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# Blockade of Capacitive $Ca^{2+}$ Influx by $Cl^-$ Channel Blockers Inhibits Secretion from Rat Mucosal-Type Mast Cells

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## SUMMARY

Whole-cell patch-clamp recordings of membrane currents were performed in combination with measurements of mediator secretion from mucosal-type mast cells (rat basophilic leukemia cells, subline 2H3), to determine the involvement of membrane conductances induced upon depletion of intracellular $Ca^{2+}$ stores. In patch-clamp experiments, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid-induced depletion of internal $Ca^{2+}$ stores led to activation of two distinct membrane conductances, a $Ca^{2+}$ current and a $Cl^-$ current. The $Ca^{2+}$ current was blocked by 100 μM $La^{3+}$, which did not affect the $Cl^-$ current. In contrast, 500 μM 4,4′-diisothiocyanato-2,2′-disulfonic acid produced selective blockade of the $Cl^-$ current. Remarkably, the $Cl^-$ channel blockers 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), niflumic acid, and *N*-phenylanthranilic acid (NPAA) inhibited not only the $Cl^-$ current but also the $Ca^{2+}$ current. $IC_{50}$ values for the blockade of the $Ca^{2+}$ inward current by NPPB, niflumic acid, and NPAA were determined to be 23, 150, and 190 μM, respectively. In secretion experiments, thapsigargin-induced depletion of internal $Ca^{2+}$ stores stimulated serotonin release, which was found to be strictly dependent on extracellular $Ca^{2+}$. In the presence of 100 μM $La^{3+}$ secretion was almost completely inhibited. In contrast, only 50% of secretion was suppressed by 500 μM 4,4′-diisothiocyanato-2,2′-disulfonic acid, which fully blocked the $Cl^-$ current without affecting $Ca^{2+}$ influx, as monitored by electrophysiological experiments. The other $Cl^-$ channel blockers produced a very different pattern for the inhibitory dose dependence of secretion, with $IC_{50}$ values for NPPB, niflumic acid, and NPAA of 23, 60, and 180 μM, respectively. Taken together, these findings suggest that $Ca^{2+}$ store depletion leads to concomitant activation of $Cl^-$ and $Ca^{2+}$ currents. Blockade of the latter is apparently an additional mode of action for diarylaminocarboxylate-type $Cl^-$ channel blockers inhibiting mast cell secretory responses.

One of the early steps coupling receptor stimulus to response in mast cells and many nonexcitable cells is the mobilization of $Ca^{2+}$ from intracellular and extracellular sources, leading to an increase in the concentration of free cytosolic $Ca^{2+}$ ions (1–3). $Ca^{2+}$ release from intracellular stores is linked to its entry (4). In particular, the filling state of $Ca^{2+}$ stores apparently regulates one mechanism, known as "capacitive" $Ca^{2+}$ entry (2), whereas depletion of internal stores apparently activates $Ca^{2+}$ influx by an as yet unidentified signal (5–7). Recently, electrophysiological evidence has been presented for this $Ca^{2+}$ influx pathway in mast cells being activated upon depletion of internal $Ca^{2+}$ stores (8, 9).

The present study aimed at characterizing changes in membrane conductance of RBL-2H3 mast cells in relation to secretion, both of which are induced by depletion of intracellular $Ca^{2+}$ pools. Furthermore, pharmacological agents were used in these assays to evaluate the functional significance of depletion-induced currents with respect to the secretory response. We found that activation of a $Ca^{2+}$ current occurred concomitantly with that of a $Cl^-$ current. Screening of several pharmacological agents revealed that $Cl^-$ channel blockers of the diarylaminocarboxylate type were potent inhibitors of both $Ca^{2+}$ and $Cl^-$ currents, whereas DIDS selectively blocked only the latter. Correlating these electrophysiological results with the inhibition of secretion suggested blockade of $Ca^{2+}$ entry as an additional relevant mechanism of these diarylaminocarboxylate-type $Cl^-$ channel blockers. Part of this work was previously presented in preliminary form (10).

## Experimental Procedures

**Materials.** Niflumic acid and NPAA were purchased from Aldrich (Vienna, Austria). NPPB was kindly provided by Prof. Greger (Physiologisches Institut, Albert-Ludwigs-Universität, Freiburg, Germany).

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**ABBREVIATIONS:** RBL, rat basophilic leukemia; DIDS, 4,4′-diisothiocyanato-2,2′-disulfonic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; NPAA, *N*-phenylanthranilic acid; TG, thapsigargin; $I_{CRAC}$, $Ca^{2+}$ release-activated $Ca^{2+}$ current; DMSO, dimethylsulfoxide; $IP_3$, inositol trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

DIDS and econazole were supplied by Sigma (Munich, Germany). All inhibitors were dissolved in DMSO at concentrations of 10–200 mM and were used at final DMSO concentrations of <0.3%, which had no effect either on cell membrane conductances or on secretion. TG was purchased from Sigma and further dilutions were prepared from a 10 mM stock solution in DMSO. [$^3$H]Serotonin ([$^3$H]hydroxytryptamine binoxalate) was purchased from New England Nuclear (Vienna, Austria).

