Journal of Physiology (2002), **539.2**, *pp.* 445–458
DOI: 10.1013/jphysiol.2001.013361

www.jphysiol.org

Dissociation of the store-operated calcium current I_{CRAC} and the Mg–nucleotide-regulated metal ion current MagNuM

Meredith C. Hermosura, Mahealani K. Monteilh-Zoller, Andrew M. Scharenberg*, Reinhold Penner and Andrea Fleig

*Laboratory of Cell and Molecular Signaling, Center for Biomedical Research at The Queen's Medical Center and John A. Burns School of Medicine at the University of Hawaii, Honolulu, HI 96813 and * Department of Pediatrics, University of Washington and Children's Hospital and Regional Medical Center, Seattle, WA 98195, USA*

Rat basophilic leukaemia cells (RBL-2H3-M1) were used to study the characteristics of the store-operated Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) and the magnesium–nucleotide-regulated metal cation current (MagNuM) (which is conducted by the LTRPC7 channel). Pipette solutions containing 10 mM BAPTA and no added ATP induced both currents in the same cell, but the time to half-maximal activation for MagNuM was about two to three times slower than that of I_{CRAC}. Differential suppression of I_{CRAC} was achieved by buffering free $[Ca^{2+}]_i$ to 90 nM and selective inhibition of MagNuM was accomplished by intracellular solutions containing 6 mM Mg·ATP, 1.2 mM free $[Mg^{2+}]_i$ or 100 μM GTP-γ-S, allowing investigations on these currents in relative isolation. Removal of extracellular Ca^{2+} and Mg^{2+} caused both currents to be carried significantly by monovalent ions. In the absence or presence of free $[Mg^{2+}]_i$, I_{CRAC} carried by monovalent ions inactivated more rapidly and more completely than MagNuM carried by monovalent ions. Since several studies have used divalent-free solutions on either side of the membrane to study selectivity and single-channel behaviour of I_{CRAC}, these experimental conditions would have favoured the contribution of MagNuM to monovalent conductance and call for caution in interpreting results where both I_{CRAC} and MagNuM are activated.

(Received 4 October 2001; accepted after revision 23 November 2001)
Corresponding author A. Fleig: Center for Biomedical Research, The Queen's Medical Center, University Tower 8, 1301 Punchbowl Street, Honolulu, HI 96813, USA. Email: afleig@hawaii.edu

Store-operated or capacitative calcium entry is a widespread mechanism used by electrically non-excitable cells to support sustained elevations in intracellular calcium concentration (Berridge, 1995; Lewis & Cahalan, 1995; Thomas *et al.* 1996; Parekh & Penner, 1997). It is mediated by store-operated ion channels in the plasma membrane that are activated through an unknown mechanism following InsP_3-mediated release of Ca^{2+} from intracellular stores. Experimentally, any manoeuvre that causes store depletion through either active Ca^{2+} release (e.g. InsP_3, ionomycin) or passive release by impeding refilling (e.g. thapsigargin, intracellular Ca^{2+} buffers) will activate these channels (Hoth & Penner, 1992; Zweifach & Lewis, 1993; Parekh & Penner, 1996; Broad *et al.* 1999). The prototypical and best-characterized store-operated mechanism is the so-called 'calcium release-activated calcium' current (I_{CRAC}) (Hoth & Penner, 1992, 1993; Zweifach & Lewis, 1993; Lewis & Cahalan, 1995; Parekh & Penner, 1997). This current is characterized by its high selectivity for Ca^{2+} ions, an inwardly rectifying current–voltage relationship, and an extremely small single-channel conductance in the sub-picoSiemens range (Hoth & Penner, 1993; Zweifach & Lewis, 1993). Even whole-cell currents carried by CRAC channels are very small and specific experimental conditions need to be established in order to reliably measure I_{CRAC} (Hoth & Penner, 1992; Parekh & Penner, 1996). I_{CRAC} can be carried by monovalent ions when all divalent ions are removed from the extracellular solution (Hoth & Penner, 1993; Kerschbaum & Cahalan, 1998). Recently, several investigations have taken advantage of this feature to arrive at the first single-channel recordings through CRAC channels (Kerschbaum & Cahalan, 1999; Braun *et al.* 2000; Fomina *et al.* 2000). It was found that single-channel conductance of CRAC channels carrying monovalent ions is around 30–40 pS and the number of channels per cell was estimated to be around 100–400 in Jurkat lymphocytes (Kerschbaum & Cahalan, 1999), 260–500 in RBL cells (Braun *et al.* 2000) and as few as 15 in resting human lymphocytes (Fomina *et al.* 2000). These investigations suggest that CRAC channels can now be studied at the single-channel level.

We have recently cloned and characterized LTRPC7, a novel ion channel of the LTRPC family of ion channels (Nadler *et al.* 2001). LTRPC7 is regulated by intracellular levels of Mg^{2+}-complexed nucleotides and underlies a current that is strongly activated when Mg·ATP falls below 1 mM (designated MagNuM for magnesium–nucleotide-

regulated metal current). LTRPC7 is a widely expressed ion channel that conducts Ca^{2+} and Mg^{2+} at negative membrane potentials. However, at potentials above +50 mV, where the driving force for divalent ions to enter the cell is small, the permeation block exerted by divalent ions is reduced and MagNuM is effectively carried by monovalent cations moving in the outward direction, a property that provides a signature for identifying LTRPC7. The permeation block of divalent ions at negative membrane potentials can be removed by omitting divalent ions from the extracellular solution, and under these conditions, MagNuM, like I_{CRAC}, can significantly be carried by monovalent ions moving inwards. Since I_{CRAC} and MagNuM are both present in lymphocytes and RBL cells (Nadler *et al.* 2001), this provides for some ambiguity in whole-cell and single-channel studies of I_{CRAC}, as those have been performed under experimental conditions that would also favour activation of MagNuM. We have therefore carried out experiments in RBL cells to assess the relative contributions of I_{CRAC} and MagNuM to monovalent cation currents by selectively dissociating the currents based on their differences in activation mechanism, Mg^{2+} dependence and pharmacology. We conclude that many of the reported properties ascribed to I_{CRAC} carried by monovalent ions may require re-evaluation, since MagNuM contributes significantly to membrane currents under experimental conditions previously thought to enhance I_{CRAC} carried by monovalent ions alone.

METHODS

For patch-clamp experiments, RBL-2H3-M1 cells (a clonal mast cell stably transfected with the muscarinic M1 receptor) grown on glass coverslips were transferred to the recording chamber and kept in a standard modified Ringer solution of the following composition (mM): NaCl 145, KCl 2.8, CsCl 10, $CaCl_2$ 10, $MgCl_2$ 2, glucose 10, Hepes-NaOH 10, pH 7.2. In some experiments, the above solution was temporarily replaced by an otherwise identical extracellular solution in which Ca^{2+} was omitted (0 Ca^{2+}) and in others both Ca^{2+} and Mg^{2+} were omitted and 2 mM EDTA was added (divalent-free, DVF). Solution changes were performed by pressure ejection from a wide-tipped pipette. In experiments reported in Fig. 1, external Ca^{2+} concentration was 1 mM throughout. Intracellular pipette-filling solutions contained (mM): caesium glutamate 145, NaCl 8, caesium BAPTA 10, Hepes-CsOH 10, pH 7.2. This solution was considered divalent free. Mg·ATP and/or $MgCl_2$ were added as indicated in the figure legends and free $[Mg^{2+}]_i$ was calculated by WEBMAXC v. 2.10 (http://www.stanford.edu/~cpatton/webmaxc2.htm). In all experiments that required buffering of free $[Ca^{2+}]_i$ to 90 nM, BAPTA was replaced by a mixture of 10 mM EGTA and 3.6 mM $CaCl_2$.

Patch-clamp experiments were performed in the tight-seal whole-cell configuration at 24 ± 2 °C. High-resolution current recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA, Lambrecht, Germany). Patch pipettes had resistances between 2 and 4 MΩ after filling with the standard intracellular solution. Immediately following establishment of the whole-cell configuration, voltage ramps of 50 ms duration spanning the voltage range of −100 to +100 mV were delivered from a holding potential of 0 mV at a rate of 0.5 Hz over a period of 200 to 400 s. All voltages were corrected for a liquid junction potential of 10 mV between external and internal solutions. Currents were filtered at 2.3 kHz and digitized at 100 μs intervals. Capacitive currents and series resistance were determined and corrected before each voltage ramp using the automatic capacitance compensation of the EPC-9. For analysis, the very first ramps prior to current activation were digitally filtered at 2 kHz, pooled and used for leak subtraction of all subsequent current records. The low-resolution temporal development of currents at a given potential was extracted from the leak-corrected individual ramp current records by measuring the current amplitudes at voltages of −80 and +80 mV. Effects of DVF solutions on background currents other than I_{CRAC} and MagNuM were negligible (0.52 ± 0.13 pA pF^{-1}), as determined in experiments in which neither current was allowed to activate (i.e. $[Ca^{2+}]_i$ was buffered to 90 nM and 6 mM Mg·ATP was added to the intracellular solution).

Ionomycin and GTP-γ-S were purchased from Sigma. 2-Aminoethoxydiphenyl borate (2-APB) was kindly provided by Dr Mikoshiba (Department of Molecular Neurobiology, Tokyo University, Tokyo, Japan).

Where applicable, data are given as means ± S.E.M. with n determinations and statistical significance was assessed by Student's t test.

