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2-Aminoethoxydiphenyl Borane Activates a Novel Calcium-Permeable Cation Channel

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ABSTRACT

The membrane-permeable, noncompetitive inositol 1,4,5-trisphosphate (IP_3)-receptor inhibitor 2-aminoethoxydiphenyl borane (2-APB) has been widely used to probe for IP_3-receptor involvement in calcium signaling pathways. However, a number of recent studies in different cell types revealed other sites of action of 2-APB. In this study, we examined the influence of 2-APB on capacitative calcium entry and intracellular Ca^{2+} concentrations in rat basophilic leukemia (RBL-2H3 m1) cells. 2-APB was found to inhibit capacitative calcium entry, but at concentrations greater than 50 μM, a new effect of 2-APB was observed. When capacitative calcium entry was blocked with Gd^{3+}, 2-APB caused an increase in cytoplasmic Ca^{2+}. This increase in intracellular Ca^{2+} was not caused by altered buffering of cytoplasmic Ca^{2+} and was not caused by or in any way affected by the depletion of intracellular Ca^{2+} stores. Associated with the increase in intracellular Ca^{2+}, in the presence of 2 mM Ca^{2+}, 2-APB activated single channels in the plasma membrane with a conductance of ~50 pS. These channels seem to be nonselective cation channels; monovalent cations are the major carriers of current, but finite permeability to Ca^{2+} leads to a significant intracellular Ca^{2+} signal. Experiments with excised patches indicate that 2-APB activates these channels from the outer aspect of the cell membrane. This effect of 2-APB further illustrates the complex actions of this compound and reveals the presence in RBL-2H3 m1 cells of a novel, ligand-gated calcium-permeable channel.

2-Aminoethoxydiphenyl borane (2-APB) was originally described as a membrane-permeable inhibitor of inositol 1,4,5-trisphosphate (IP_3) receptors (Maruyama et al., 1997b). Accordingly, 2-APB has been used to investigate the role of IP_3 receptors in the regulation of capacitative calcium entry (Ma et al., 2000) and other processes (Maruyama et al., 1997a; Ascher-Landsberg et al., 1999; Wu et al., 2000). Capacitative or store-operated calcium entry is a process whereby the depletion of intracellular Ca^{2+} stores in some manner signals the opening of Ca^{2+}-permeable channels in the plasma membrane (Putney, 1986, 1997). One proposed mechanism for this signaling is the conformational coupling model, according to which IP_3 receptors on the endoplasmic reticulum in close proximity with plasma membrane capacitative calcium entry channels interact with those channels and, in response to a reduction in endoplasmic reticulum Ca^{2+} content, signal their activation (Irvine, 1990; Berridge, 1995). The ability of 2-APB to inhibit capacitative calcium entry was originally interpreted as supportive of the conformational coupling mechanism (Ma et al., 2000). However, subsequently we (Braun et al., 2001) and others (Bakowski et al., 2001; Dobrydneva and Blackmore, 2001; Gregory et al., 2001; Iwasaki et al., 2001; Prakriya and Lewis, 2001) demonstrated that 2-APB inhibits capacitative calcium entry channels at an extracellular site by a mechanism not involving IP_3 receptors. 2-APB inhibits other channels as well, in particular the Mg^{2+}- or Mg^{2+}-ATP–inhibitable channels believed to be encoded by LTRPC7/TRPM7 (Hermosura et al., 2002; Kozak et al., 2002; Prakriya and Lewis, 2002). In addition, low concentrations of 2-APB have been reported to potentiate rather than inhibit capacitative calcium entry channels (Prakriya and Lewis, 2001; Ma et al., 2002).

In the current study, we investigated the action of 2-APB in the mast cell line RBL-2H3 m1. We discovered that, in addition to its previously described actions, 2-APB activates a calcium-permeable cation channel in the plasma membrane by an action at the outer surface. This channel seems to be a novel, "orphan" ligand-gated channel that may be involved in the regulation of cellular cation fluxes by unknown extracellular factors.

Materials and Methods

Cell Culture. Rat basophilic leukemia cells (RBL-2H3 m1), an immortalized mucosal mast cell line expressing m1 muscarinic receptors, were obtained from Dr. M. Beaven (National Institutes of Health, Bethesda, MD). The cells were cultured in Earle's minimal essential medium with Earle's salts, 10% fetal bovine serum, 4 mM

ABBREVIATIONS: 2-APB, 2-aminoethoxydiphenyl borane; IP_3, inositol 1,4,5-trisphosphate; I_{crac}, calcium release-activated calcium current; NMDG, *N*-methyl-D-glucamine; RBL, rat basophilic leukemia; I-V, current-voltage; CRAC, Ca^{2+}-release–activated Ca^{2+}; MagNuM, Mg^{2+}-nucleotide–inhibited metal; MIC, Mg^{2+}-inhibited cation; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

L-glutamine, 50 units/ml penicillin, and 50 mg/ml streptomycin (37°C, 5% CO_2). For experiments, cells were plated onto glass coverslips and were used 12 to 24 h thereafter.

Fura-2 Loading and Fluorescence Measurements. Coverslips with attached cells were mounted in a Teflon chamber and incubated at room temperature for 30 min in culture medium containing 1 μM Fura-2 AM (Molecular Probes, Eugene, OR). Cells were then washed and bathed in HEPES-buffered saline solution (140 mM NaCl, 10 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2) for at least 15 min before Ca^{2+} measurements were made.

Fluorescence was monitored by placing the Teflon chamber with the coverslip of Fura-2–loaded cells onto the stage of a Nikon Diaphot microscope (40x Neofluor objective; Nikon, Melville, NY). Cells were excited alternately by light (340 and 380 nm) from a Deltascan D101 (Photon Technology International, Monmouth Junction, NJ) light source equipped with a filter changer. Emitted fluorescence (510 nm) was collected by a photomultiplier tube (Omega Optical, Brattleboro, VT). All experiments were conducted at room temperature (20 to 22°C). All measurements shown are means ± S.E.M. or are representative of a minimum of three independent experiments.