**Solutions.** Tyrode buffer used in release experiments consisted of 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM Na-HEPES, and 0.1% bovine serum albumin, pH 7.4. In electrophysiological experiments the following solutions were used: pip/Cl solution, 10 mM EGTA, 145 mM CsCl, 10 mM HEPES; pip/0 Cl solution, 10 mM EGTA, 125 mM aspartic acid, 10 mM tetraethylammonium hydroxide, 10 mM HEPES; extracellular cesium solution, 130 mM NaCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM glucose, 5 mM CsCl, 10 mM HEPES; Ca/Cl solution, 10 mM $CaCl_2$, 240 mM glucose, 10 mM HEPES; Ca/0 Cl solution, 10 mM calcium gluconate, 240 mM glucose, 10 mM HEPES. In the pip/Cl pipette solution the pH was adjusted with CsOH; in all other solutions *N*-methylglucamine was used to set the pH to 7.4. The osmolarity of each solution was measured to be 310 ± 15 mOsM. Use of mannitol instead of glucose in the Ca/Cl and Ca/0 Cl extracellular solutions yielded identical results. In all experiments except for those in Fig. 1, A and B, the pip/0 Cl pipette solution was used to largely eliminate contaminating ion fluxes out of the cell. This was accomplished by substituting $Cl^-$ with aspartate; additionally, tetraethylammonium was included to eliminate outward currents through cation channels.

**Cell culture.** All experiments were performed on a secreting subline (2H3) of RBL cells maintained in monolayer culture (11). The cells were grown in stationary flasks using minimal essential medium Earle's salts (GIBCO) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics, in a humidified atmosphere with 2.5% $CO_2$ at 37°. Further cell handling was performed as described (12). Electrophysiological experiments as well as serotonin release assays were carried out with RBL cells of passages 3–40.

**Electrophysiology.** Electrophysiological experiments were performed at 20–24°, using the patch-clamp technique (13) in the whole-cell recording configuration. An Ag/AgCl electrode in combination with a 3 M KCl-filled agar bridge was used as reference electrode. The stability of electrode potentials, particularly in $Cl^-$-free solutions, was carefully checked at the end of each experiment, to exclude those showing a drift greater than ±5 mV within 20 min. Liquid-junction potentials were corrected according to the method reported in Ref. 14. Soft glass pipettes immersed in extracellular cesium solution exhibited resistances between 6 and 10 MΩ when filled with pip/0 Cl pipette solution. Voltage ramps and steps were repetitively (0.2 Hz) applied from a holding potential of 0 mV, covering a range of −95 mV to +85 mV. Durations of ramps and steps were 1996 msec and 292 msec, respectively. Current signals were detected using an L/M-EPC7 amplifier (List Medical, Darmstadt, Germany) and either digitized (44 kHz) with a modified pulse-code modulator and stored on videotape or directly transferred to a computer hard disk. For off-line computer-supported analysis, current signals in response to voltage ramps and voltage steps were digitized at 1 and 6.6 kHz, respectively, after low-pass filtering at 500 Hz (−3 dB). Current signals were not corrected for capacitive and leakage currents. Errors due to series resistances were estimated to be <4 mV and thus were not taken into account. For presentation of current responses to voltage ramps, data points were usually reduced by averaging 10 digitization points, except for Fig. 1D.

**Serotonin release assay.** Secretion from RBL cells was monitored in duplicate by measurement of released [$^3$H]serotonin, as described (12). In brief, a 10-min preincubation period at 37° was followed by a 30-min incubation period at 37°. In inhibition experiments inhibitors were present during both incubation periods. Stimulation of serotonin release was performed with TG at concentrations between 200 nM and 1000 nM, yielding a maximal secretory response (15) of 40 ± 2% (11 experiments) of total serotonin content. In some secretion experiments performed at room temperature, TG-induced serotonin release amounted to 12 ± 1% (four experiments) of total serotonin content. Aliquots (200 μl) of supernatant were taken after 30 min and monitored by liquid scintillation counting. All presented results are net percentage serotonin release values normalized to total serotonin content (100%). Net release was calculated by subtracting the basal release of serotonin without stimulation by TG from that obtained in its presence. Basal release at 37° usually amounted to 3–10% of total serotonin content and was not significantly affected by the presence of inhibitors. Means ± standard errors are usually presented throughout this paper.

## Results

**Activation of both $Ca^{2+}$ and $Cl^-$ currents upon depletion of intracellular $Ca^{2+}$ stores.** Changes in membrane conductances of RBL-2H3 cells were recorded in the whole-cell configuration (13), by monitoring current responses to voltage ramps or steps from −95 mV to +85 mV. Using TG to deplete internal $Ca^{2+}$ pools (16) did not allow resolution of currents at an intracellular $Ca^{2+}$ concentration high enough to prevent spontaneous current activation. Thus, $Ca^{2+}$ store depletion was induced either passively with 10 mM EGTA or actively by additional inclusion of 6.5 μM $IP_3$ in the pipette solution. In initial experiments (Fig. 1, A and B) performed to reproduce (8, 9) activation of $I_{CRAC}$ upon active store depletion, the compositions of pipette and bath solutions were specifically chosen to isolate $I_{CRAC}$ from interfering cation currents. However, activation of two current components was clearly observed ($n$ = 4) (Fig. 1, A and B). Specifically, activation of an inward current preceded that of an outward current, usually starting within 20–30 sec after the whole-cell configuration was obtained. The difference in the time courses of current development suggested the involvement of at least two distinct current components.

