23. A DNA sequence encoding residues 905 to 981 from EphB2 was amplified by polymerase chain reaction from a human renal microvascular endothelial cell cDNA library and subcloned into a modified pet vector optimized for enhanced expression. The vector-encoded sequence Met-Glu-Lys-Thr-Arg was added to the NH2-terminus of the SAM domain so that in our numbering scheme, residue 6 corresponds to residue 905 in the full-length receptor. The protein was purified to homogeneity with anion and cation exchange chromatography and gel-filtration chromatography. The protein was crystallized by hanging drop crystallization at 4°C. The drop contained a 1:1 mixture of reservoir buffer and protein at 20 mg/ml. The reservoir contained 100 mM Heps (pH 7.0), 10 mM tris, 20 mM dithiothreitol, 30 mM LiSO4, and 26 to 37% PEG 1000.
26. The conserved residues in the family of SAM domains as defined by Shultz et al. (4) are all buried within a monomer, with two exceptions. The first exception is Gly77, which is found in a tight turn between helices 3 and 4. This residue adopts a positive ë– angle and glycine is probably needed to facilitate the otherwise unfavorable backbone conformation. The second exception is Tyr96 on the NH2-terminal loop. Tyrosine-8 is the key residue in one of the oligomeric interfaces.
30. In 31 Eph receptor sequences, Asp48 is invariant, position 16 is always a negatively charged residue, position 28 is a negatively charged residue in 30 sequences, and position 47 is a negatively charged residue in 29 sequences.
36. We thank M. Phillips for performing the sedimentation experiments, T. Daniel for clones and helpful discussions, and D. Eisenberg, T. Yeates, D. Casco, S. Faham, R. Landgraf, F. Pettit, C. Kim, E. Toth, C. Colovos, E. Mar- cotte, and M. Saper for advice and comments on the manuscript. Supported by an NSF National Young Inv estigator and a Pew Scholar Award to [JUB] and an NIH training grant to C.D.T. This work is dedicated to the memory of Marcia Robb Whitt.

13 November 1998; accepted 29 December 1998
level. CRAC channels, like voltage-dependent Ca\(^{2+}\) channels, are permeable to monovalent cations (including organic cations up to 6 A in diameter) when the concentration of extracellular divalent ions is reduced (5–7).

To detect the activity of single CRAC channels, we lowered the concentration of external divalent cations to the micromolar range to enable Na\(^{+}\) to serve as the charge carrier, eliminated Mg\(^{2+}\) from the pipette solution to prevent inactivation of the monovalent current (7), and measured current over a range of potentials to −120 mV during whole-cell recording (8). CRAC channels were activated either by passive Ca\(^{2+}\) store depletion with the Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'\(^{-}\)-tetraacetic acid (BAPTA) or by store depletion in response to added inositol 1,4,5-triphosphate (IP\(_3\)). When carrying either Ca\(^{2+}\) or Na\(^{+}\) current, CRAC channels opened with a similar biphasic time course during passive store depletion initiated by whole-cell dialysis, but macroscopic whole-cell Na\(^{+}\) currents were typically 40 times as large (Fig. 1, A and B). Current-voltage (I–V) relations revealed weak inward rectification of the current with a very positive reversal potential for the Ca\(^{2+}\) current and a reversal potential near 0 mV for the Na\(^{+}\) current through CRAC channels (Fig. 1, C and D). The initial activation of Na\(^{+}\) current was composed of discrete current steps, with a succession of identical steps leading to the macroscopic current (Fig. 1E). The steps are multiples of a unitary current of 4.6 pA at −120 mV, corresponding to a conductance of 38 pS. Single-channel currents observed during voltage-ramp stimuli exhibited the same reversal potential and weakly rectifying I–V shape as those of the macroscopic current (Fig. 1F). Dialysis with 10 μM IP\(_3\) more rapidly elicited the same 36 to 40 pS single-channel activity (9).