Electrophysiology. Patch-clamp experiments were performed at 20 to 22°C in the tight-seal whole-cell and cell-attached configurations. Patch pipettes were pulled from borosilicate glass (Corning glass, 7052; Corning Glassworks, Corning, NY) and fire-polished. Membrane currents, filtered at 1 to 2 kHz, were recorded using an Axopatch-200B amplifier (Axon Instruments Inc., Union City, CA). Voltage-clamp protocols were implemented, and data acquisition was performed with pCLAMP 8.2 software (Axon Instruments). Solution changes were accomplished by bath perfusion. All voltages for creating I-V curves were corrected for liquid junction potential.

For whole-cell experiments with Ca^{2+} as the charge carrier, unless stated otherwise, the patch pipette (2 to 5 MΩ) solution had the following composition: 140 mM Cs^+ aspartate, 2 mM $MgCl_2$, 1 mM MgATP, 10 mM Cs^+-BAPTA (with free calcium set to 100 nM, calculated using MaxChelator software, version 6.60), and 10 mM HEPES; pH adjusted to 7.2 with CsOH. The bath solution contained 140 mM NaCl, 4.7 mM KCl, 10 mM CsCl, 1.13 mM $MgCl_2$, 10 mM glucose, 10 mM $CaCl_2$, and 10 mM HEPES; pH adjusted to 7.2 with NaOH. In experiments examining monovalent and divalent selectivity of 2-APB–activated channels (Fig. 6A), the NaCl was increased to 150 mM, and KCl, CsCl, and $MgCl_2$ were omitted from the bath solution. Divalent-free bath solutions, with Na^+ as the charge carrier, contained 150 mM Na^+ methane sulfonate or NaCl, 2 mM EDTA, and and 10 mM HEPES; pH adjusted to 7.2 with NaOH. CRAC channels were opened by passive store-depletion with 1 μM thapsigargin added to the bath. Cells were held at a potential of 0 mV. Every 1, 2, or 5 s, either voltage ramps from −100 to +60 mV or voltage steps from 0 to −100 mV were delivered for 200 ms. Currents were sampled at 5 kHz during voltage ramps and at 25 kHz during voltage steps.

During cell-attached recordings, data were collected from 10- or 60-s records at the given membrane potential, digitized at 5 or 10 kHz, and filtered digitally for analysis and presentation. The pipette (5 to 10 MΩ) solutions contained 150 mM NaCl, 2 mM EDTA, and 10 mM HEPES, pH 7.2 (with NaOH). The bath solutions contained 145 mM KCl, 5 mM NaCl, 10 mM $MgCl_2$, and 10 mM HEPES, pH 7.2 (with KOH) to nullify the cell's resting potential. When patches were excised, the bath solutions contained 145 mM K-glutamate, 5 mM NaCl, 2 mM EDTA, and 10 mM HEPES. Single-channel analysis was performed with the pCLAMP 6 software. P_o values were calculated for 10- (Fig. 8) and 60-s (Fig. 7) periods according to the relationship

$$P_o = \Sigma_N^1 (i \times t_i) / (N \times t_t)$$

where $i = 1$ to N, N is the maximum number of channels open simultaneously, t_t is the total time of the recording, and t_i is the cumulative time during which exactly i channels are open. When multichannel behavior was not monitored across the complete voltage range, activity is expressed as $N \times P_o$.

Materials. Thapsigargin was from LC laboratories (Woburn, MA). Cs_4-BAPTA and Fura-2 were from Molecular Probes. 2-APB was purchased from Paradigm Organics (Raleigh, NC).

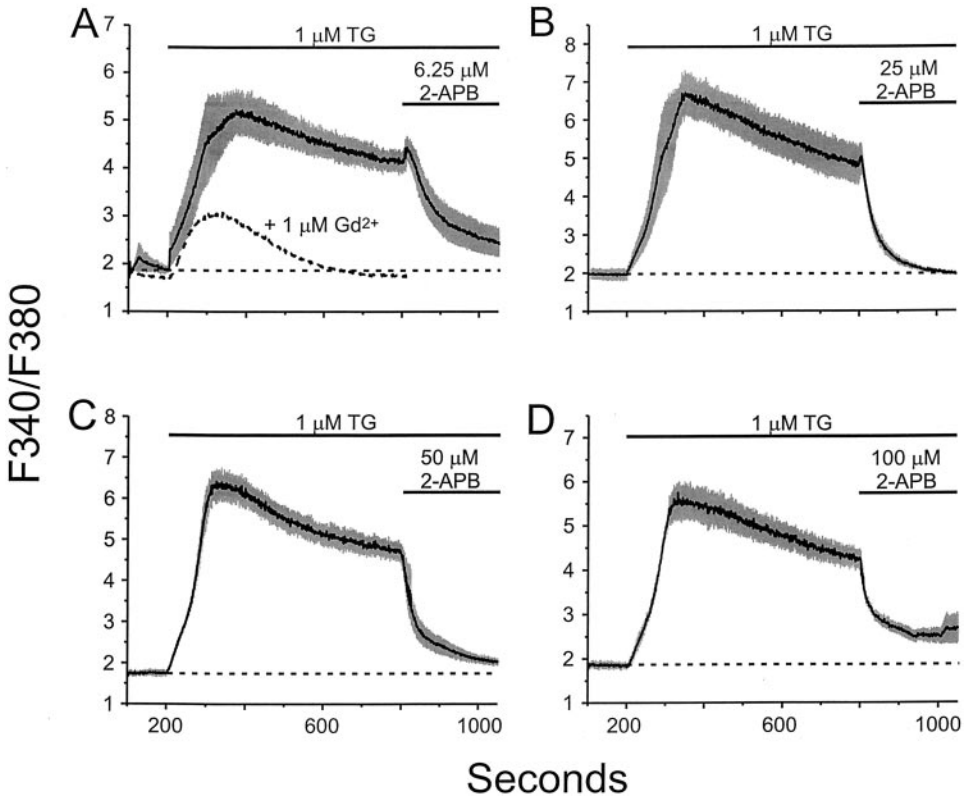

Fig. 1. Inhibition of capacitative calcium entry by increasing concentrations of 2-APB. Capacitative calcium entry was initiated by 1 μM thapsigargin (TG) as indicated. 2-APB was added, where indicated, at concentrations of 6.25 μM (A), 25 μM (B), 50 μM (C), or 100 μM (D). The $t_{1/2}$ values for the inhibitory phase were 48, 27, 27, and 14 s for 6.25, 25, 50, and 100 μM 2-APB, respectively. In A, the time course of the response to 1 μM thapsigargin when Ca^{2+} entry is blocked by Gd^{3+} is also shown (taken from mean data in Fig. 2B).