Because in these initial experiments $Cs^+$ ions were substituted for $K^+$ in both extra- and intracellular solutions, a contribution of currents through $K^+$ channels appeared to be unlikely (17). Whereas the inward current might be related to activation of $I_{CRAC}$ (9), activation of $Cl^-$ currents, as occasionally observed in rat peritoneal mast cells (9), could contribute to the increase in outward currents. A $Cl^-$ conductance characterized at the single-channel level has been observed consistently in RBL cells (12). To test the assumption that the inward current is carried mainly by $Ca^{2+}$, whereas the outward current might arise from an influx of $Cl^-$ ions, the following two-step protocol was used to sequentially change the ion composition of extracellular solutions. First, substitution of all extracellular ions except for $CaCl_2$ (10 mM) by an equiosmolar amount of glucose leaves $Ca^{2+}$ and $Cl^-$ ions as the main current carriers (see Experimental Procedures). Then, complete substitution of extracellular $Cl^-$ ions by impermeable gluconate should enable isolation of $Ca^{2+}$ currents from $Cl^-$ currents. The results obtained in these ion substitution experiments are shown in Fig. 1, C and D. Here, passive instead of active depletion of intracellular $Ca^{2+}$ stores with 10 mM EGTA was used to induce activation of currents. Activation of the inward current occurred gradually, resulting in a time course similar to that observed for the outward current component (Fig. 1C). The slower activation of the $Ca^{2+}$ inward current upon passive versus active store depletion is in accordance with results reported previously (9). In all of the 60 cells studied, reproducible activation