RESULTS

Activation kinetics of I_{CRAC} and MagNuM

In a first series of experiments, we established conditions that would cause activation of both I_{CRAC} and MagNuM. This was achieved by using a standard intracellular solution supplemented with 10 mM BAPTA and devoid of added ATP. The extracellular solution contained 1 mM Ca^{2+}, which is sufficient to carry significant current through CRAC channels without unduly suppressing outward MagNuM currents. Immediately following break-in, voltage ramps from −100 to +100 mV were delivered at a rate of 0.5 Hz to continuously monitor membrane currents.

As shown in Fig. 1*A*, both inward and outward currents (measured at −80 and +80 mV, respectively) developed over 100–300 s. The characteristic current–voltage relationship of the conductance under these conditions is seen in Fig. 1*B*, which illustrates a high-resolution current record in response to a voltage ramp recorded at 300 s in a representative experiment. Under these experimental conditions, at least initially, the inward current at −80 mV is predominantly I_{CRAC}, which activates passively as BAPTA prevents reuptake of Ca^{2+} into the stores. The outward current at +80 mV is primarily MagNuM, which activates with a delay as cytosolic ATP decreases due to diffusional exchange between the cytosol and the ATP-free pipette solution. Given that there is also an inward MagNuM current, it is likely that the inward current progressively reflects a mixture of I_{CRAC} and MagNuM components.

Kinetic analysis yields a time to half-maximal activation ($t_{½}$) of 91 ± 8 s for I_{CRAC} and 220 ± 6 s for MagNuM (n = 8), suggesting that the two current components are carried by distinct sets of ion channels.

Since I_{CRAC} is activated by store depletion and MagNuM is activated by Mg·ATP depletion, we reasoned that under otherwise identical conditions, the inclusion of InsP_3 in the pipette solution, due to its active Ca^{2+} release activity, would selectively accelerate store-dependent I_{CRAC} with little or no effect on the MagNuM time course. As illustrated in Fig. 1*C* this is indeed the case. The half-maximal activation times in the presence of 20 μM InsP_3 were 31 ± 7 s for I_{CRAC} and 200 ± 15 s for MagNuM (n = 3). The acceleration of inward I_{CRAC} was statistically significant ($P < 0.002$), whereas time courses of outward currents were not significantly different. This confirms that inward currents carried by I_{CRAC} are store dependent, whereas outward currents are not.

Since both I_{CRAC} and MagNuM are regulated by $[Mg^{2+}]_i$, we carried out experiments similar to those of Fig. 1*A*, but omitted Mg^{2+} from the pipette solutions (Fig. 1*D*). This resulted in a large increase in outward current amplitude and a smaller increase in inward current, suggesting that MagNuM is more susceptible to $[Mg^{2+}]_i$ regulation than I_{CRAC}. In terms of kinetics, $t_{½}$ was 84 ± 6 s and was not significantly different from that observed in Fig. 1*A*, where free $[Mg^{2+}]_i$ was calculated to be 780 μM. However, the time course of MagNuM was accelerated under $[Mg^{2+}]_i$-free conditions with $t_{½}$ = 151 ± 3 s (n = 5), and this shift was statistically significant ($P < 1 \times 10^{-6}$) with respect to the time course of MagNuM shown in Fig. 1*A*. We attribute this acceleration to a faster decrease in cytosolic Mg·ATP levels when perfusing cells with $[Mg^{2+}]_i$-free intracellular solutions.

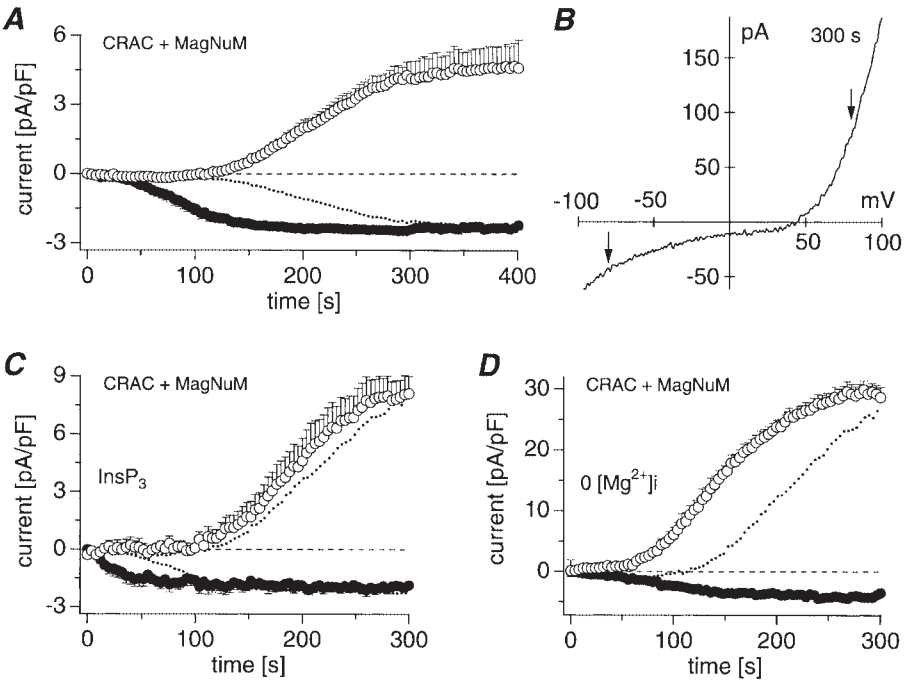

Figure 1. Differential activation time course of I_{CRAC} and MagNuM

A, average inward (●) and outward (○) I_{CRAC} and MagNuM at −80 and +80 mV, respectively (n = 8). I_{CRAC} and MagNuM were activated passively by omission of ATP and inclusion of 10 mM BAPTA in the pipette solution ($[MgCl_2]_i$ = 1 mM, free $[Mg^{2+}]_i$ = 780 μM, extracellular $[Ca^{2+}]$ = 1 mM). The dotted line represents the inverted and scaled outward current to illustrate the difference in activation time course. *B*, current–voltage relationship derived from a high-resolution current record in response to a voltage ramp of 50 ms duration that ranged from −100 to +100 mV, taken at 300 s from a representative cell under experimental conditions described in *A*. Arrows indicate −80 and +80 mV. Inward currents are predominantly I_{CRAC} and outward currents are predominantly MagNuM. *C*, average inward (●) and outward (○) I_{CRAC} and MagNuM at −80 and +80 mV, respectively (n = 3). Experimental conditions and solutions as in *A*, except that 20 μM InsP_3 was added to the pipette solution to accelerate activation of I_{CRAC}. The dotted lines represent the scaled inward and outward currents depicted in *A* to illustrate the selective acceleration of I_{CRAC} activation time course. *D*, average inward (●) and outward (○) I_{CRAC} and MagNuM at −80 and +80 mV, respectively (n = 5). Experimental conditions and solutions as in *A*, except that the pipette solution lacked $MgCl_2$. The dotted line represents the scaled outward current taken from *A* to illustrate the difference in MagNuM activation time course.

Dissociation of I_{CRAC} and MagNuM by selective inhibition of activation

Based on their different activation mechanisms, we designed experimental conditions that would enable us to activate either of the two conductances in isolation. Since MagNuM is regulated by Mg·ATP, we performed experiments under similar conditions to Fig. 1, but included 6 mM Mg·ATP to suppress MagNuM (Fig. 2*A*). Under these conditions, cells developed an inward current that had the current–voltage (*I*–V) signature of I_{CRAC}, whereas outward MagNuM currents were absent (Fig. 2*B*). Thus, the presence of high levels of Mg·ATP selectively suppressed MagNuM and unmasked I_{CRAC}.

In order to activate MagNuM in isolation, we omitted ATP from the pipette solution and suppressed I_{CRAC} by clamping cytosolic free Ca^{2+} to 90 nM using appropriate mixtures of EGTA and Ca^{2+}. As can be seen in Fig. 2*C*, this resulted in selective activation of predominantly outward currents with the *I*–V signature of MagNuM (Fig. 2*D*). Note, however, that MagNuM also has an inward component, which develops in parallel with the outward component.

Based on the differential kinetics and the selective suppression of currents, the experiments so far suggest that the major portions of inward and outward currents are carried by different ion channel mechanisms, namely I_{CRAC} and MagNuM, respectively. However, one might argue that these differences are due to changes in conductance properties of only one mechanism, namely I_{CRAC}, and that MagNuM reflects an unusual behaviour of outward currents through CRAC channels. We have therefore performed experiments in which we first allowed MagNuM to fully activate under experimental conditions that suppress I_{CRAC} and then applied the Ca^{2+} ionophore ionomycin to actively induce store depletion. As illustrated in Fig. 2*E*, this resulted in a selective increase in inward current without a corresponding increase in outward current. The *I*–*V* relationships of cells prior to and following ionomycin-induced store depletion, illustrated in Fig. 2*F*, demonstrate in more detail the selective recruitment of an inwardly rectifying conductance with the signature of I_{CRAC}. The additivity of I_{CRAC} and MagNuM establishes, without reasonable doubt, that the two conductances are indeed distinct entities.