Results

2-APB Inhibits Capacitative Calcium Entry but Activates an Intracellular Ca^{2+}-Increase. To examine the effects of 2-APB on capacitative calcium entry in RBL-2H3 m1 cells, we first activated Ca^{2+} entry in Fura-2–loaded cells with 1 μM thapsigargin, a membrane-permeable inhibitor of the endoplasmic reticulum Ca^{2+} ATPase (Thastrup, 1990). In the presence of 2 mM Ca^{2+} in the bath, the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) on thapsigargin addition was biphasic because of Ca^{2+} release from internal stores followed by Ca^{2+} influx through capacitative calcium entry channels (Takemura et al., 1989) (Fig. 1A; see also Fig. 2B wherein thapsigargin-induced release and entry are separated with the use of a capacitative calcium entry blocker, Gd^{3+}).

As shown in Fig. 1, the application of 2-APB inhibited capacitative calcium entry in RBL-2H3 m1 cells in a concentration-dependent manner. Low doses of 2-APB, 6.25 μM (Fig. 1A) and 25 μM (Fig. 1B), caused a transient elevation of $[Ca^{2+}]_i$ preceding the inhibitory phase, with the lowest concentration producing a more pronounced potentiation of Ca^{2+} influx. This potentiation of influx by low concentrations of 2-APB is caused by augmentation of capacitative calcium influx, as reported previously by others (Prakriya and Lewis, 2001; Ma et al., 2002). A concentration of 25 μM 2-APB was able to fully inhibit Ca^{2+} entry and bring the $[Ca^{2+}]_i$ back to baseline within 250 s (Fig. 1B). Within the same time frame of 250 s after the addition of 2-APB, concentrations of this drug lower than and unexpectedly also greater than 25 μM resulted in incomplete inhibition (Fig. 1, A, C, and D). A possible explanation for the incomplete inhibition at higher concentrations of 2-APB could be that in addition to inhibiting capacitative calcium entry channels, higher concentrations of 2-APB may inhibit Ca^{2+} extrusion. However, if higher concentrations of 2-APB were to block Ca^{2+}-extrusion, then the decay times of $[Ca^{2+}]_i$ would be increased. From the results in Fig. 1 precisely the opposite was observed; the $t_{1/2}$ after 25 μM 2-APB was 27 s versus 14 s after100 μM 2-APB.

To address the nature of the residual $[Ca^{2+}]_i$ elevation in response to 2-APB, we examined the effects of 2-APB on $[Ca^{2+}]_i$ in the absence of capacitative calcium entry. For the experiments shown in Fig. 2A, the same protocol was used as for Fig. 1, except that all solutions contained 1 μM Gd^{3+} to inhibit capacitative calcium entry (Broad et al., 1999; Luo et al., 2001). In the presence of Gd^{3+}, although Ca^{2+} was included in the bath, thapsigargin-addition now resulted only in a transient increase in $[Ca^{2+}]_i$, caused by Ca^{2+} release from intracellular stores. Challenging the cells subsequently with 6.25 μM 2-APB did not cause any increase in $[Ca^{2+}]_i$, indicating that the response seen in Fig. 1 at this concentration results from capacitative calcium entry. However, 100 μM 2-APB caused a significant increase in $[Ca^{2+}]_i$ that was insensitive to Gd^{3+}. The level of $[Ca^{2+}]_i$ reached after the addition of 2-APB, with capacitative calcium entry channels blocked by Gd^{3+}, was similar to the level of $[Ca^{2+}]_i$ when capacitative calcium entry was fully blocked by 2-APB, in the absence of Gd^{3+} (Fig. 2B). Therefore, what seemed to be incomplete inhibition of capacitative calcium entry at supramaximal concentrations of the inhibitor 2-APB results from simultaneous activation of a $[Ca^{2+}]_i$ increase by 2-APB, which is independent of calcium flux through capacitative calcium entry channels.

2-APB Activates Ca^{2+}-Permeable Channels in the Plasma Membrane Independently of Store Depletion. We next attempted to determine the source of the intracellular Ca^{2+} increase activated by the addition of 2-APB. Figure 3 shows that after the discharge of Ca^{2+} stores by thapsigargin and in the absence of extracellular Ca^{2+}, 2-APB does not cause any detectable Ca^{2+} release. This indicates that 2-APB does not release Ca^{2+} from thapsigargin-insensitive stores and that the 2-APB $[Ca^{2+}]_i$ signal depends on extracellular Ca^{2+}.

The most likely scenario is that 2-APB activates Ca^{2+} channels in the plasma membrane independently of store depletion. To further establish independence of store depletion, 100 μM 2-APB was added to cells with and without prior store depletion. For this experiment, we depleted stores by incubating the cells for 11 min in Ca^{2+}-free buffer with 2 mM EDTA added. This depletion protocol resulted in robust Ca^{2+}