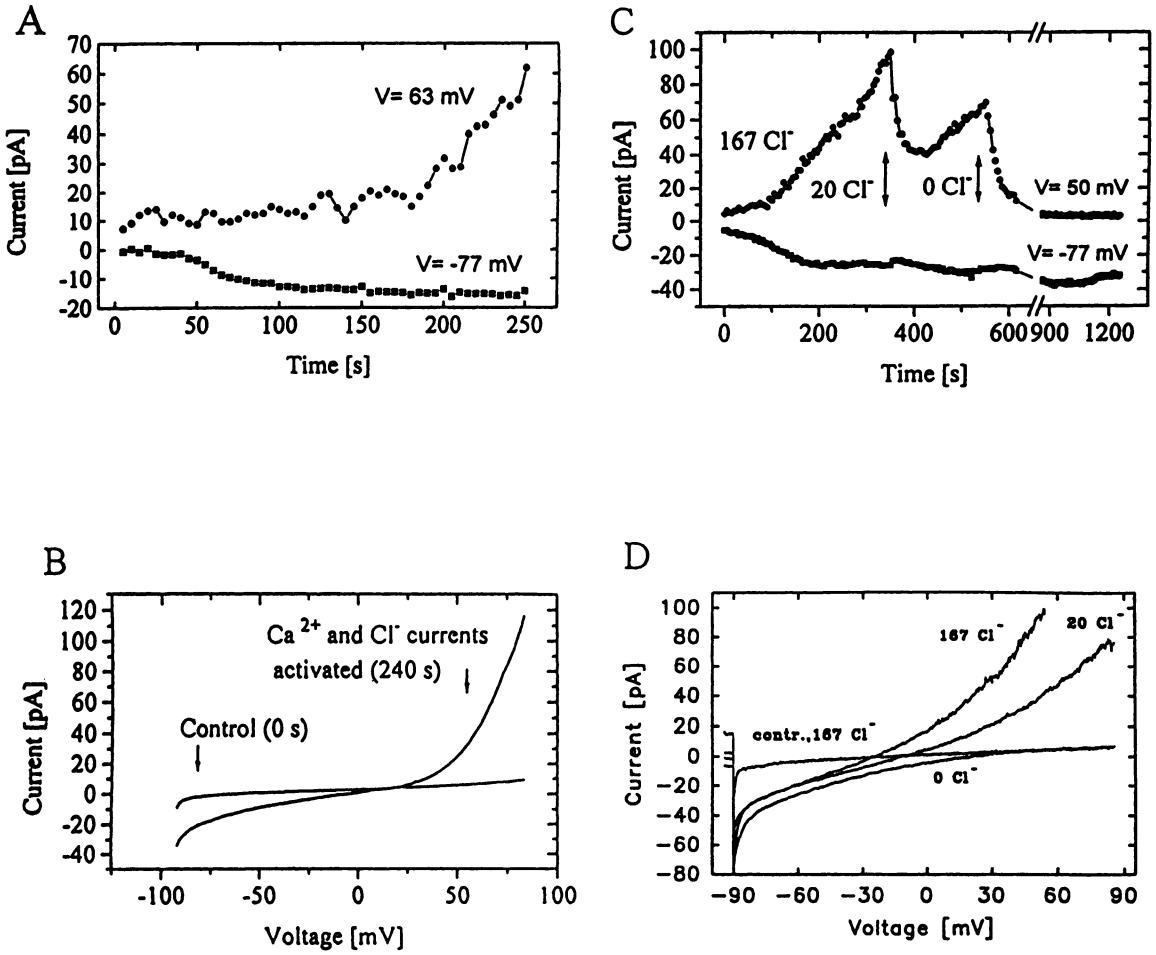



**Fig. 1.** Activation of $Ca^{2+}$ and $Cl^-$ currents upon depletion of intracellular $Ca^{2+}$ pools in RBL-2H3 cells. Depletion was induced either actively by additional inclusion of 6.5 $\mu$M $IP_3$ (A and B) or passively in the presence of 10 mM EGTA in the pipette solution (C and D). A, Time course of current amplitudes (at −77 mV and 63 mV) determined from current responses to voltage ramps from −95 mV to +85 mV; B, two current traces recorded immediately after whole-cell access (0 sec) and 240 sec later; C, time course of current amplitudes determined at −77 mV and +50 mV initially in extracellular cesium solution (*167 $Cl^-$*), followed by changes to Ca/Cl (*20 $Cl^-$*) and Ca/0 Cl (*0 $Cl^-$*) solutions; D, corresponding current traces in response to voltage ramps from −95 mV to +85 mV, recorded under conditions as described for C, at 0 sec, 355 sec (*167 $Cl^-$*), 420 sec (*20 $Cl^-$*), and 820 sec (*0 $Cl^-$*). The pip/Cl pipette solution supplemented with 6.5 $\mu$M $IP_3$ and the Ca/Cl extracellular solution supplemented with 5 mM CsCl were used in A and B. C and D show one experiment using pip/0 Cl pipette solution.

of both current components was observed. The inward current reached an almost steady state activation at 308 ± 131 sec (mean ± standard deviation, $n$ = 19), varying in current amplitude between −14 pA and −50 pA, as determined at a ramp potential of −72 mV, whereas the outward current component showed larger variations in steady state current amplitudes, from 10 pA to 105 pA at +45 mV, which were reached within 250–500 sec. Reduction of the extracellular $Cl^-$ concentration from 167 mM to 20 mM in the first substitution step resulted in a clear decline in the outward current (Fig. 1C). Concomitant removal of all extracellular cations except 10 mM $Ca^{2+}$ produced only a small effect on the inward current (Fig. 1C, *20 $Cl^-$*), suggesting a high selectivity for $Ca^{2+}$ over monovalent cations, as reported for $I_{CRAC}$ (9). Equimolar substitution of $Ca^{2+}$ by $Na^+$ ions consistently resulted in an approximately 90% reduction in $Ca^{2+}$ inward current, whereas substitution by $Ba^{2+}$ reduced this current to about 50%. Activation of both currents proceeded until complete removal of extracellular $Cl^-$ ions eliminated the outward current, leaving the $Ca^{2+}$ inward current isolated (Fig. 1C, *0 $Cl^-$*). The clear sensitivity of the outward current to extracellular $Cl^-$ concentrations suggested that this current is mainly carried by $Cl^-$ ions. Comparison of the current response to a voltage ramp recorded at the beginning of the experiment (assuming no activation of currents) with that observed in the absence of $Cl^-$ ions (Fig. 1D, *0 $Cl^-$*) clearly revealed a reversal potential above +50 mV (Fig. 1D), as expected for an inward current carried by $Ca^{2+}$ ions. Steady state currents (at 250 msec) in response to voltage steps from −70 mV to +85 mV exhibited similar current amplitudes, as determined during a corresponding voltage ramp (data not shown), suggesting an appropriate representation of current behavior by the ramp protocol used.

To confirm that the observed currents were indeed evoked by store emptying and not, for instance, by cellular dialysis, we performed careful control experiments in which $Ca^{2+}$ store depletion was prevented by supplementing the pipette solution with $Ca^{2+}$ to a concentration of about 60 nM free $Ca^{2+}$ (5). A total of eight experiments were performed in strictly paired comparison with four experiments under each condition, either to promote or to prevent depletion of internal $Ca^{2+}$ stores. In the presence of 60 nM free $Ca^{2+}$, no