Monovalent I_{CRAC} and MagNuM

I_{CRAC} is a highly specific Ca^{2+} current, but can support a large monovalent current when divalents are removed from the extracellular solution (Hoth & Penner, 1993; Kerschbaum & Cahalan, 1998). Figure 3*A* illustrates this phenomenon under conditions where MagNuM has been suppressed by inclusion of 6 mM intracellular Mg·ATP. Under these conditions I_{CRAC}, but not MagNuM, activates due to passive depletion of Ca^{2+} from intracellular stores. Upon removal of divalents, there is a transient increase in inward I_{CRAC} as previously described (Hoth & Penner, 1993). Note, however, that outward currents do not increase proportionally so that inward rectification of I_{CRAC} is preserved (Fig. 3*B*). Also note the rather strong inactivation of the monovalent inward current, whose underlying mechanism is at present not understood but has been proposed to be related to the presence of $[Mg^{2+}]_i$ (Kerschbaum & Cahalan, 1998). However, as will be shown below, our data do not support this interpretation.

We have previously shown that MagNuM can also sustain a large monovalent current upon removal of all divalent ions from the extracellular solution (Nadler *et al.* 2001). Figure 3*C* illustrates this under experimental conditions where buffering of $[Ca^{2+}]_i$ to 90 nM suppresses I_{CRAC} activation, while omission of ATP selectively leads to MagNuM activation. During application of divalent-free extracellular solution, there is a large increase in inward MagNuM current, but in contrast to the behaviour of I_{CRAC}, there is also a significant increase in outward current even at potentials below +50 mV, which we interpret to be due to the removal of permeation block of divalent ions. As outward currents are no longer impeded by divalent ions, the MagNuM *I*–*V* relationship becomes almost linear (Fig. 3*D*). Furthermore, monovalent MagNuM currents also exhibit some inactivation, most prominently of the outward current, albeit to a lesser degree than the inactivation of monovalent I_{CRAC}.

Effects of intracellular Mg^{2+} on I_{CRAC} and MagNuM

The majority of experiments presented so far were carried out under conditions where intracellular Mg^{2+} concentrations were kept in the physiological range of 0.5–0.8 mM (Romani & Scarpa, 2000). Previous studies of I_{CRAC} have suggested that $[Mg^{2+}]_i$ contributes to inactivation and inward rectification of I_{CRAC} through a voltage-dependent block of outward currents (Kerschbaum & Cahalan, 1998). We have therefore attempted to directly compare monovalent I_{CRAC} and MagNuM under similar free $[Mg^{2+}]_i$. The left panel of Fig. 4*A* illustrates that I_{CRAC} carried by Ca^{2+}, in this case activated by 20 μM InsP_3, activates rapidly and maximally, while MagNuM remains suppressed by 6 mM Mg·ATP. Upon switching to divalent-free extracellular solution, there is significant increase in inward current that rapidly inactivates to a steady-state level that is similar to the current amplitude seen with Ca^{2+} as charge carrier. As illustrated in the right panel of Fig. 4*A*, the same level of 1.1 mM free $[Mg^{2+}]_i$ largely suppressed activation of MagNuM and consequently there is little monovalent current when establishing divalent-free conditions.

We next reduced the free $[Mg^{2+}]_i$ to 550 μM, which represents the minimal concentration achievable when using 6 mM Mg·ATP to suppress MagNuM and study I_{CRAC} in isolation. Figure 4*B* illustrates that under these conditions, InsP_3-induced I_{CRAC} carried by Ca^{2+} is not affected in any significant way compared to 1.1 mM free $[Mg^{2+}]_i$. When

switching to divalent-free solution, there is a large increase in inward current, which, as with 1.1 mM $[Mg^{2+}]_i$, inactivates rapidly and strongly. Thus, the reduction in $[Mg^{2+}]_i$ increases the monovalent current through I_{CRAC}, but has little impact on inactivation. The corresponding experiment, in which MagNuM was activated by ATP depletion while $[Mg^{2+}]_i$ was fixed to 550 μM and $[Ca^{2+}]_i$ was buffered to 90 nM to avoid activation of I_{CRAC}, is illustrated in the right panel of Fig. 4*B*. It demonstrates that exposing cells to divalent-free extracellular solution greatly amplifies monovalent MagNuM currents, confirming that $[Mg^{2+}]_i$ regulates this conductance as well. The magnitude of monovalent inward MagNuM current is about half of that of I_{CRAC}, but exhibits less-pronounced inactivation.

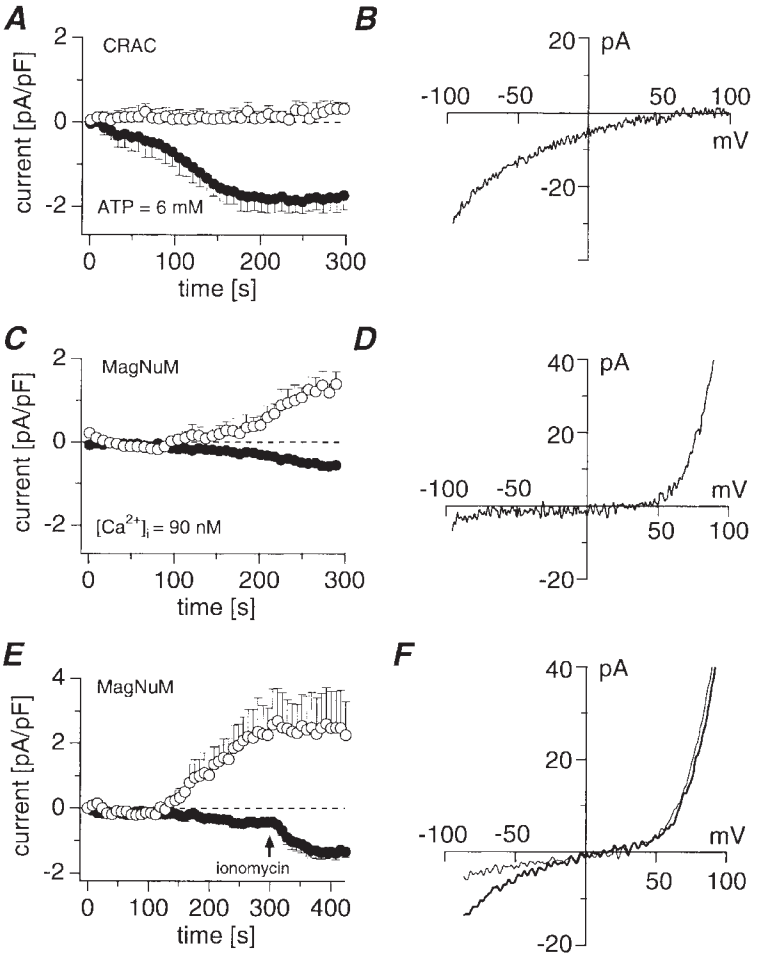

Figure 2. Selective activation of I_{CRAC} and MagNuM

A, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 4$), recorded under experimental conditions that suppress MagNuM and favour I_{CRAC} activation. I_{CRAC} was activated passively by inclusion of 10 mM BAPTA in the pipette solution and MagNuM was suppressed by 6 mM Mg·ATP ($[MgCl_2]_i = 0$, free $[Mg^{2+}]_i = 550$ μM). *B*, *I–V* relationship of I_{CRAC} derived from high-resolution current records in response to voltage ramps of 50 ms duration that ranged from −100 mV to +100 mV. Data are taken from a representative cell under experimental conditions described in *A*. *C*, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 7$), recorded under experimental conditions that suppress I_{CRAC} and favour MagNuM activation. MagNuM was activated passively by omission of ATP from the pipette solution and I_{CRAC} was suppressed by buffering $[Ca^{2+}]_i$ to 90 nM using 10 mM EGTA and 3.6 mM $CaCl_2$ ($[MgCl_2]_i = 1$ mM, free $[Mg^{2+}]_i = 760$ μM). *D*, *I–V* relationship of MagNuM derived from high-resolution current records measured with the same pulse protocol as in *B*. Data are taken from a representative cell under experimental conditions described in *C*. *E*, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 5$), recorded under experimental conditions that suppress I_{CRAC} and favour MagNuM activation. MagNuM was activated passively by omission of ATP from the pipette solution and I_{CRAC} was suppressed by buffering $[Ca^{2+}]_i$ to 90 nM using 10 mM EGTA and 3.6 mM $CaCl_2$ (free $[Mg^{2+}]_i = 0$). I_{CRAC} was activated by brief application (2–3 s) of 20 μM ionomycin applied at the time indicated by the arrow. *F*, *I–V* relationships derived from high-resolution current records, measured as in *B*. Data are taken from a representative cell under experimental conditions described in *E* before ionomycin application (300 s, thin line) and after I_{CRAC} had fully developed (400 s, thick line).

A further increase in monovalent MagNuM currents was achieved by omitting Mg^{2+} from the intracellular solution, as illustrated in the right panel of Fig. 4*C*. It should be noted that in order to suppress I_{CRAC}, the intracellular solution was buffered to 90 nM free $[Ca^{2+}]_i$, and this presumably limited the maximal amount of monovalent MagNuM current, since $[Ca^{2+}]_i$ causes some inhibition of MagNuM (Nadler *et al.* 2001). Nevertheless, it is clear that omitting $[Mg^{2+}]_i$ significantly increases monovalent inward MagNuM currents.