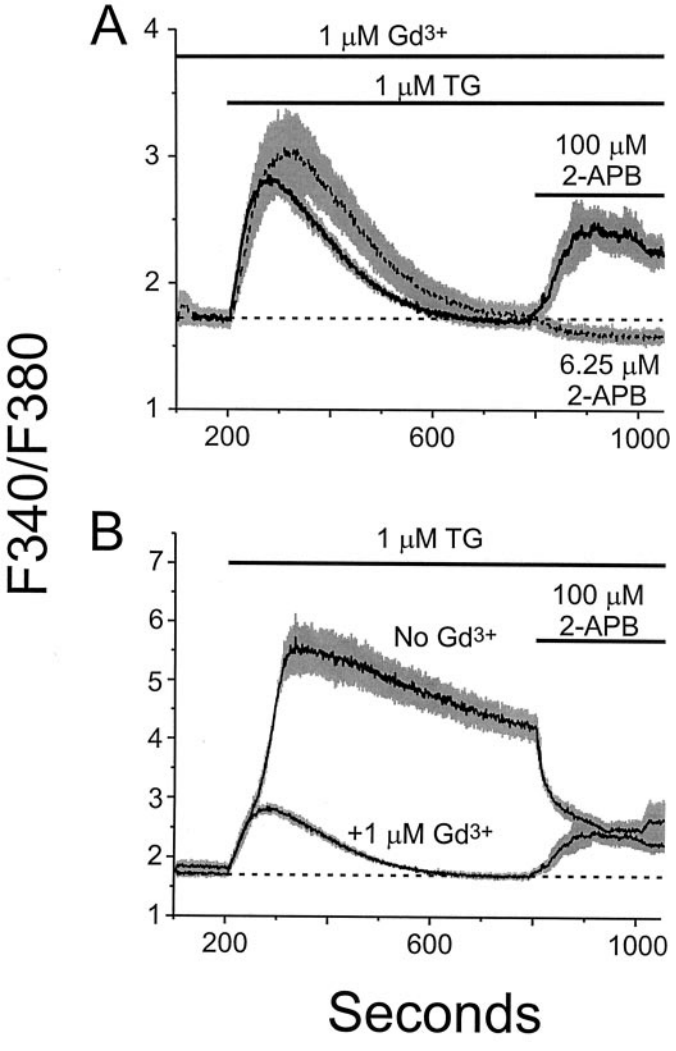

Fig. 2. 2-APB inhibits capacitative calcium entry and activates an intracellular Ca^{2+} increase. A, when capacitative calcium entry channels are inhibited with 1 μM Gd^{3+} and only the transient Ca^{2+} release phase upon thapsigargin addition can be seen, 2-APB at a concentration of 100 μM (solid line), but not at 6.25 μM (broken line) caused a significant increase in $[Ca^{2+}]_i$. B, application of 100 μM 2-APB to thapsigargin-treated cells in the presence or absence of Gd^{3+} results in a similar $[Ca^{2+}]_i$ level.

entry when Ca^{2+} was restored to the bath (Fig. 4A). This Ca^{2+} entry was genuine capacitative calcium entry because it was inhibited by 1 μM Gd^{3+} (control + Gd^{3+}). With Gd^{3+} present to prevent the activation of capacitative calcium entry, the addition of 2-APB along with Ca^{2+} activated Ca^{2+} entry that was indistinguishable in the presence or absence of prior store-depletion (Fig. 4B).

Currents through Capacitative Calcium Entry Channels and 2-APB–Activated Channels. We first used the whole-cell recording configuration of the patch-clamp technique to establish that >50 μM 2-APB indeed activates a store-depletion–independent conductance across the plasma membrane. The results of the $[Ca^{2+}]_i$ measurements predict that the channel activity and properties induced by 2-APB should be the same with or without prior store depletion. The results depicted in Fig. 5 confirm that this is indeed the case, and they lead to a few interesting observations. First, in a modified Ringer solution containing 10 mM Ca^{2+}, capacitative calcium entry currents (I_{crac}) (Hoth and Penner, 1992) showed the well-described development upon thapsigargin-addition, and as shown previously (Braun et al., 2001) 100 μM 2-APB was able to inhibit this current (Fig. 5A). However, the inhibition of capacitative calcium entry channels seemed incomplete, consistent with the findings shown in Figs. 1 through 4. The residual and somewhat unstable current after 2-APB addition (plus thapsigargin trace) showed behavior similar to the current activated by treating the cells with 2-APB alone (without thapsigargin trace). In both cases, after the addition of 2-APB, single channel openings were observed (Fig. 5B), and these channels had an average slope conductance of 40 pS (Fig. 5, C and D). These channels were not activated by an intracellular Ca^{2+} increase because the cytoplasmic solution in the pipette was strongly clamped to 100 nM $[Ca^{2+}]_i$ with 10 mM BAPTA. The 2-APB–activated single channels could easily be resolved in the whole-cell configuration, indicating either a low number of those channels per cell or channels that have a low open probability under these conditions. The latter explanation is favored by the fact that similar channels were seen in the majority of measurements in the cell-attached configuration (Fig. 9).

The observation of 2-APB–activated single-channel events in the whole-cell configuration explains the rather unstable pattern of measured current values plotted versus time compared with the more stable I_{crac} (Fig. 5A). During I_{crac} development, thousands of channels having a very small conductance of approximately 0.2 pS but with high open probability opened sequentially (Prakriya and Lewis, 2002).

Na^+ Is the Main Charge Carrier through 2-APB–Activated Channels. The next step was to determine the permeability of the 2-APB–activated channels for Ca^{2+}, because the addition of 2-APB at concentrations greater than 50 μM always caused a significant increase in $[Ca^{2+}]_i$, as measured with the Ca^{2+} indicator dye Fura-2 (Figs. 1 through 4). The addition of 100 μM 2-APB to cells bathed in Ringer's solution containing 10 mM Ca^{2+} resulted in immediate single-channel events with a channel conductance of 40 pS (Fig. 6, A and B), as established in Fig. 5. When the Na^+ ions in the bath were substituted with the impermeable cation *N*-methyl-D-

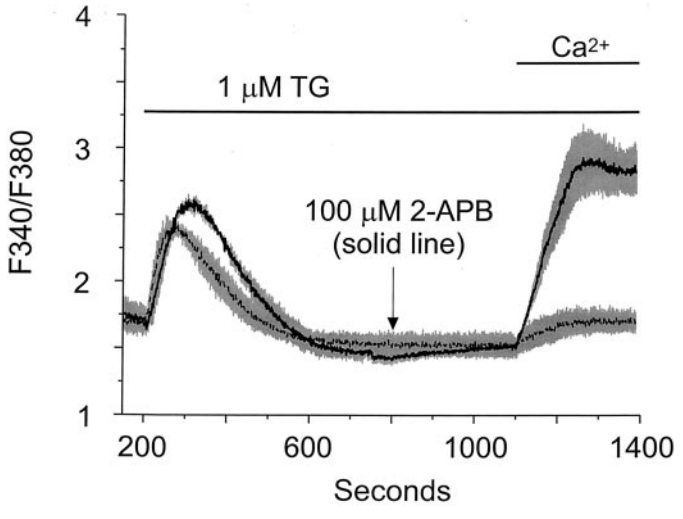

Fig. 3. 2-APB activates Ca^{2+} entry across the plasma membrane. Cells were treated with thapsigargin, as indicated, in the absence of extracellular Ca^{2+} and in the presence of 1 μM Gd^{3+}. 2-APB (100 μM) was added where indicated for the trace with the solid line. The readdition of Ca^{2+} reveals Ca^{2+} entry in the 2-APB–treated but not untreated cells.