Single channels at the onset of CRAC channel activation initially exhibited very brief openings before suddenly stabilizing in the open configuration (Fig. 2, A to C). In an examination of 800 data segments (sweeps), each lasting 0.2 s, from 20 cells during which 120 channels began to conduct, the vast majority of single-channel events occurred in equal-sized increments, consistent with unitary increases in the number of conducting channels rather than a gradual increase in their open probability \(P_o\) during the activation of CRAC channels. Of thousands of single-channel events, 10 were initially twice the amplitude of the single-channel events because two channels appeared to activate simultaneously within the resolution of the measurement (1 kHz); larger amplitudes were never observed. Brief closures from the open state were voltage-dependent; the duration of these closures increased with depolarization from 3 ± 1 ms at −120 mV to 10 ± 5 ms at −60 mV (\(n = 3\) cells). This voltage dependence may contribute to the inwardly rectifying shape of the I–V relation. At −120 mV, \(P_o\) was estimated to be 0.94 ± 0.04 (\(n = 41\) sweeps recorded at the time of a single conducting channel in four cells). Longer closures caused fluctuations in the number of conducting channels, but the progressive appearance of new single-channel events led to peak macroscopic currents that varied from 447 to 1539 pA in 45 cells, corresponding to 100 to 360 channels per cell, with an average surface density of 0.36 channels square per/ micrometer.

With internal Mg\(^{2+}\) present, monovalent current through CRAC channels inactivates slowly over several tens of seconds after the removal of external divalent ions (5–7). The activation of monovalent CRAC currents occurred with a similar time course with or without internal Mg\(^{2+}\) present, and similar single-channel events were observed (Fig. 2, D to F), but current densities were one-tenth those when Mg\(^{2+}\) was absent in the pipette solution; this suggested that about 90% of the available CRAC channels were inactivated (10). When external divalent cations were removed shortly after the onset of CRAC channel activation, the time course of inactivation was revealed as a progressive closure of single channels (Fig. 3). Evaluated at −120 mV, the initial 40- to 45-pA current represented the activity of eight or nine ion channels, and successive traces showed a stepwise decline in current, finally stabilizing at 4.9 pA, representing the activity of one ion channel. These results demonstrate that changes in the number of conducting channels underlie both the activation and inactivation processes.

We used channel blockers to confirm that

![Fig. 1. Divalent and monovalent current through a store-operated Ca\(^{2+}\) channel in Jurkat cells.](image)
the observed steps of current represent Na\(^+\) current through single CRAC channels. 

Ca\(^2+\), Mg\(^2+\), Ni\(^2+\), and Gd\(^3+\), all of which inhibit current through CRAC channels, blocked the monovalent current detected from single channels (9). Single channels exhibited long-duration openings that were interrupted by rapid blocking and unblocking events when external Mg\(^2+\) was buffered to 3 \(\mu\)M (Fig. 4, A to D). Blockade of channels by external Mg\(^2+\) was voltage-dependent, as reflected in both the ensemble average of single-channel records and macroscopic I-V curves (Fig. 4, E and F).

We observed single-channel activity of a store-operated Ca\(^2+\) channel as a succession of long-duration, independent single-channel events during activation of the macroscopic current. Macroscopic and single-channel Na\(^+\) currents had the same reversal potential and I-V shape and were blocked by the same polyvalent cations. Extracellular Mg\(^2+\) rapidly blocked and was released from the channel in discrete events, with concentration and voltage dependence similar to those of macroscopic currents, indicating a binding site deep within the electric field of the pore. With internal Mg\(^2+\) present, inactivation of Na\(^+\) current proceeded as single channels progressively closed. We conclude that the single-channel activity described reflects monovalent current through single CRAC channels. These observations support suggestions that CRAC is an ion channel and not a pump (3–7).