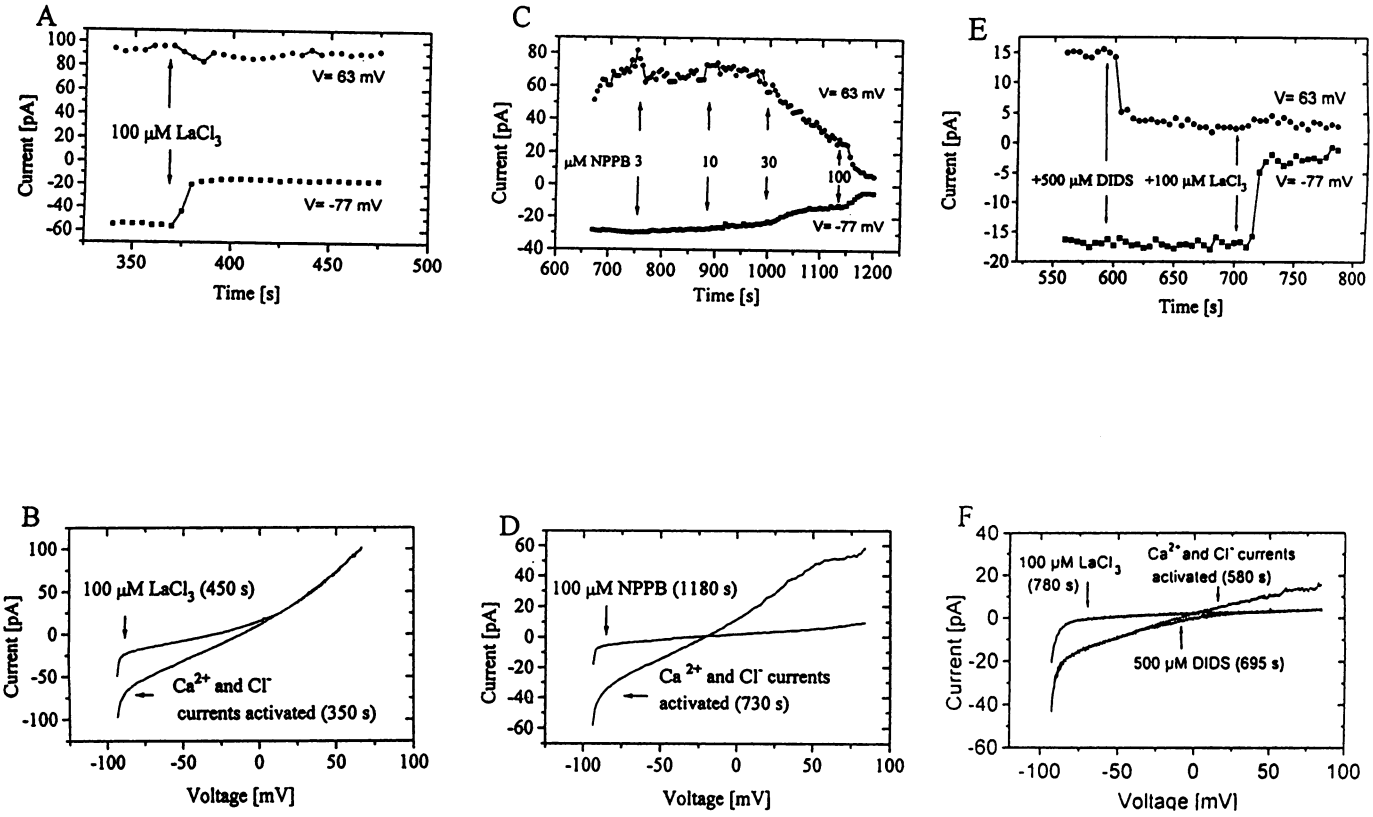



**Fig. 2.** Sensitivity of $Ca^{2+}$ and $Cl^-$ currents to $La^{3+}$, NPPB, and DIDS. A, C, and E, Representative time courses of current amplitudes determined from voltage ramps at the indicated voltages, upon application of $LaCl_3$ (A), NPPB (C), and DIDS (E); B, D, and F, corresponding current signals in response to voltage ramps. Compounds were applied after steady state activation of $Ca^{2+}$ and $Cl^-$ currents was reached. Intracellular and extracellular solutions were pip/0 Cl and Ca/Cl, respectively.

activation of whole-cell currents occurred within 400 sec, whereas both $Ca^{2+}$ and $Cl^-$ currents were activated as usual (see Fig. 1) upon depletion of internal $Ca^{2+}$ pools.

**Pharmacological resolution of $Ca^{2+}$ and $Cl^-$ currents.** Further characterization of both current components was performed using well known blockers of $Ca^{2+}$ and $Cl^-$ currents. Fig. 2 presents an overview of the effects of $La^{3+}$, NPPB, and DIDS on both current types. The inorganic $Ca^{2+}$ channel blocker $La^{3+}$, applied extracellularly at a concentration of 100 $\mu$M, led to a rapid block of the $Ca^{2+}$ inward current without having any effect on the outward current carried by $Cl^-$ ions ($n = 6$) (Fig. 2, A and B). $La^{3+}$ has been consistently identified as the most effective blocker of $I_{CRAC}$, yielding 94% inhibition at 10 $\mu$M (9). Attempts to estimate the reversal potential for the $La^{3+}$-sensitive current gave no clear intersection with an asymptotic superposition above +25 mV. No recovery of $Ca^{2+}$ current was obtained upon extensive washout of $La^{3+}$ ions. This might be attributed to the very strong binding of $La^{3+}$ ions to the channel, as similarly observed with the voltage-dependent L-type $Ca^{2+}$ channel (18). The $Cl^-$ channel blocker NPPB, which was shown earlier to block single $Cl^-$ channels in RBL cells (12), also showed no selective inhibition of either current component, eliminating both at a concentration of 100 $\mu$M ($n = 6$) (Fig. 2, C and D). A quite similar effect was caused by the $Cl^-$ channel blocker NPAA, which was found to inhibit both currents but at a 5-fold higher concentration ($n = 3$), and niflumic acid ($n = 3$). In contrast, DIDS (500 $\mu$M) produced a selective blockade of the $Cl^-$ current without affecting the $Ca^{2+}$ current ($n = 4$) (Fig. 2, E and F). The DIDS-sensitive component exhibited a reversal potential below −50 mV, as expected for a current carried by $Cl^-$ ions. Subsequent application of 100 $\mu$M $La^{3+}$ consistently yielded the expected blockade of the remaining $Ca^{2+}$ inward current, as depicted in Fig. 2, A and B. Application of econazole, a compound widely used as an inhibitor of capacitive $Ca^{2+}$ entry (19), resulted also in blockade of both $Ca^{2+}$ and $Cl^-$ currents. Inhibition of either current type occurred with a slower time course, compared with that caused by $La^{3+}$, and reached its maximal effect at a concentration of 10 $\mu$M.[1]