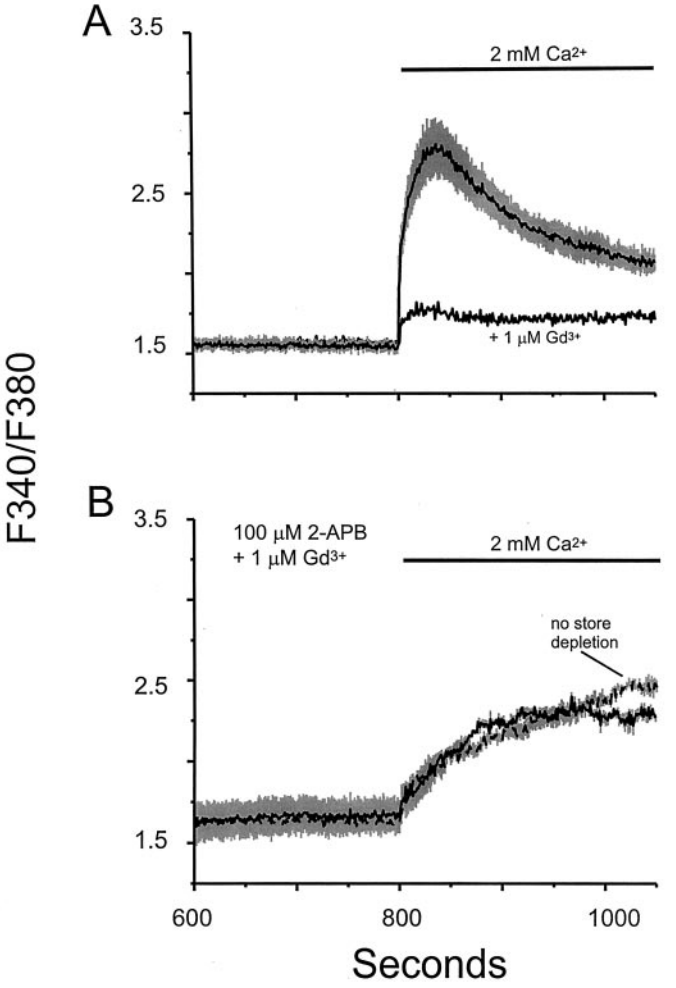

Fig. 4. 2-APB activates Ca^{2+} entry independently of store depletion. Cells were incubated for 11 min in a divalent-free buffer with 2 mM EDTA added (except for the trace indicated as "no store depletion"). A, this procedure depleted intracellular Ca^{2+} stores, with the outcome that the readdition of Ca^{2+} resulted in calcium entry. This Ca^{2+} increase was caused by capacitative calcium entry, because 1 μM Gd^{3+} blocked this increase (+ Gd^{3+}). B, 100 μM 2-APB, applied in the presence of 1 μM Gd^{3+}, activated entry to the same extent with (solid line) or without (broken line) prior store depletion.

glucamine (NMDG), leaving all the other ions unchanged, single-channel currents were no longer detectable (Fig. 6B). This indicates that the channels mainly conduct monovalent cations. However, the Fura-2 experiments indicate sufficient Ca^{2+} permeability to increase $[Ca^{2+}]_i$ measurably, suggesting that under physiological conditions the Ca^{2+} conductance of the channels is small but finite and is only detectable with Ca^{2+}-indicating dyes (Figs. 1 through 4). In support of this conclusion, in the presence of an NMDG-substituted extracellular solution, no current is detected, yet Ca^{2+} entry is still observed (Fig. 6C). We attempted measurement of Ca^{2+} currents with isotonic Ca^{2+} solutions, but we still could not observe single channels (data not shown); however, extracellular Ca^{2+} seems to negatively regulate these channels (see below). Thus, the inability to observe measurable current carried by Ca^{2+} does not necessarily mean that Ca^{2+} does not permeate the channels; for example, in the case of the store-operated channels underlying I_{crac}, it is possible to produce sufficient Ca^{2+} entry to increase $[Ca^{2+}]_i$ significantly, yet no associated current is detected. Only strategies that reduce feedback inhibition of CRAC channels result in detectable current (Huang et al., 1998).

Because Na^+ seems to be the main ion passing through 2-APB–activated channels, we next determined whether divalent cations might influence or regulate Na^+ conductance of the channels. When all divalent cations were omitted from the bath solution, single channels with an average conductance of 170 pS were activated immediately upon 2-APB addition, and this effect was rapidly reversed after the removal of 2-APB (Fig. 6, D and E). We next compared the appearance of 2-APB–activated channels in bath solutions containing 2 mM Ca^{2+}, containing no added divalent cations but without a divalent chelator present, and containing no added divalent cations plus 2 mM EDTA. As shown in Fig. 7, with decreasing concentrations of divalent cations in the bath, the conductance, as well as the open probability of the channels, increased. The open probabilities with 2 mM Ca^{2+} and with no divalent cations added to the bath (which equals approximately 10 μM Ca^{2+}) were not significantly different. However, the reduction of divalent cations to very low levels with EDTA resulted in an approximately 4-fold increase in open probability (Fig. 7B). The conductance of the channels is even more closely connected to the presence of extracellular divalent cations. The conductance more than tripled from 50 to 165 pS when the bath was switched from a solution containing 2 mM Ca^{2+} to a bath that was free of divalent cations