Unfortunately, it is not possible to completely remove all free Mg^{2+} from the intracellular solution and measure I_{CRAC} in isolation from MagNuM, since MagNuM suppression requires Mg·ATP (Nadler *et al.* 2001). However, we have found that GTP-γ-S at 100 μM can inhibit MagNuM in a Mg^{2+}- and ATP-independent manner, possibly through a G protein-mediated regulation, and this allowed us to study monovalent I_{CRAC} in isolation from MagNuM under $[Mg^{2+}]_i$-free conditions. In this experiment, illustrated in the left panel of Fig. 4*C*, intracellular solutions contained 100 μM Na-GTP-γ-S in the absence of Mg^{2+} and I_{CRAC} was activated by 20 μM InsP_3. Under these conditions, we observed only a very small, transient increase in outward MagNuM current activation, which peaked at 0.6 pA pF^{-1} around 150 s into the experiment and subsequently decayed to 0.1 pA pF^{-1} due to GTP-γ-S block. Thus the inhibition of MagNuM by GTP-γ-S was essentially complete by the time DVF solution was applied, since the magnitude of the corresponding outward MagNuM current under Mg^{2+}-free conditions is 3.2 pA pF^{-1} (right panel of Fig. 4*C*).

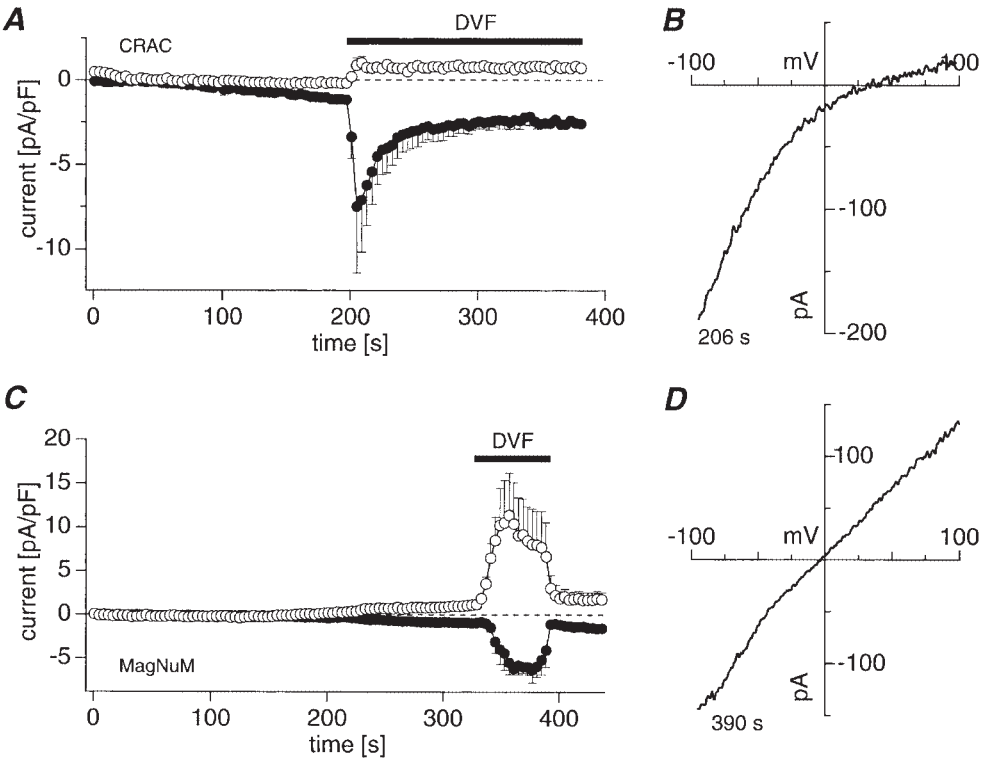

Figure 3. Monovalent I_{CRAC} and MagNuM

A, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 3$), recorded under experimental conditions that suppress MagNuM and favour I_{CRAC} activation. I_{CRAC} was activated passively by inclusion of 10 mM BAPTA in the pipette solution and MagNuM was suppressed by 6 mM Mg·ATP ($[MgCl_2]_i = 1$ mM, free $[Mg^{2+}]_i = 1.1$ mM). Cells were perfused with divalent-free (DVF) extracellular solution for the time indicated by the bars. *B*, *I–V* relationship derived from a high-resolution current record in response to a voltage ramp of 50 ms duration that ranged from −100 to +100 mV, taken at the peak of monovalent inward current (206 s) from a representative cell under experimental conditions described in *A*. Note the strong inward rectification of monovalent I_{CRAC}. *C*, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 4$), recorded under experimental conditions that suppress I_{CRAC} and favour MagNuM activation. MagNuM was activated passively by omission of ATP from the pipette solution and I_{CRAC} was suppressed by buffering $[Ca^{2+}]_i$ to 90 nM using 10 mM EGTA and 3.6 mM $CaCl_2$ ($[MgCl_2]_i = 1$ mM, free $[Mg^{2+}]_i = 760$ μM). Cells were perfused with DVF extracellular solution for the time indicated by the bars. *D*, *I–V* relationship derived from a high-resolution current record in response to a voltage ramp as detailed in *B*, taken at the peak of monovalent inward current (390 s) from a representative cell under experimental conditions described in *C*. Note the linear *I–V* relationship of monovalent MagNuM currents.