**Inhibition of the $Ca^{2+}$ current to similar extents by the $Cl^-$ channel blocker NPPB and $La^{3+}$.** The dose dependence of $Ca^{2+}$ inward current inhibition by NPPB was determined in $Cl^-$-free solutions, to eliminate the outward $Cl^-$ current component (Fig. 3). After steady state activation of the $Ca^{2+}$ current was achieved, increasing concentrations of NPPB were applied, followed by a washout. This removal of NPPB led to a partial recovery of the $Ca^{2+}$ current, which was then blocked again by application of $La^{3+}$. A similar extent of inhibition of the inward current by either agent occurred at a concentration of 100 $\mu$M, whereas the outward leakage current remaining in $Cl^-$-free solutions was unaffected (Fig. 3A). Voltage steps between −75 and +85 mV, yielding an inward current at potentials in the range of −75 mV to less than +45 mV (Fig. 3B, *upper*), confirmed a similar

[1] M. H. Rohn and C. Romanin, unpublished observations.

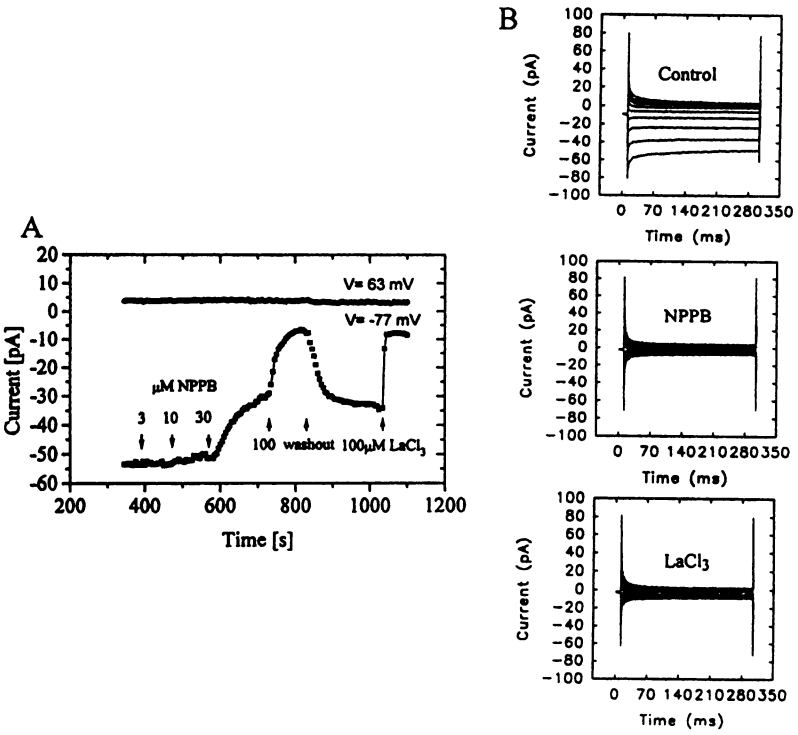



**Fig. 3.** Effects of NPPB and $La^{3+}$ on the isolated $Ca^{2+}$ current. A, Time course of current amplitudes (at −77 mV and 63 mV) determined from current responses to repetitively applied voltage ramps from −95 mV to +85 mV, upon application of NPPB and $La^{3+}$ at the indicated concentrations. B, Corresponding current traces in response to voltage steps (−85 mV to +85 mV at 20-mV intervals) in the absence and in the presence of 100 μM NPPB and 100 μM $LaCl_3$. Intracellular and extracellular solutions were pip/0 Cl and Ca/0 Cl, respectively.

current inhibition, which was independent of the voltage and blocker applied (Fig. 3B, compare *middle* and *lower*).

**Parallel inhibition of $Ca^{2+}$ inward current and TG-induced serotonin secretion.** To evaluate the relationship between the depletion-induced $Ca^{2+}$ and $Cl^-$ currents and the stimulus-secretion coupling cascade of the cells, measurements of serotonin release from TG-stimulated RBL cells were performed as a functional assay for the coupling between depletion of intracellular $Ca^{2+}$ stores and secretion. TG has been shown to induce an $IP_3$-independent increase of intracellular $Ca^{2+}$ in RBL cells (14). TG-induced serotonin release was strictly dependent on the influx of extracellular $Ca^{2+}$, because omission of these ions from the extracellular medium reduced secretion to basal values, as measured in the absence of TG (three experiments). Inclusion of 100 μM $La^{3+}$ in $Ca^{2+}$-containing medium similarly led to an almost complete inhibition of the net serotonin secretion (to 3 ± 1% of control, three experiments). Thus, selective blockade of $Ca^{2+}$ entry by $La^{3+}$ without an effect on $Cl^-$ currents, as characterized by the present electrophysiological experiments, is apparently sufficient for inhibition of TG-induced secretion. Therefore, the concentration dependence of $Ca^{2+}$ current blockade was determined for the nonselective $Cl^-$ channel blockers inhibiting both $Ca^{2+}$ and $Cl^-$ currents and was then compared with that of the inhibition of secretion (Fig. 4). Dose-response relationships in electrophysiological experiments were determined in $Cl^-$-free solutions, for isolated (see Fig. 3) $Ca^{2+}$ inward currents. $IC_{50}$ values for inhibition of $Ca^{2+}$ currents by NPPB, niflumic acid, and NPAA were found to be 23 μM, 150 μM, and 190 μM, respectively (Fig. 4A). These $Cl^-$ channel blockers exhibited similar $IC_{50}$ values for inhibition of $Cl^-$ currents, e.g., for NPPB the $IC_{50}$ was 22 μM and for niflumic acid the $IC_{50}$ was 120 μM. All of these compounds, which were shown by electrophysiological experiments to block depletion-activated $Ca^{2+}$ and $Cl^-$ currents, were then also identified as inhibitors of TG-induced secretion (Fig. 4B). Moreover, the $IC_{50}$ values of NPPB (23 μM) and NPAA (180 μM) were similar to those determined in electrophysiological experiments. Niflumic acid was, however, about 2.5-fold more potent as an inhibitor of secretion ($IC_{50}$ = 60 μM), compared with its blockade of the $Ca^{2+}$ inward current. This difference was not caused by the different temperatures at which electrophysiological (room temperature) and secretion (37°) experiments were performed, because concentrations of NPPB, NPAA, and niflumic acid giving half-maximal inhibition of secretion at 37° produced similar inhibition (53%, 35%, and 56%, respectively) at room temperature. Inhibition of serotonin release by DIDS, which was found to selectively block $Cl^-$ currents, clearly showed a distinct concentration dependence. The dose-response curve was considerably less steep and yielded 50% inhibition of secretion at a concentration of 500 μM DIDS, which completely blocked $Cl^-$ currents.