The single-channel conductance of 36 to 40 pS for the CRAC channel carrying Na\(^+\) is 1500 times the estimated 24 fS for the Ca\(^2+\) current through CRAC channels, but is close to the conductance of Na\(^+\) through single L-type voltage-gated Ca\(^2+\) channels (11). CRAC channels and voltage-gated Ca\(^2+\) channels share several properties of ion permeation, including a common pore dimension of 6 Å and selectivity for Ca\(^2+\) that depends on selective binding and interactions between Ca\(^2+\) ions at the selectivity filter (7, 12). In both channel types, the smaller conductance of Ca\(^2+\) compared to that of Na\(^+\) results from a much higher affinity of Ca\(^2+\) ions for the selectivity filter, which limits throughput but guarantees selectivity for the divalent ion.

From the measured macroscopic and single-channel currents and the average \(P_o\),
we conclude that there are 100 to 400 CRAC channels per cell, fewer than a previous estimate of more than 10,000 per cell derived from an analysis of conductance fluctuations (13). During the activation process, single CRAC channels opened abruptly and rapidly stabilized to a state of very high $P_o$, indicating an underlying mechanism that switches the single channels from a nonconducting state to a state in which $P_o$ is near unity. The identification of unitary CRAC channel currents provides a single-channel signature for identifying candidate genes and may facilitate the study of CRAC channel regulation.

References and Notes
8. The human leukemia T cell line Jurkat E6-1 was cultured in RPMI 1640 with fetal calf serum (10%), 1 mM glutamate, and 25 mM Hepes in a 5% CO$_2$ incubator at 37°C. Cells were plated on polylysine-coated glass cover slips immediately before whole-cell recording with Syglgard-coated pipettes fire-polished to a resistance of 2 to 5 megohms. (Sylgard was purchased from Dow Corning; all other chemicals were from Sigma.) All experiments were done at room temperature (20°C). Membrane currents were recorded with an EP9-9 patch clamp amplifier (HEKA, Lambrecht, Germany). Voltage stimuli lasting 200 ms were delivered from a holding potential of 0 mV every second, using either voltage ramps in which voltage increased from -120 to +40 mV or voltage steps from 0 to -120 mV. Currents were sampled at 5 kHz during voltage ramps and at 25 kHz during voltage steps. Currents were digitally filtered off-line at 1 kHz. CRAC channels were opened by passive depletion of Ca$_2^+$ stores with 12 mM BAPTA or by addition of 10 µM IP$_3$, with ionic conditions chosen to eliminate currents through K- or Cl- channels. Traces recorded before the activation of CRAC channels were used as a template to subtract leak current from each trace. 

Supported by NIH grants NS-14609 and GM-41514.

References and Notes
10. Whole-cell Na$^+$ current densities after complete activation of CRAC channels averaged 153 ± 44 pA/pF with no Mg$^{2+}$ inside ($n$ = 45 cells) and 16 ± 6 pA/pF with internal Mg$^{2+}$ ($n$ = 10 cells).
13. Conductance fluctuation analysis is likely to have underestimated the single-channel conductance and overestimated the number of CRAC channels per cell, because the $P_o$ value of single channels is very high, as soon as the CRAC channels begin to conduct. In stationary noise analysis, the single-channel conductance can be estimated by measuring the mean conductance values and the standard deviation divided by the number of currents. If the $P_o$ value also applies to high-divalent conditions, the variance and mean conductance values of conductance estimated in (4) would correspond to a single-channel Ca$^{2+}$ conductance of -400 fS. High-frequency block and unbinding by divalent ions above 1 kHz may also have saturated the variance and hence the single-channel Ca$^{2+}$ conductance estimated by noise analysis. An alternative approach to estimate the Ca$^{2+}$ conductance through a single CRAC channel is to divide the single-channel Na$^+$ conductance of 36 to 40 pS by the ratio of macroscopic Na$^+$ to Ca$^{2+}$ currents when divalents are withdrawn and then Ca$^{2+}$ reapplied, a ratio that averaged 25 (7). This approach assumes a constant number of open channels immediately before and after addition of Ca$^{2+}$ and yields an estimate of 1.6 pS for the CRAC channel carrying Ca$^{2+}$ at a concentration of 20 mM.