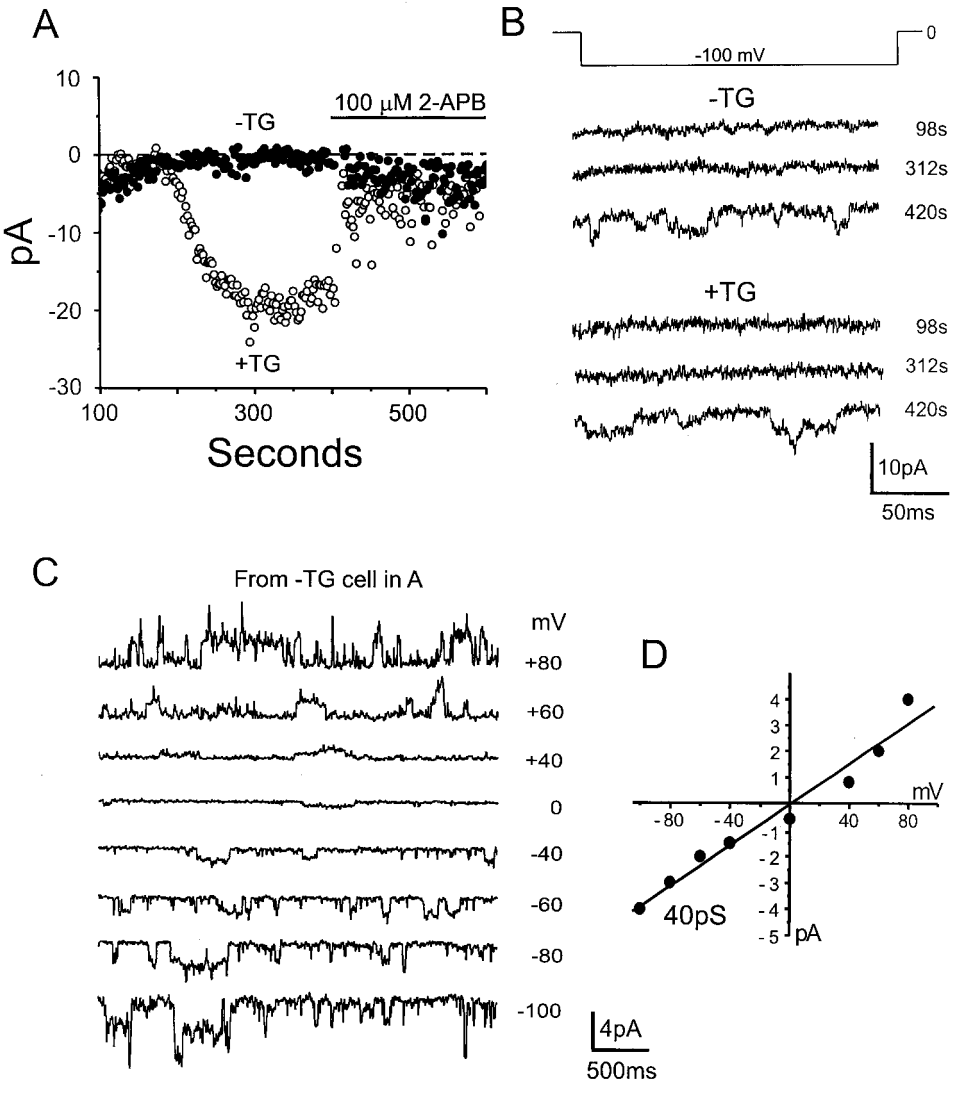

Fig. 5. Currents through capacitative calcium entry channels (I_{crac}) and 2-APB–activated channels. A, shown are membrane currents, recorded in the whole-cell mode, after steps to −100 mV from a holding potential of 0 mV and with 10 mM Ca^{2+} in the bath. Addition of 1 μM thapsigargin (○) activates I_{crac}, and this current is inhibited by 100 μM 2-APB. The addition of 2-APB results in less stable membrane current in both the presence and absence (●) of thapsigargin. B, inspection of current records during steps to −100 mV reveals unitary channel openings after the addition of 2-APB. C and D, from current measurements at different voltages (C) the current-voltage relationship (D) was constructed, which yielded a slope conductance of 40 pS.

(Fig. 7C). However, in all cases, the I-V relationships remained linear and crossed the voltage axis near 0 mV.

From the demonstrated Na^+ permeability (Fig. 6), the Ca^{2+} increases in the Fura-2 measurements (Fig. 1 to 4), as well as the linear I-V curves with reversal potentials of approximately 0 mV with and without extracellular Ca^{2+} (Figs. 5D and 7C), the 2-APB–activated channels seem to be Ca^{2+}-permeable nonselective cation channels.

Sensitivity of the Channels to 2-APB. We next addressed the dose-response properties of the channels for 2-APB. These experiments were carried out in divalent cation-free bath solutions to take advantage of the larger Na^+ conductance. The goal was to compare the results from these measurements with those for 2-APB–activated intracellular Ca^{2+} increases in Fura-2 experiments, as shown in Fig. 1. When the open probability of the channels, conducting Na^+, was examined as a function of increasing concentrations of 2-APB (Fig. 8), the concentrations of 2-APB activating the single channels were found to be similar to those that activated Ca^{2+} influx measured with Fura-2 (Fig. 1). 2-APB (50 μM) was a threshold concentration to observe the stimulatory effect on the channels. 2-APB (100 μM) resulted in an approximate 4-fold increase in influx under conditions in which the release of Ca^{2+} by 2-APB was still minimal. Concentrations of greater than 100 μM 2-APB were not examined because Ca^{2+} release becomes more prominent in this range (data not shown). The similarity of the thresholds of 2-APB concentration for activating Na^+ currents and Ca^{2+} influx suggest that the same channels underlie both phenomena.

We also observed the activation of single channels by 2-APB in the cell-attached mode (Fig. 9). In these experiments, the pipette contained 100 μM 2-APB, and single-channel activity was observed in 15 of 16 experiments. When 2-APB was not included in the pipette, channels were never seen (>100 experiments). After excision of the 2-APB–treated patches, activity was maintained for several minutes, although often at a somewhat diminished open probability. In seven experiments in which 2-APB was added outside of the pipette after establishing the cell attached configuration, five cells showed no channel activity and two showed channels with very low open probability that appeared after a very long latency (minutes). In 14 of the excised patches and to which 2-APB was added after excision to the cytoplasmic side of the membrane, 2-APB–activated channels were never seen; rather, the low divalent cation conditions led to the appearance of lower-conducting (40 pS) channels (data not