Comparison of the chemical structures of the $Cl^-$ channel blockers used (Fig. 4C) shows that the compounds that inhibit both $Ca^{2+}$ and $Cl^-$ currents belong to the diarylamine-2-carboxylate class. The similarity in structure of NPPB, niflumic acid, and NPAA suggests that a common binding site may be involved in their inhibitory actions on the capacitive $Ca^{2+}$ entry pathway.

## Discussion

**General conclusions.** The main results of this study are that depletion of intracellular $Ca^{2+}$ stores in RBL-2H3 mast cells leads to activation of two distinct membrane conductances, i.e., a $Ca^{2+}$ current and a $Cl^-$ current. Three compounds, NPPB, niflumic acid, and NPAA, that are usually used as $Cl^-$ channel blockers inhibited not only the $Cl^-$ current but also the $Ca^{2+}$ current. In contrast, selective in-

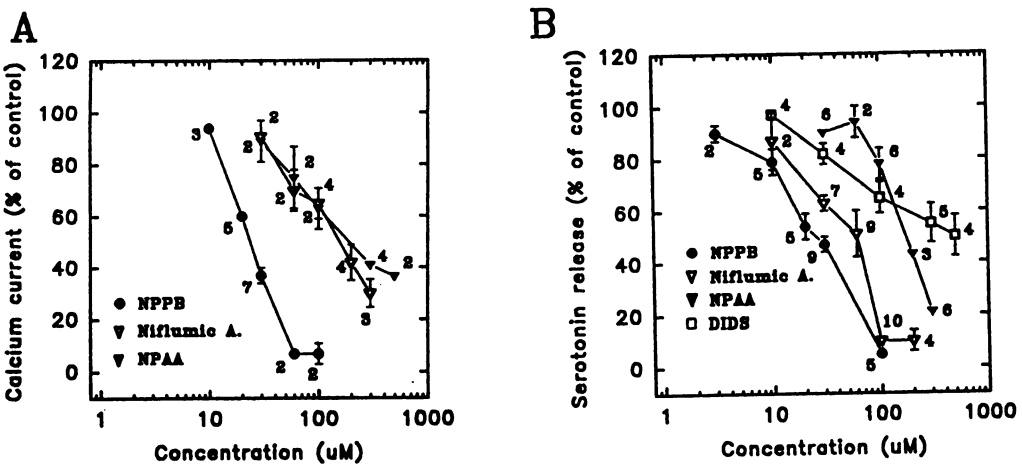
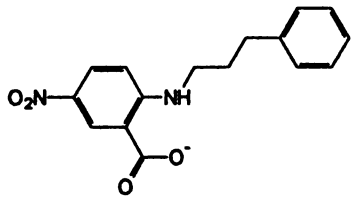
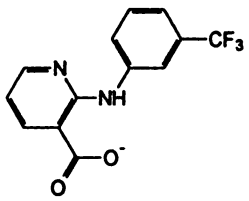



**Fig. 4.** Inhibition of $Ca^{2+}$ currents and serotonin secretion by $Cl^-$ channel blockers. A and B, Dose dependence of the inhibition of $Ca^{2+}$ currents (A) and of serotonin release (B) by NPPB, niflumic acid, NPAA, and DIDS (only shown in B). C, Chemical structures of the $Cl^-$ channel blockers tested. Complete current inhibition (0% of control) was taken as the amount of current remaining in the presence of saturating blocker concentrations plus 100 $\mu$M $La^{3+}$. The limited solubility of NPAA might account for a blocking effect smaller than that expected for 500 $\mu$M NPAA. TG (200 nM) was used to stimulate secretion. Serotonin release (percentage of control) was calculated from net serotonin release values. Numbers next to data points in A and B correspond to independent experiments.