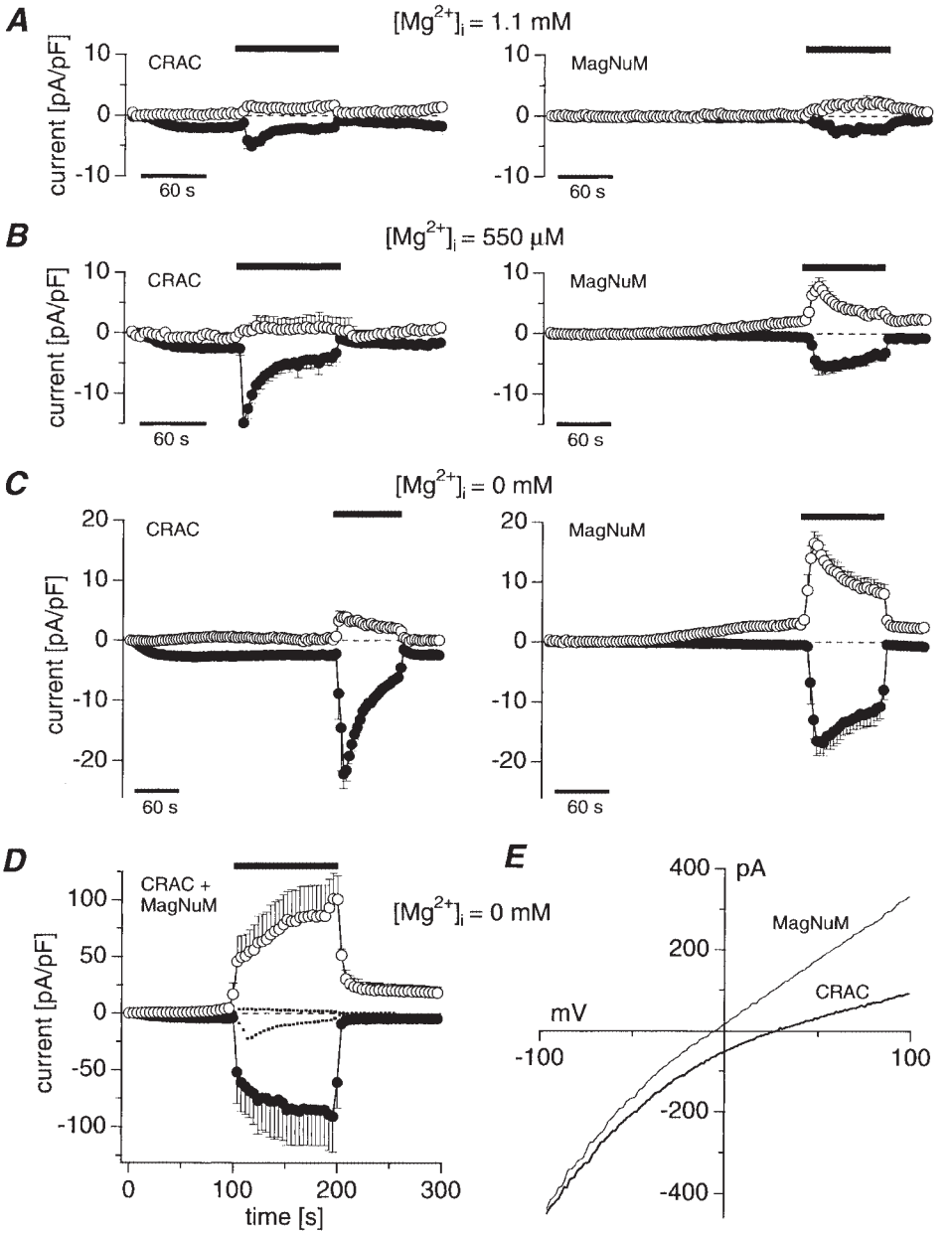

Figure 4. Monovalent I_{CRAC} and MagNuM in reduced intracellular Mg^{2+}

In all panels, average inward (●) and outward (○) currents at −80 and +80 mV were acquired under defined free $[Mg^{2+}]_i$. Left panels represent experimental conditions that suppress MagNuM and favour I_{CRAC} activation by inclusion of 6 mM Mg·ATP, 20 μM InsP_3 and 10 mM BAPTA in the pipette solution, whereas right panels represent conditions in which I_{CRAC} was suppressed by buffering $[Ca^{2+}]_i$ to 90 nM using 10 mM EGTA and 3.6 mM $CaCl_2$. MagNuM developed by omission of ATP. In all experiments, appropriate amounts of $MgCl_2$ were added to arrive at defined $[Mg^{2+}]_i$, as indicated above each pair of panels. Cells were perfused with divalent-free extracellular solution for the times indicated by the bars. *A*, left panel: average responses of five cells ($[MgCl_2]_i$ = 1 mM, free $[Mg^{2+}]_i$ = 1.1 mM). Right panel: average responses of nine cells ($[MgCl_2]_i$ = 1.4 mM, free $[Mg^{2+}]_i$ = 1.1 mM). *B*, left panel: average responses of four cells ($[MgCl_2]_i$ = 0, free $[Mg^{2+}]_i$ = 550 μM). Right panel: average responses of eight cells ($[MgCl_2]_i$ = 740 μM, free $[Mg^{2+}]_i$ = 550 μM). *C*, left panel: average responses of five cells in $[Mg^{2+}]_i$-free conditions where ATP and $MgCl_2$ were omitted from the pipette solution and MagNuM was suppressed by 100 μM GTP-γ-S. I_{CRAC} was activated by 20 μM InsP_3. Right panel: average responses of nine cells in $[Mg^{2+}]_i$-free conditions. *D*, average responses of four cells in $[Mg^{2+}]_i$-free conditions where ATP and $MgCl_2$ were omitted from the pipette solution. Therefore, both InsP_3-mediated I_{CRAC} and ATP-dependent MagNuM were activated. The dotted trace represents data taken from the left panel of *C* to illustrate the contribution of I_{CRAC}. *E*, *I–V* relationships derived from high-resolution current records in response to a voltage ramp of 50 ms duration that ranged from −100 to +100 mV, taken at the peak of monovalent inward currents from representative cells under experimental conditions described in *C*. Note the linear *I–V* relationship of monovalent MagNuM currents and inward rectification of I_{CRAC}.

Upon exposure to divalent-free extracellular solution, there was again a large increase in inward current, which also inactivated rather quickly. The degree of inactivation was significantly larger than the one observed with MagNuM, amounting to 73 % at the end of the DVF challenge, whereas the corresponding decrease in MagNuM was only 35 % (right panel of Fig. 4*C*). Monovalent I_{CRAC} retained considerable inward rectification, whereas the corresponding monovalent MagNuM currents were rather linear (Fig. 4*E*). Finally, we performed experiments in which both I_{CRAC} and MagNuM were allowed to activate under $[Mg^{2+}]_i$-free conditions and perfused cells with internal solutions that included InsP_3 but were devoid of Mg^{2+} and ATP (Fig. 4*D*). Here, the activation of I_{CRAC} proceeded rapidly and when the extracellular solution was changed to divalent-free there was a sudden massive increase in both inward and outward monovalent current, which was probably due to the combined contributions of I_{CRAC} and MagNuM, followed by a slower, steady increase during the application period of divalent-free solution. We interpret this slow increase to be primarily due to MagNuM, since its activation proceeds during the divalent-free episode, as witnessed by the increased levels of outward current upon readmission of divalent-containing extracellular solution. This suggests that the contribution of MagNuM masks that of I_{CRAC}, which is likely to be similar to the response seen in the left panel of Fig. 4*C* and which is superimposed here for comparison as a dotted line. Since I_{CRAC} is subject to inactivation and MagNuM continues to increase, the latter is likely to carry the major component of monovalents under these experimental conditions.

Selectivity of I_{CRAC} and MagNuM

We next took advantage of differences in selectivity as a further means to distinguish I_{CRAC} and MagNuM. I_{CRAC} is well known for the high selectivity for Ca^{2+} over any other divalent ion, including Mg^{2+} (Hoth & Penner, 1993). MagNuM, on the other hand, is due to divalent-selective cation channel that conducts both Ca^{2+} and Mg^{2+} at membrane potentials below 0 mV (Nadler *et al.* 2001). The permeation of divalent ions such as Ca^{2+} and Mg^{2+} obstructs any significant monovalent conductance at negative membrane voltages. Only above 0 mV will MagNuM be carried by monovalent cations, since relief from this divalent permeation block gradually decreases as the reversal potential for Ca^{2+} is approached. Figure 5 illustrates that I_{CRAC} and MagNuM indeed behave differently when exposed to extracellular solutions that are Ca^{2+} free, but contain the standard Mg^{2+} levels. In the case of I_{CRAC}, illustrated in Fig. 5*A*, removal of Ca^{2+} completely blocks any inward current and there is no change in outward current. Conversely, when suppressing I_{CRAC} and activating MagNuM in isolation (Fig. 5*B*), the removal of extracellular Ca^{2+} suppresses the inward current only partially, since Mg^{2+} can sustain inward MagNuM currents. At the same time, there is a significant augmentation of monovalent outward current, which is consistent with a reduced permeation block as Ca^{2+} permeation is reduced.

Pharmacology of I_{CRAC} and MagNuM

Finally, we sought to dissociate I_{CRAC} and MagNuM pharmacologically. 2-Aminoethoxydiphenyl borate (2-APB)

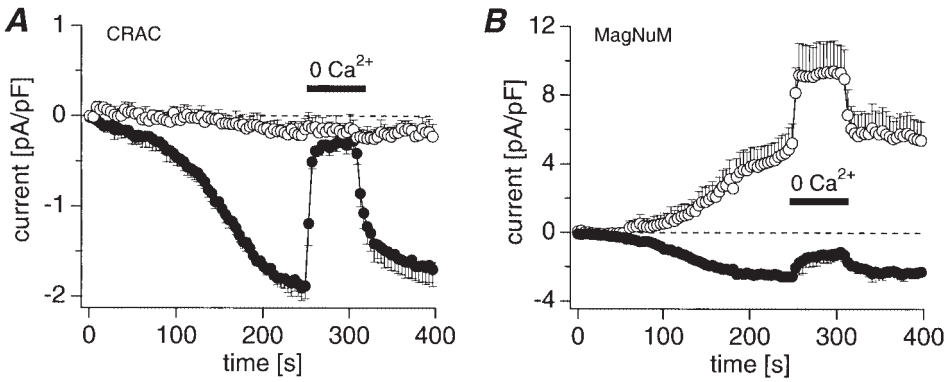

Figure 5. Differential permeation of Ca^{2+} and Mg^{2+} through I_{CRAC} and MagNuM

A, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 3$), recorded under experimental conditions that suppress MagNuM and favour I_{CRAC} activation. I_{CRAC} was activated passively by inclusion of 10 mM BAPTA in the pipette solution and MagNuM was suppressed by 6 mM Mg·ATP ($[MgCl_2]_i = 1$ mM, free $[Mg^{2+}]_i = 1.1$ mM). Cells were perfused with nominally Ca^{2+}-free, but Mg^{2+}-containing extracellular solution for the time indicated by the bars. Note the complete block of I_{CRAC} when Ca^{2+} was removed. *B*, average inward (●) and outward (○) currents at −80 and +80 mV, respectively ($n = 3$), recorded under experimental conditions that suppress I_{CRAC} and favour MagNuM activation. MagNuM was activated passively by omission of ATP and $MgCl_2$ from the pipette solution and I_{CRAC} was suppressed by buffering $[Ca^{2+}]_i$ to 90 nM using 10 mM EGTA and 3.6 mM $CaCl_2$ (free $[Mg^{2+}]_i = 0$). Cells were perfused with nominally Ca^{2+}-free, but Mg^{2+}-containing extracellular solution for the time indicated by the bars. Note that MagNuM inward current is not abolished, but carried by Mg^{2+} and that outward currents are enhanced.

has been reported to be a selective inhibitor of store-operated Ca^{2+} entry (Ma *et al.* 2000). Its inhibition of InsP_3 receptors has been interpreted in support of the conformational coupling model of I_{CRAC} activation, where InsP_3 receptors are hypothesized to directly interact with and gate CRAC channels (Irvine, 1990; Berridge, 1995). By the same token, the inhibitory effect of 2-APB on single channels of RBL, recorded under divalent-free conditions, has been invoked as major supporting evidence for identifying these channels as being CRAC channels (Braun *et al.* 2000). We have re-investigated the effects of 2-APB under conditions where I_{CRAC} and MagNuM are activated in the same cell. Figure 6*A* illustrates the InsP_3-mediated activation of I_{CRAC} carried by 10 mM Ca^{2+}. As I_{CRAC} is almost fully activated, the delayed activation of MagNuM starts, as evidenced by the small increase in outward current. After the inward I_{CRAC} reached its maximum, 2-APB (100 μM) was applied extracellularly, and this resulted in a strong and irreversible inhibition of I_{CRAC}. At the same time, the outward MagNuM current, which had just started to activate, was also inhibited. This inhibition, however, was not irreversible, since outward MagNuM currents recovered from 2-APB block. After significant activation of MagNuM, we re-applied 2-APB and again observed a significant, albeit not complete, inhibition of outward currents.

Thus, aside from a slightly lower efficacy in suppressing MagNuM, the major difference of 2-APB-mediated effects on I_{CRAC} and MagNuM appears to be the degree of reversibility. Furthermore, although we are not concerned with the activation mechanism of I_{CRAC}, this result demonstrates that 2-APB can inhibit I_{CRAC} after it has been fully activated and it does so with a rapidity that is consistent with a direct pharmacological inhibition (Braun *et al.* 2000). We observed the same type of inhibition in cells where I_{CRAC} was activated in an InsP_3-independent manner through passive depletion (data not shown). Furthermore, the 2-APB-induced block of I_{CRAC} only occurred when the compound was applied extracellularly and inclusion of the same concentration of 2-APB in the pipette solution was ineffective, consistent with an interpretation that 2-APB acts as an extracellular pore blocker of CRAC channels (Braun *et al.* 2000). In any event, the 2-APB-induced block of I_{CRAC} is not selective and can therefore not be considered an identifying signature for CRAC channels.