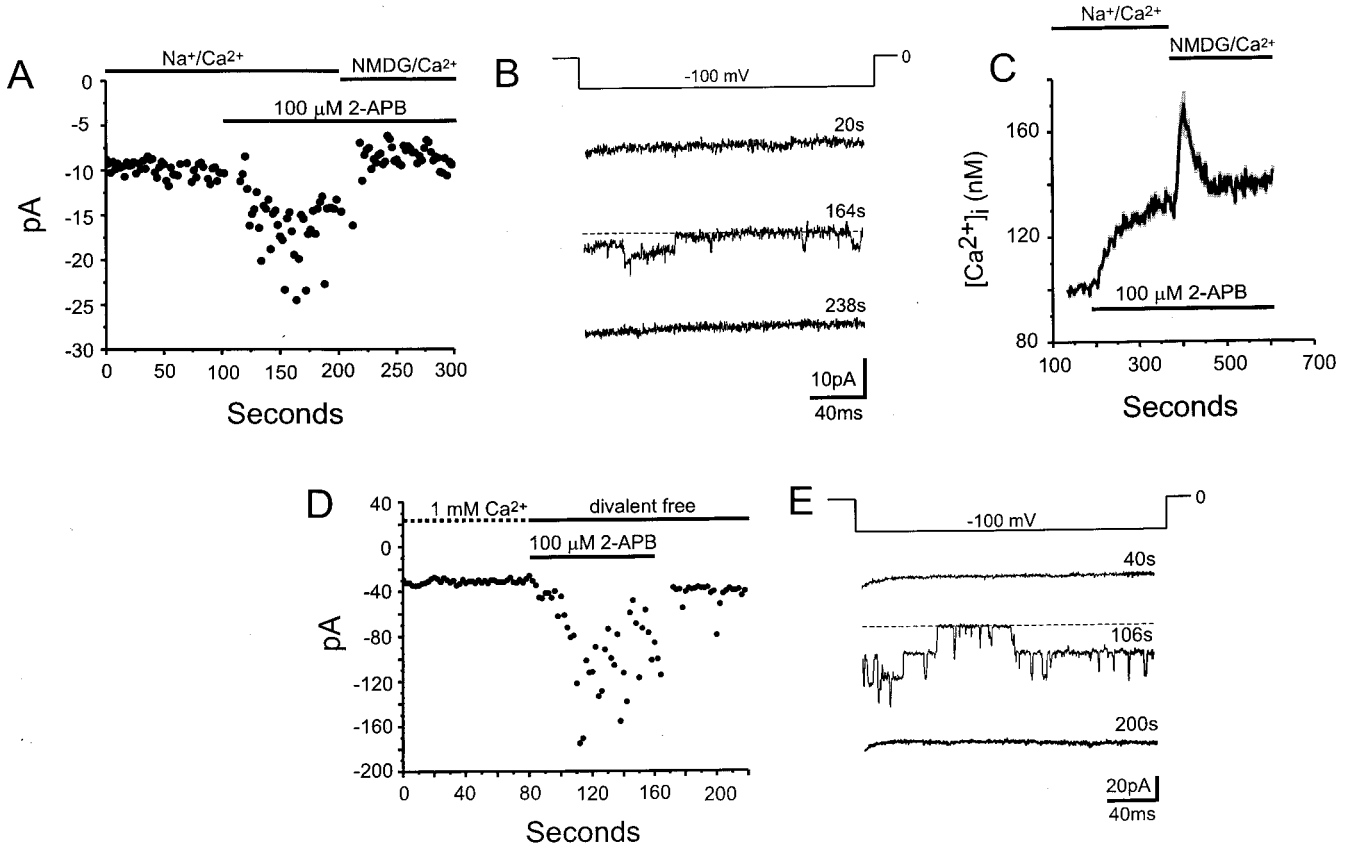

Fig. 6. Na^+ is the main charge carrier through 2-APB–activated channels. A, addition of 100 μM 2-APB to cells bathed in Ringer's solution containing 10 mM Ca^{2+} resulted in an unstable increase in the whole-cell current and the appearance of immediate single-channel events (B) with a channel conductance of 40 pS when probed at −100 mV. Na^+ ions in the bath were substituted by the impermeable cation $NMDG^+$ where indicated, leaving all the other ions unchanged. The bath solution contained 140 mM NMDG-Cl, 4.7 mM KCl, 10 mM CsCl, 1.13 mM $MgCl_2$, 10 mM glucose, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.2 (with NaOH). This resulted in the disappearance of single-channel activity. C, NMDG does not interfere with 2-APB activation of Ca^{2+}-permeable channels. The protocol was similar to that shown in A, except cells were not voltage-clamped and $[Ca^{2+}]_i$ rather than current was measured. When the Na^+-containing bath solution was changed to one in which Na^+ was replaced with NMDG, 2-APB–activated Ca^{2+} entry was not impaired; rather, entry transiently increased. Means ± S.E.M. from 216 cells are given. D and E, in the absence of divalent cations, single-channel conductance increased ~4-fold to an average of 170 pS (Fig. 7).

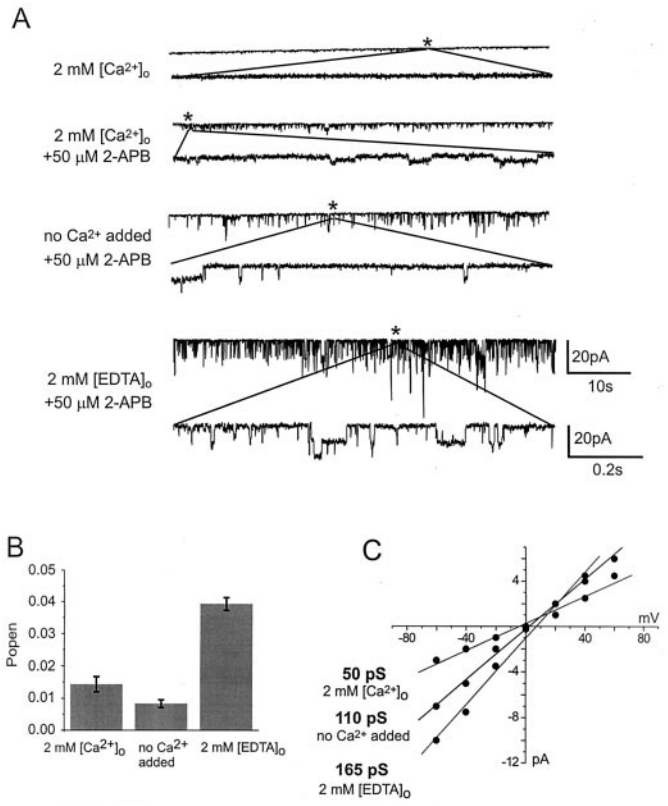

Fig. 7. Regulation of the 2-APB–activated channels by extracellular divalent cations. A, single-channel activity was observed in whole-cell mode in cells treated with 50 μM 2-APB. The figure shows Na^+ inward currents during 60-s sweeps at a holding potential of −60 mV with 1 s expanded under conditions whereby the bath medium contained no Mg^{2+} and 2 mM Ca^{2+}, no added Ca^{2+} (~10 μM [Ca^{2+}]), or no added Ca^{2+} plus 2 mM EDTA (< 1 nM [Ca^{2+}]). B, average open probabilities of the channels under the three conditions. C, current-voltage relationships under the three conditions.

