$. *G*: a low dose of CIF (1:500 dilution) cannot reverse the inhibition of I_{CRAC} by INAD ($n = 5$). *H*: this low dose of CIF (1:500 dilution), however, accelerates the activation of I_{CRAC}: $\tau_{delay} = 20.2 \pm 2.4$ s, $\tau = 97.96 \pm 3.9$ s, $n = 5$ ($P < 0.001$ compared with data in *B*). *I*: purification of recombinant INAD from *E. coli*. Protein samples (10 μl each) from various stages of INAD purification were electrophoresed on a 15% SDS-polyacrylamide gel and stained with Coomassie blue. *Lane 1*, 30% ammonium sulfate precipitate; *lane 2*, Ni^{2+} affinity column chromatography of 30% ammonium sulfate precipitate after resolubilization; *lane 3*, 40% ammonium sulfate precipitate; *lane 4*, Ni^{2+} affinity column chromatography of 40% ammonium sulfate precipitate after resolubilization.

complex to the cytoskeleton (52). In the intact cell, Ca^{2+} and MINAD function to maintain CRAC channels in the closed state until a diffusible store-depletion signal, CIF, causes the complex to dissociate and CRAC channels to open. After refilling of the stores and the decay of CIF, the complex is reestablished and CRAC channels close. In the absence of CIF, low local [Ca^{2+}] either physiologically or experimentally due to passive dialysis may also result in disassociation of MINAD from the CRAC channel and, thus, I_{CRAC} activation. In this light, it is interesting to note the putative EF-hand Ca^{2+}-binding motif (G279-I291) in the second PDZ domain of INAD. We hypothesize that dissociation of Ca^{2+} from this site due to low local Ca^{2+} levels induces a conformational change in MINAD and, thus, dissociation of MINAD from the CRAC channels. This may well explain the finding by Krause et. al (19) that I_{CRAC} can be activated by low levels of cytoplasmic Ca^{2+} independently of store depletion. Therefore, CIF might serve as a coarse regulator of CRAC channels while the local cytoplasmic Ca^{2+} serves as a fine tuner.

This model has some very attractive features that add to its credibility. First, TRP mammalian homologues have PDZ domain-binding motifs, as mentioned before. Second, INAD homologues exist in mammalian cells (31). Most interestingly, a PDZ domain-containing mammalian protein, NHERF, which interacts with the cytoskeleton, was recently found to bind to the TRP4 and TRP5 proteins (46). Third, the association/dissociation of CRAC channels and INAD occurs at physiologically relevant [Ca^{2+}] (~100 nM). Fourth, overexpression of INAD inhibits the activation of TRP channels by store depletion (10).

These results also provide further strong support for the existence of CIF. The interaction with INAD may well provide a means to purify and identify this elusive substance. In addition, the "secretory vesicle/kiss and run" model might now be explained by the necessity of delivering vesicles producing CIF to the vicinity of the CRAC channel/INAD-like complex. Finally, results on some SOC with agents that affect the cytoskeleton (30, 55) may well be due to alterations in PDZ domain interactions with cytoskeletal elements, which are known to occur with INAD and other PDZ domain-containing proteins (52).

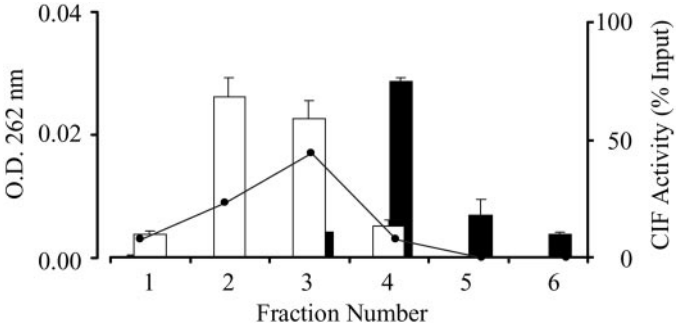

Fig. 5. Elution profile of CIF activity from an INAD-Sepharose column. After HPLC strong anion exchange chromatography, fractions containing CIF activity were dried down and reextracted with methanol. Active fractions were solubilized in 0.1 N acetic acid and buffered to pH 6.0 with NaOH. They were then applied to either an INAD-Sepharose column or to a comparable column without coupled INAD but cyanogen bromide-activated and blocked with Tris buffer. Bed volume (0.5 ml) fractions were collected and assessed for optical density at 262 nm (OD_{262}) and CIF activity using the *Xenopus* oocyte bioassay. Open bars, activity profile using the uncoupled Sepharose column; filled bars, CIF activity eluted from INAD-Sepharose. Solid line is the OD profile.