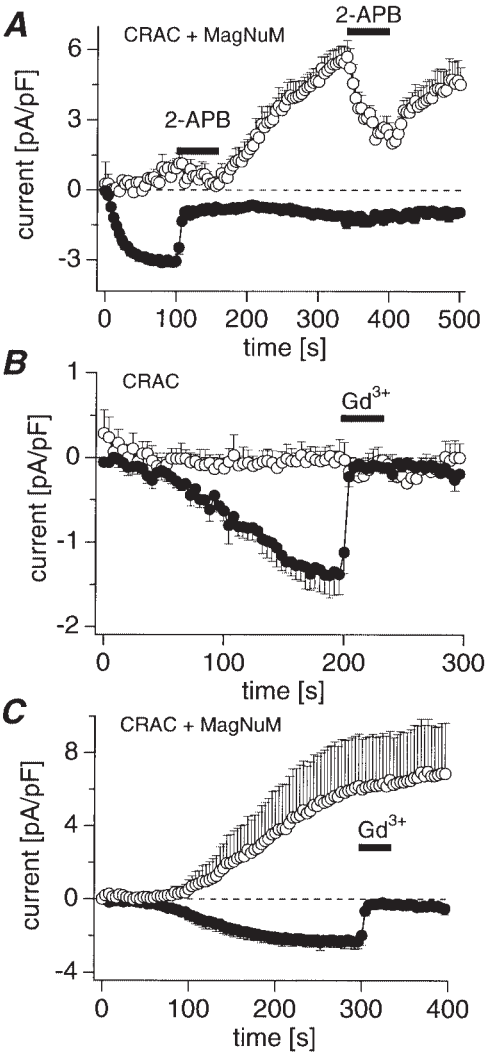

Figure 6. Differential pharmacology of I_{CRAC} and MagNuM

A, average inward (●) and outward (○) I_{CRAC} and MagNuM at −80 and +80 mV, respectively (n = 4). I_{CRAC} and MagNuM were activated passively by omission of ATP and inclusion of 20 μM InsP_3 and 10 mM BAPTA in the pipette solution ($[MgCl_2]_i$ = 1 mM, free $[Mg^{2+}]_i$ = 780 μM). After full activation of I_{CRAC}, 100 μM 2-APB was applied extracellularly for the time indicated by the bar and a second application was performed after MagNuM had developed. Note the differential reversibility of 2-APB-mediated inhibition of outward and inward currents. *B*, average inward (●) and outward (○) currents at −80 and +80 mV, respectively (n = 3), recorded under experimental conditions that suppress MagNuM and favour I_{CRAC} activation. I_{CRAC} was activated passively by inclusion of 10 mM BAPTA in the pipette solution and MagNuM was suppressed by 6 mM Mg·ATP ($[MgCl_2]_i$ = 1 mM, free $[Mg^{2+}]_i$ = 1.1 mM). After full activation of I_{CRAC}, 10 μM Gd^{3+} was applied extracellularly for the time indicated by the bar. *C*, average inward (●) and outward (○) I_{CRAC} and MagNuM at −80 and +80 mV, respectively (n = 3). I_{CRAC} and MagNuM were activated passively by omission of ATP and $MgCl_2$ and inclusion of 10 mM BAPTA in the pipette solution. After full activation of both I_{CRAC} and MagNuM, 10 μM Gd^{3+} was applied extracellularly for the time indicated by the bar. Note the complete inhibition of inward current and the lack of effect on outward current.

We sought to establish a more discriminating pharmacological assay to distinguish between I_{CRAC} and MagNuM and tested for selective Gd^{3+} sensitivity of the two conductances. As illustrated in Fig. 6*B*, I_{CRAC} is potently inhibited by low concentrations of Gd^{3+} (10 μM). However, Gd^{3+} at this concentration did not significantly affect outward MagNuM currents (Fig. 6*C*), while the inward current, in this case both I_{CRAC} and MagNuM, was completely suppressed. Additionally, we confirmed that 10 μM Gd^{3+} could partially block inward but not outward currents in HEK-293 cells over-expressing recombinant LTRPC7 (data not shown). Thus, although Gd^{3+} does not inhibit outward MagNuM currents, it does not seem to be an ideal tool to distinguish between I_{CRAC} and MagNuM, since inward MagNuM currents, as with I_{CRAC}, are suppressed by Gd^{3+}.

DISCUSSION

The present study was designed to distinguish the Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) from the recently discovered magnesium–nucleotide-regulated metal ion current (MagNuM) (Nadler *et al.* 2001). The underlying ion channels are encoded by the *LTRPC7* gene, a member of TRP family of ion channel genes (Harteneck *et al.* 2000). *LTRPC7* is widely expressed in numerous tissues and cells (Nadler *et al.* 2001). MagNuM shares some features with I_{CRAC}, most notably a large monovalent conductance in the absence of divalent charge carriers such as Ca^{2+} and Mg^{2+}. Furthermore, MagNuM is activated under experimental conditions that have traditionally been used to study I_{CRAC}. Finally, since MagNuM is also present in RBL cells and lymphocytes (Nadler *et al.* 2001), the model systems used to study I_{CRAC}, it is important to establish experimental conditions that rule out ambiguities arising from the parallel activation of I_{CRAC} and MagNuM in these cells. This is particularly important for recently developed protocols designed to measure single-channel currents through CRAC channels (Kerschbaum & Cahalan, 1999; Braun *et al.* 2000; Fomina *et al.* 2000), as they involve Mg·ATP and divalent-free solutions, which greatly favour the activation of MagNuM. In the following, we shall discuss the salient features of I_{CRAC} and MagNuM.

I_{CRAC} is a store-operated current that is activated by Ca^{2+} release from intracellular stores (Hoth & Penner, 1992; Parekh & Penner, 1997). Physiologically, this is achieved by receptor-mediated InsP_3 formation that causes store depletion. MagNuM, due to its relatively low sensitivity to Mg·ATP (millimolar), is constitutively active at low levels in resting cells, but is strongly activated by cytosolic ATP depletion below 1 mM (Nadler *et al.* 2001). Given the differential activation mechanisms, it is possible to isolate both conductances by appropriately composed intracellular solutions. Thus, I_{CRAC} can be effectively suppressed by buffering $[Ca^{2+}]_i$ to resting levels around 100 nM (Fasolato *et al.* 1993; Krause *et al.* 1999) and MagNuM can be inhibited by increasing Mg·ATP and/or $[Mg^{2+}]_i$ (Nadler *et al.* 2001). However, studies on I_{CRAC} originating from our own, as well as numerous other, laboratories have traditionally used low Mg·ATP concentrations or have completely omitted ATP from intracellular solutions. Therefore, I_{CRAC} recordings in most of these studies have probably been contaminated to various degrees with MagNuM currents.

Kinetics of activation of I_{CRAC} and MagNuM

Experimentally, one can trigger I_{CRAC} activation by a variety of conditions that lead to store depletion (Hoth & Penner, 1992; Parekh & Penner, 1996). MagNuM is activated by depletion of Mg·ATP and appears to be store independent, since active store depletion by InsP_3 does not significantly affect its activation kinetics (see Fig. 1). Several electrophysiological studies have used a passive store depletion protocol, where I_{CRAC} is activated by perfusing cells with pipette solutions that contain high concentrations of Ca^{2+} chelators such as EGTA and BAPTA. This results in the gradual depletion of intracellular stores due to basal leakage of Ca^{2+} out of the stores into the cytosol and the inability to refill these stores, since released Ca^{2+} is immediately captured by the buffers. As a result, store-operated I_{CRAC} is activated and reaches half-maximal amplitude at around 80 s in RBL cells (see Fig. 1). In these cells, ATP-free internal solutions cause activation of MagNuM with a considerably longer delay. The average half-maximal activation times of I_{CRAC} and MagNuM differ by about 140 s (see Fig. 1). There is certainly some degree of variability in the kinetics of activation of both conductances, as would be expected from their activation mechanisms (store depletion and ATP depletion, respectively). It is conceivable that both currents might activate in parallel, depending on how efficiently the two activation mechanisms are engaged under various experimental conditions. Factors that may influence this include (i) the metabolic state (e.g. cells with reduced Mg·ATP levels could activate MagNuM earlier), (ii) the cell type under investigation (e.g. lymphocytes have a significantly smaller cytosol volume compared to RBL cells and may equilibrate more rapidly with the pipette solution), (iii) the series resistance of a particular patch-clamped cell (e.g. higher resistances will slow diffusional exchange of cytosol and pipette solutions). One study has attempted to correlate inward and outward currents activated by store depletion and this study reported a parallel activation of inward and outward currents (Hoth, 1996). Our present data provide no evidence for parallel activation and although, on rare occasions, we have seen similar activation time courses, in no case was there an identical correspondence; MagNuM consistently lagged behind activation of I_{CRAC}. It is difficult to directly compare our data with those of the above investigation, since that study did not provide a quantitative assessment of average

current kinetics and illustrated only anecdotal records from individual cells. Kerschbaum & Cahalan (1998) have also considered kinetic aspects of inward and outward currents through I_{CRAC} and observed parallel activation. However, those experiments were carried out under conditions that excluded divalent ions from either side of the membrane and, as will be discussed below, are not unambiguous as they do not directly address the issue of whether the monovalent currents were carried by I_{CRAC} and/or MagNuM.