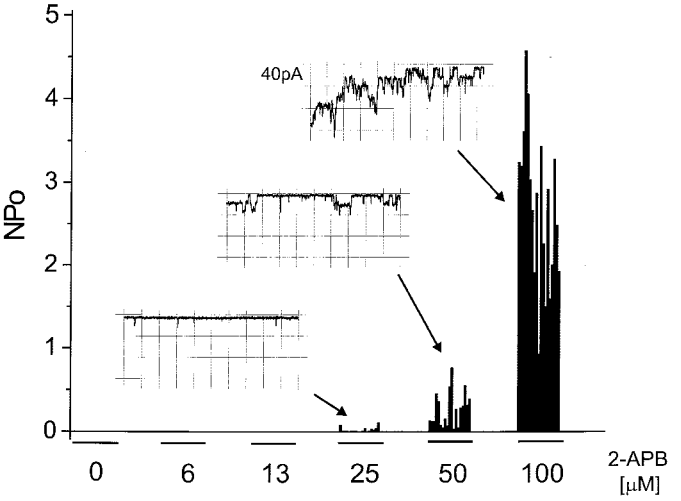

Fig. 8. Concentration-dependent activation of Na^+-conducting channels by 2-APB. The concentration dependence for 2-APB–activating Na^+ conducting single channels in RBL-2H3 cells reveals a minimal concentration of ~50 μM 2-APB to observe significant channel activity. The open probability was measured for 10-s traces at a holding potential of −50 mV.

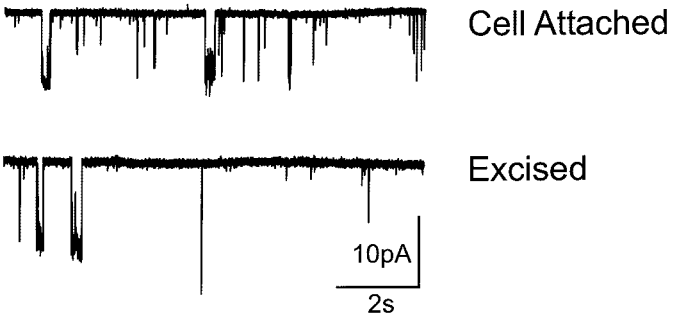

Fig. 9. Activation of single channels by 100 μM 2-APB in cell-attached and excised-patch mode. The patch pipette contained 100 μM 2-APB. Channel activity was observed in the cell-attached configuration in 15 of 16 experiments and continued after excision (six of nine cells).

shown) (Braun et al., 2001) believed to reflect MagNuM/MIC channels (Hermosura et al., 2002).

Discussion

The findings in the current study reveal the presence in RBL-2H3 m1 cells of a novel, calcium-permeable cation channel that can be activated by the organoborane, 2-APB. 2-APB activates this channel from the extracellular side of the membrane, raising the possibility that the channel is a ligand-gated one. The channel seems to be a nonselective cation channel with limited permeability to Ca^{2+}; yet, sufficient Ca^{2+} is passed to elevate $[Ca^{2+}]_i$ measurably. Whether the physiological function of the channel is to produce a $[Ca^{2+}]_i$ signal or to regulate the fluxes of cations cannot be determined at present. The open probability seems to be very low in response to 100 μM 2-APB, suggesting that a more powerful activator could produce very large $[Ca^{2+}]_i$ responses. The conductance and open probability of the channels are regulated, or at least affected, by the presence of extracellular divalent cations. This does not seem to be a channel-blocking effect, however, because the reversal potential and the linear nature of the current-voltage relationships were not altered by the presence or absence of extracellular divalent cations.

Two other important channels that have been shown to be modulated by 2-APB are CRAC channels (Hoth and Penner, 1992; Braun et al., 2001) and the Mg^{2+}- or Mg^{2+}-ATP–regulated MagNuM/MIC channels (Nadler et al., 2001; Runnels et al., 2001; Hermosura et al., 2002; Kozak et al., 2002; Prakriya and Lewis, 2002). Both of these channels are inhibited by 2-APB, and CRAC channels can also be potentiated at low concentrations of 2-APB. We observed these actions of 2-APB in RBL cells, including the inhibition of I_{crac} (Braun et al., 2001; Broad et al., 2001), potentiation of I_{crac} (Fig. 1), and inhibition of MagNuM/MIC (Braun et al., 2001). In the latter case, we originally described the inhibition by 2-APB of channels observed in the absence of divalent cations that we believed were CRAC channels; however, recent findings indicate that these channels are more likely to be MagNuM/MIC channels (Hermosura et al., 2002; Kozak et al., 2002; Prakriya and Lewis, 2002). It is clear that the channels observed in the current study activated by higher concentrations of 2-APB are distinct from both CRAC and MagNuM/MIC channels. The distinct properties of the three channel types are summarized in Table 1. There are reports of other cation-permeable channels in mast cells or RBL cell lines (Fasolato et al., 1993; Obukhov et al., 1995). However, none has the single-channel conductance, current-voltage relationship, or other properties found for the 2-APB–activated chan-

TABLE 1

Characteristics distinguishing 2-APB–activated channels, CRAC, and TRPM7/MagNuM/MIC channels

I_{MIC} data are from Prakriya and Lewis (2002).

	I_{APB}	I_{CRAC}	I_{MIC}
Effect of 2-APB (>50 μM)	Activation	Inhibition	Inhibition
Activation by store depletion	No	Yes	No
Conductance for Ca^{2+}	Minimal	Yes	Yes
Sensitivity to 1 μM Gd^{3+}	No	Yes	Unknown
Conductance for Na^+ (with 2 mM Ca^{2+} present)	Yes	No	No
Single-channel conductance in divalent-free buffers	170 pS	~0.2 pS	44 pS
P_o in divalent-free buffers	Low	?	High
Influence of intracellular divalent ions on Na^+ permeability	No	Yes	Yes

nels. Therefore we conclude that the action of this drug has revealed the presence of a previously undetected cation channel capable of significant regulation of cation fluxes and/or Ca^{2+} signaling. The challenge for future work is to determine the regulator or regulators and the physiological function of this channel in RBL and mast cells.

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