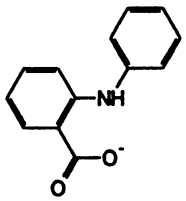
5-Nitro-2-(3-phenylpropylamino)-benzoic acid



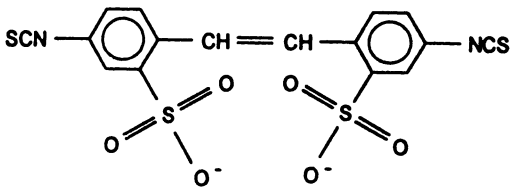

Niflumic Acid




N-Phenylanthranilic acid




DIDS




hibition was observed with $La^{3+}$ and DIDS, which blocked $Ca^{2+}$ and $Cl^-$ currents, respectively. Furthermore, TG-induced secretion from RBL-2H3 cells was inhibited in parallel with blockade of the $Ca^{2+}$ current, suggesting the latter as an additional target for $Cl^-$ channel blockers of the diarylaminocarboxylate type.

**$Ca^{2+}$ and $Cl^-$ currents in mast cells.** The $Ca^{2+}$ current observed in the present experiments appears to be indistinguishable from the $I_{CRAC}$ (5, 8) recently characterized in peritoneal mast cells (8, 9). Consistent results were obtained concerning its activation behavior, permeability, and blockade by $La^{3+}$ ions. In addition, concomitant with $Ca^{2+}$ current activation in RBL cells a $Cl^-$ conductance was induced, which could be clearly distinguished by its distinct activation behavior and sensitivity to $La^{3+}$, DIDS, and $Cl^-$ ions. In peritoneal mast cells, $Cl^-$ current activation has only occasionally been observed under conditions used to study $I_{CRAC}$ (9). The $Cl^-$ current observed here might be related to single $Cl^-$ channel currents recently characterized in RBL cells (12). An increasing open state probability with increasing depolarization determined for these single $Cl^-$ channels might correspond to the outward rectification observed here for the $Cl^-$ current. Because $La^{3+}$ blocked $Ca^{2+}$ currents without affecting $Cl^-$ currents, the latter are apparently not directly regulated by $Ca^{2+}$ ions. Single $Cl^-$ channels have consistently been found (12) not to be under direct modulation by $Ca^{2+}$ and are inhibited by NPPB with comparable $IC_{50}$ values. How far activation of both $Ca^{2+}$ and $Cl^-$ currents could be mediated by similar mechanisms is still to be resolved. However, induction of either current was found to occur under conditions where the other current was blocked.[2]

**$Cl^-$ channel blockers as inhibitors of the $Ca^{2+}$ current.** Pharmacological characterization of the $Ca^{2+}$ inward current is currently only beginning. Recently, nonselective

[2] M. H. Rohn and C. Romanin, unpublished observations.

inhibition of $Ca^{2+}$ entry in rat peritoneal mast cells by econazole, SKF 96365, tenidap, and ketotifen has been reported (20), showing similar inhibitory potencies of the aforementioned compounds also for nonspecific 50-pS cation channels and cAMP-activated $Cl^-$ channels. In accordance, econazole was identified here as a nonspecific inhibitor of both $Ca^{2+}$ and $Cl^-$ currents in RBL cells. Similarly, no selectivity was found for the $Cl^-$ channel blockers NPPB, niflumic acid, and NPAA. Therefore, these diarylaminocarboxylate-type compounds should be used and their effects interpreted with caution, because they may also directly interfere with capacitive $Ca^{2+}$ entry found in many nonexcitable cells. In particular, conclusions drawn from the sensitivity of sustained $Ca^{2+}$ entry to the aforementioned $Cl^-$ channel blockers (e.g., NPPB and NPAA), as reported from fluorescence experiments (21, 22,), should be reconsidered in light of the observed direct inhibitory effect on $Ca^{2+}$ influx. Whether these compounds act on similar binding domains present in both channel types or on a regulatory factor (7) common to both channels remains to be determined.

**Functional significance of current inhibition by $Cl^-$ channel blockers.** To examine the relationship between the inhibitory effects characterized in electrophysiological experiments and the secretory response of these cells, serotonin release from RBL-2H3 cells upon stimulation by TG was monitored (15). Inhibition of secretion by the $Cl^-$ channel blockers NPPB, niflumic acid, and NPAA occurred in parallel with their blocking effects on $Ca^{2+}$ and $Cl^-$ currents, as characterized in electrophysiological experiments. Because blockade of $Ca^{2+}$ currents without effects on $Cl^-$ currents, as found with $La^{3+}$, was sufficient to completely inhibit secretion, the more relevant action of these $Cl^-$ channel blockers is apparently their direct, rather than indirect, inhibition of $Ca^{2+}$ entry. The indirect inhibition may take place by blockade of $Cl^-$ currents (12). This in turn would cause a reduction in the driving force for $Ca^{2+}$ influx (12, 23, 24) by favoring cell depolarization (25). DIDS, which selectively blocked the $Cl^-$ current, consistently led to only partial inhibition of secretion, compared with the $Ca^{2+}$ entry-blocking compounds. Hence, other repolarizing currents (26) might additionally contribute to the regulation of capacitive $Ca^{2+}$ entry as well as of secretion. In conclusion, development of selective inhibitors of capacitive $Ca^{2+}$ entry is a major future task in the pursuit of specific pharmacological interventions in $Ca^{2+}$ homeostasis in nonexcitable cells.

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