It is important to note that previous studies have, in fact, observed kinetic differences in the activation of I_{CRAC} carried by Ca^{2+} ions and inward currents carried by monovalent ions in the absence of divalents (Kerschbaum & Cahalan, 1999; Braun *et al.* 2000). Thus, Cahalan and colleagues illustrate I_{CRAC} carried by Ca^{2+} that require about 80 s for half-maximal activation, whereas monovalent currents in the same study consistently activated half-maximally at around 200 s (Kerschbaum & Cahalan, 1999). Both of these values are in very close correspondence to the kinetics observed in our present study. Similarly, Putney and colleagues observed characteristic kinetic delays of about 1.5- to 2-fold for monovalent currents compared to Ca^{2+} currents (Braun *et al.* 2000). In the light of the newly discovered MagNuM conductance, which supports large monovalent currents and matches the delayed activation kinetics of monovalent currents in the above studies, it is difficult to avoid the conclusion that at least part of the monovalent currents in those studies, if not all, reflect MagNuM rather than I_{CRAC}.

Selectivity of I_{CRAC} and MagNuM

MagNuM comprises inward current in the voltage range where I_{CRAC} is carried by Ca^{2+}, i.e. in the range of −100 mV to 0 mV. Thus, depending on the expression levels of a given cell type and the specific experimental conditions established by the composition of extra- and intracellular solutions, MagNuM can contribute significantly to inward currents and this contribution develops with a delay that coincides with the peak activation of I_{CRAC}. This makes it very difficult to assess accurately the contribution of each current. The typical size of total inward current carried by divalent ions at −80 mV in RBL cells under conditions that are normally used to study I_{CRAC} amounts to about 3 pA pF^{-1}. In the absence of Mg·ATP and under conditions that suppress I_{CRAC}, MagNuM typically generates around 0.5–1 pA pF^{-1}, and, although these conditions do not match exactly, we can estimate that MagNuM can contribute around 15–30 % of total inward current under standard conditions used to study I_{CRAC}.

MagNuM currents become relatively prominent above +50 mV, where they are carried by monovalent ions in the outward direction, but many investigations of I_{CRAC} cut off data presentation obtained at these positive voltages. We are aware of one publication that specifically addresses the outward currents of RBL cells in relation to I_{CRAC} (Hoth, 1996). This study suggested that outward currents recorded above +50 mV were carried by monovalent ions through CRAC channels. These outward currents were only observed when the intracellular solution contained K^+, but not Cs^+, as the primary monovalent cation and it was concluded that CRAC channels were permeable to K^+ at positive voltages. This investigation was carried out with intracellular solutions containing sub-millimolar ATP levels (0.5 mM), which in our hands will lead to significant activation of MagNuM. Our present study was exclusively conducted with Cs^+-based intracellular solutions and this cation is clearly permeant through LTRPC7 to the same extent as K^+ (Nadler *et al.* 2001). At present, we cannot offer an explanation for the discrepancies between these findings. However, since we can suppress all outward currents by including Mg·ATP in our intracellular solutions without suppressing I_{CRAC}, we conclude that CRAC channels do not carry significant outward currents *per se* and that under our experimental conditions the predominant part of the outward currents is MagNuM.

I_{CRAC} is normally a highly selective Ca^{2+} current (Hoth & Penner, 1992, 1993). However, removal of divalent ions from the extracellular solution leads to a characteristic loss in selectivity, so that Na^+ ions can permeate (Hoth & Penner, 1993; Kerschbaum & Cahalan, 1998). This typically results in a large increase in inward current. However, this increase is only transient and monovalent currents quickly inactivate by an unknown mechanism (Hoth & Penner, 1993). It has been proposed that this inactivation of I_{CRAC} is regulated by intracellular Mg^{2+}, since removal of $[Mg^{2+}]_i$ reduces the amount of inactivation (Kerschbaum & Cahalan, 1998), but, as discussed below, this notion may need revision, as it is based on experiments in which MagNuM may have contributed to monovalent inward currents.

MagNuM is normally also a divalent-selective cation current, at least over the physiological voltage range of −100 to +50 mV, where it is carried by both Ca^{2+} and Mg^{2+}. This characteristic difference between I_{CRAC} and MagNuM is highlighted by our experiments illustrated in Fig. 5, where the selective removal of extracellular Ca^{2+} completely suppresses I_{CRAC}, while the remaining extracellular Mg^{2+} partially supports inward MagNuM currents. Only at very positive potentials, where the permeation block of these divalents is reduced, MagNuM is carried by monovalent ions outwardly (physiologically this would be K^+, but experimentally Cs^+ permeates equally well). MagNuM, like I_{CRAC}, can lose its selectivity for divalents when both Ca^{2+} and Mg^{2+} are removed from the extracellular solution and this results in a significant augmentation of inward currents. Unlike I_{CRAC}, however, MagNuM supports large outward currents, and this occurs even when intracellular Mg^{2+} is present, whereas outward I_{CRAC} is virtually absent

(see Fig. 4). Given that several investigations have studied monovalent currents in the absence of $[Mg^{2+}]_i$, we need to consider how intracellular Mg^{2+} affects I_{CRAC} and MagNuM.

Effects of $[Mg^{2+}]_i$ on I_{CRAC} and MagNuM

Previous studies have suggested that I_{CRAC} is regulated by intracellular Mg^{2+}, since monovalent inward currents are greatly enhanced and their inactivation is reduced when $[Mg^{2+}]_i$ is removed (Kerschbaum & Cahalan, 1998, 1999; Braun *et al.* 2000; Fomina *et al.* 2000). Data presented in Fig. 4 of the present investigation confirm that monovalent currents carried by I_{CRAC} are larger than those carried by Ca^{2+} when free $[Mg^{2+}]_i$ is lowered from 1.1 mM to 550 μM. However, inward currents carried by Ca^{2+} are not significantly inhibited by $[Mg^{2+}]_i$ in this concentration range. We even failed to observe a significant inhibition of I_{CRAC} carried by Ca^{2+} at concentrations up to 2.8 mM (data not shown). By contrast, free $[Mg^{2+}]_i$ levels above 1 mM essentially abolish both inward and outward MagNuM currents, irrespective of the ion species carried. We therefore conclude that MagNuM is significantly more sensitive to inhibition by $[Mg^{2+}]_i$ than I_{CRAC}, at least with respect to currents carried by divalent ions.

An important result of the present study is that GTP-γ-S can suppress MagNuM in a Mg^{2+}- and ATP-independent fashion. Although GTP-γ-S has also been reported to inhibit the activation of I_{CRAC} (Fasolato *et al.* 1993), this effect requires prolonged pre-exposure to GTP-γ-S and delayed store depletion, whereas GTP-γ-S is completely ineffective in suppressing I_{CRAC} once it has been activated early in the experiment, e.g. when GTP-γ-S is co-perfused with InsP_3. Therefore, it is feasible to measure I_{CRAC} in isolation under Mg^{2+}-free conditions. The results presented in Fig. 4*C* directly demonstrate that I_{CRAC} remains subject to inactivation even under $[Mg^{2+}]_i$-free conditions. We can therefore assess the relative contributions of I_{CRAC} and MagNuM to monovalent membrane currents flowing upon removal of divalent ions when both conductances are activated. The peak monovalent inward I_{CRAC} and MagNuM currents are roughly equivalent under $[Mg^{2+}]_i$-free conditions (Fig. 4*C*). Importantly, the rapid inactivation of I_{CRAC} is evident even under $[Mg^{2+}]_i$-free conditions. It should be noted that the original observation of a reduction in the inactivation of monovalent currents through I_{CRAC} in $[Mg^{2+}]_i$-free solutions was made under experimental conditions that would inevitably reveal MagNuM as a contaminating, if not dominant, conductance. The experiment depicted in Fig. 4*D* reproduces this scenario and shows that, indeed, inactivation of monovalent currents is abolished in $[Mg^{2+}]_i$-free conditions. In fact, the monovalent currents continue to increase while exposing cells to divalent-free extracellular solutions. This increase may be attributed solely to MagNuM, since InsP_3-mediated activation of I_{CRAC} surely was maximal at this time. Even if we assumed that at the time of switching to divalent-free solutions, I_{CRAC} was the only pathway for monovalent ions, its contribution to inward current would have faded at the end of the divalent-free episode of the experiment due to inactivation. The dotted line in the left panel of Fig. 4*C*, representing the monovalent inward current through I_{CRAC} under $[Mg^{2+}]_i$-free conditions, illustrates the rapidly decreasing contribution of I_{CRAC} to whole-cell monovalent currents, leaving MagNuM as the primary, if not sole, monovalent influx pathway.

Pharmacology of I_{CRAC} and MagNuM

Investigations on I_{CRAC} suffer from the lack of specific pharmacological tools. However, 2-APB has been shown to inhibit I_{CRAC} (Braun *et al.* 2000; Bakowski *et al.* 2001; Prakriya & Lewis, 2001). This compound was originally suggested to represent a specific InsP_3 receptor antagonist (Maruyama *et al.* 1997) and, based on its effects on capacitative Ca^{2+} entry, was subsequently invoked to support the conformational coupling model (Ma *et al.* 2000), in which InsP_3 receptors are hypothesized to activate and maintain the activity of CRAC channels (Irvine, 1990; Berridge, 1995). However, subsequent studies have recognized that 2-APB may, in fact, be a direct blocker of I_{CRAC} and its inhibitory effect may be unrelated to its action on InsP_3 receptors (Braun *et al.* 2000; Bakowski *et al.* 2001; Prakriya & Lewis, 2001). Nevertheless, since 2-APB is ineffective in suppressing other channels such as L-type Ca^{2+} channels and Trp-3 channels, the compound has been considered as relatively selective for I_{CRAC}. Since 2-APB was found to inhibit both the whole-cell monovalent currents and single channels, this has been interpreted as additional evidence that they are both carried by CRAC channels (Braun *et al.* 2000). In the light of the present study, this assessment may need to be revised, since 2-APB was found to also inhibit MagNuM (see Fig. 6) and therefore cannot selectively identify CRAC channels.