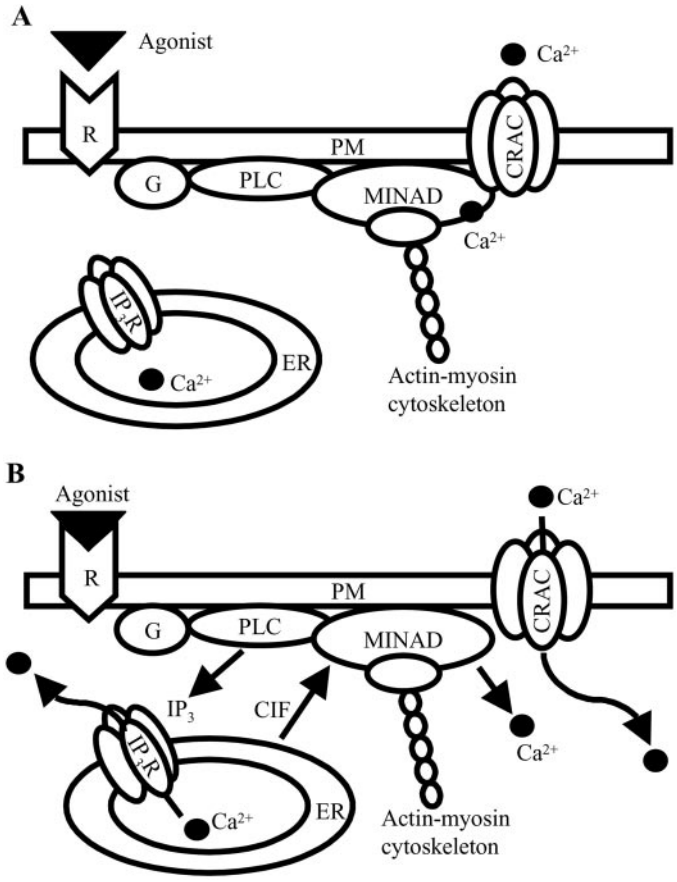

Fig. 6. Model for activation of I_{CRAC}. *A*: a mammalian INAD (MINAD) serves as the organizer of the "signalplex" (21, 48) and as an inhibitor of CRAC channels in an unstimulated cell. PDZ domains within MINAD bind phospholipase C (PLC), the actin-myosin cytoskeleton, and the CRAC channel, while the EF-hand motif of MINAD is occupied by Ca^{2+}. ER, endoplasmic reticulum; PM, plasma membrane; R, receptor; G, G protein. *B*: when the cell is activated by an agonist binding to its receptor in the plasma membrane, CIF is produced by the Ca^{2+} store-depleted ER. Binding of CIF to MINAD facilitates dissociation of MINAD from the CRAC channel and allows the channel to open. Alternatively, dissociation of MINAD from the channel can occur when intracellular [Ca^{2+}] is low, such as during whole cell recordings or at some physiological conditions. Dissociation of MINAD from the CRAC channel allows the opening of the channel even in the absence of store depletion.

We thank Dr. B. Shieh (Vanderbilt University) for providing the pCmv4/INAD clone and Dr. K. Mikoshiba (Tokyo University) for the generous gift of 2-APB.

This work was supported in part by National Institutes of Health Grants AI-37670 and HL-68806 (to J. E. Blalock) as well as DK-55647 and HL-68806 (to J. E. Blalock and R. B. Marchase), Juvenile Diabetes Foundation Grant 2000-137 (to R. B. Marchase and J. E. Blalock), and a grant from the C. C. Wu Foundation in Hong Kong (Z. Su).

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