Conclusions

In the light of the properties of the newly discovered ion channel LTRPC7, which underlies the Mg–nucleotide-regulated metal ion (MagNuM) current, we may need to re-evaluate some of the findings that were originally attributed to I_{CRAC}. In particular, this applies to those studies that obtained data under experimental conditions that favour MagNuM activation, notably ATP- and Mg^{2+}-free intracellular solutions (Kerschbaum & Cahalan, 1998, 1999; Braun *et al.* 2000; Fomina *et al.* 2000). The monovalent whole-cell and single-channel currents presented in these studies may have been due to (i) I_{CRAC} alone, (ii) MagNuM alone, or (iii) a mixture of I_{CRAC} and MagNuM, and we will consider each of these alternatives in turn.

The above studies all assumed that monovalent currents were exclusively carried by CRAC channels. Since the intracellular solutions were ATP and $[Mg^{2+}]_i$ free, there is little doubt that MagNuM must have been contributing to

monovalent currents, at least with respect to whole-cell currents, and therefore these are unlikely to represent I_{CRAC} alone. Let us next consider that monovalent currents in the absence of any divalents on either side of the membrane are exclusively MagNuM. In this context, it is important to point out that monovalent I_{CRAC} inactivates rapidly (Hoth & Penner, 1993; Kerschbaum & Cahalan, 1998) and the present study demonstrates that it does so in the absence of intracellular Mg^{2+}. Since the inactivation of monovalent I_{CRAC} proceeds faster than its activation, it seems likely that I_{CRAC} cannot support significant monovalent current and, since MagNuM is not subject to such strong inactivation, it could provide the predominant, if not exclusive, monovalent influx pathway. In fact, most features of monovalent currents recorded in the investigations above are entirely consistent with MagNuM as the sole pathway of monovalent current under ATP- and $[Mg^{2+}]_i$-free conditions, including kinetics, selectivity, current–voltage relationship and pharmacology.

Finally, we should consider a scenario in which both I_{CRAC} and MagNuM contribute to monovalent currents. In order for I_{CRAC} to do so, we would need to postulate that CRAC channels do not inactivate completely in $[Mg^{2+}]_i$-free conditions. If this were the case, then one would expect a sequential activation of monovalent currents, first I_{CRAC} and then followed by MagNuM. However, in previous studies, the activation kinetics of monovalent currents is consistent with MagNuM and not I_{CRAC}. In some circumstances, Putney and colleagues have observed an 'intermediate-sized' current that was present when cells were pre-activated by thapsigargin (Braun *et al.* 2000), which may be related to a similar intermediate-sized current Cahalan and colleagues have observed when recording monovalent currents in the presence of intracellular Mg^{2+} (Kerschbaum & Cahalan, 1999) and these currents may have been due to I_{CRAC}.

The possible contamination of I_{CRAC} with MagNuM may also have affected the study by Parekh & Penner (1995), where I_{CRAC} showed little signs of inactivation in ATP-free conditions, whereas moderate to strong inactivation was observed when perfusing cells with 2 mM ATP and ATP-γ-S, respectively. Although the general conclusions of that investigation remain valid, the overall magnitudes of the inactivation observed with these nucleotides may have been slightly overestimated, since both Mg·ATP and Mg·ATP-γ-S would have also suppressed any contaminating component of MagNuM.

In summary, the present study has demonstrated the coexistence of two potential ion channel mechanisms, I_{CRAC} and MagNuM, which both support large monovalent currents when divalent-free extracellular solutions are used. A reduction or omission of intracellular Mg^{2+} significantly augments the monovalent MagNuM. Since these latter conditions have been used to study selectivity and single-channel properties of I_{CRAC}, the results of these investigations may have to be reassessed in the light of a possible contamination with MagNuM.

REFERENCES

Bakowski, D., Glitsch, M. D. & Parekh, A. B. (2001). An examination of the secretion-like coupling model for the activation of the Ca^{2+} release-activated Ca^{2+} current I_{CRAC} in RBL-1 cells. *Journal of Physiology* **532**, 55–71.

Berridge, M. J. (1995). Capacitative calcium entry. *Biochemical Journal* **312**, 1–11.

Braun, F. J., Broad, L. M., Armstrong, D. L. & Putney, J. W. Jr. (2000). Stable activation of single CRAC-channels in divalent cation-free solutions. *Journal of Biological Chemistry* **276**, 1063–1070.

Broad, L. M., Armstrong, D. L. & Putney, J. W. (1999). Role of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} feedback Inhibition of calcium release-activated calcium current I_{CRAC}. *Journal of Biological Chemistry* **274**, 32881–32888.

Fasolato, C., Hoth, M. & Penner, R. (1993). A GTP-dependent step in the activation mechanism of capacitative calcium influx. *Journal of Biological Chemistry* **268**, 20737–20740.

Fomina, A. F., Fanger, C. M., Kozak, J. A. & Cahalan, M. D. (2000). Single channel properties and regulated expression of Ca^{2+} release-activated Ca^{2+} (CRAC) channels in human T cells. *Journal of Cell Biology* **150**, 1435–1444.

Harteneck, C., Plant, T. D. & Schultz, G. (2000). From worm to man: three subfamilies of TRP channels. *Trends in Neurosciences* **23**, 159–166.

Hoth, M. (1996). Depletion of intracellular calcium stores activates an outward potassium current in mast and RBL-1 cells that is correlated with CRAC channel activation. *FEBS Letters* **390**, 285–288.

Hoth, M. & Penner, R. (1992). Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355**, 353–356.

Hoth, M. & Penner, R. (1993). Calcium release-activated calcium current in rat mast cells. *Journal of Physiology* **465**, 359–386.

Irvine, R. F. (1990). 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates – a possible mechanism. *FEBS Letters* **263**, 5–9.

Kerschbaum, H. H. & Cahalan, M. D. (1998). Monovalent permeability, rectification, and ionic block of store-operated calcium channels in Jurkat T lymphocytes. *Journal of General Physiology* **111**, 521–537.

Kerschbaum, H. H. & Cahalan, M. D. (1999). Single-channel recording of a store-operated Ca^{2+} channel in Jurkat T lymphocytes. *Science* **283**, 836–839.

Krause, E., Schmid, A., Gonzalez, A. & Schulz, I. (1999). Low cytoplasmic $[Ca^{2+}]$ activates I_{CRAC} independently of global Ca^{2+} store depletion in RBL-1 cells. *Journal of Biological Chemistry* **274**, 36957–36962.

Lewis, R. S. & Cahalan, M. D. (1995). Potassium and calcium channels in lymphocytes. *Annual Review of Immunology* **13**, 623–653.

Ma, H. T., Patterson, R. L., van Rossum, D. B., Birnbaumer, L., Mikoshiba, K. & Gill, D. L. (2000). Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels. *Science* **287**, 1647–1651.

Maruyama, T., Kanaji, T., Nakade, S., Kanno, T. & Mikoshiba, K. (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P_3-induced Ca^{2+} release. *Journal of Biochemistry* **122**, 498–505.

Nadler, M. J. S., Hermosura, M. C., Inabe, K., Perraud, A.-L., Zhu, Q., Stokes, A. J., Kurosaki, T., Kinet, J.-P., Penner, R., Scharenberg, A. M. & Fleig, A. (2001). LTRPC7 is a Mg·ATP-regulated divalent cation channel required for cell viability. *Nature* **411**, 590–595.

Parekh, A. B. & Penner, R. (1995). Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. *Proceedings of the National Academy of Sciences of the USA* **92**, 7907–7911.

Parekh, A. B. & Penner, R. (1996). Regulation of store-operated calcium currents in mast cells. *Society of General Physiologists Series* **51**, 231–239.

Parekh, A. B. & Penner, R. (1997). Store depletion and calcium influx. *Physiological Reviews* **77**, 901–930.

Prakriya, M. & Lewis, R. S. (2001). Potentiation and inhibition of Ca^{2+} release-activated Ca^{2+} channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP_3 receptors. *Journal of Physiology* **536**, 3–19.

Romani, A. M. & Scarpa, A. (2000). Regulation of cellular magnesium. *Frontiers in Bioscience* **5**, D720–734.

Thomas, A. P., Bird, G. S., Hajnoczky, G., Robb-Gaspers, L. D. & Putney, J. W. Jr. (1996). Spatial and temporal aspects of cellular calcium signaling. *FASEB Journal* **10**, 1505–1517.

Zweifach, A. & Lewis, R. S. (1993). Mitogen-regulated Ca^{2+} current of T lymphocytes is activated by depletion of intracellular Ca^{2+} stores. *Proceedings of the National Academy of Sciences of the USA* **90**, 6295–6299.

Acknowledgements

This work was supported in part by National Institutes of Health grants AI46734, NS40927 and GM64316.