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Evidence against the functional involvement of outwardly rectifying Cl^- channels in agonist-induced mast cell exocytosis

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Abstract

In isolated rat peritoneal mast cells, an outwardly rectifying Cl^- channel has been described. Influx of Cl^- through this Cl^- channel ($I_{Cl^-(OR)}$) causes hyperpolarization, which facilitates Ca^{2+} currents through store-operated Ca^{2+} channels. The exocytotic effect of nerve growth factor (NGF) in the presence of lyso-phosphatidylserine strictly depends on the presence of extracellular $[Ca^{2+}]_o$. The aim of the present study was to assess the importance of $I_{Cl^-(OR)}$ for exocytosis induced by NGF/lyso-phosphatidylserine. Therefore, we investigated the effects on NGF/lyso-phosphatidylserine-induced exocytosis of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) in rat peritoneal mast cells: (a) of two inhibitors of $I_{Cl^-(OR)}$ (4,4′-diisothiocyanatostilbene2,2′-disulfonic acid [DIDS] and diethylstilbestrol), and (b) of replacement of extracellular Cl^- by methylsulfate. Additionally, whole-cell patch-clamp experiments (nystatin-perforated patch) were performed. Diethylstibestrol and DIDS, in concentrations sufficient to abolish the $I_{Cl^-(OR)}$ (10 μM) and the replacement of $(Cl^-)_o$ by methylsulfate, were ineffective in impairing the NGF/lyso-phosphatidylserine-induced [^{3}H]5-HT-release. These findings argue against a role of outwardly rectifying Cl^- channels in exocytosis induced by NGF/lyso-phosphatidylserine in rat peritoneal mast cells.

Keywords: Exocytosis; Secretion; Cl^- channel; NGF; Mastoparan; Mast cell; Patch-clamp; Membrane potential

1. Introduction

Mast cells are assumed to mediate type 1 allergy-related diseases (e.g. hay fever, urticaria, allergic asthma, etc.) by the exocytotic release of granule-stored mediator compounds (Metcalfe et al., 1997). Activation of mast cells in type 1 allergy is caused by antigen(Ag)-induced cross-bridging of immunglobulin E(IgE)-bound high-affinity receptors for IgE (FcεRI) (Turner and Kinet, 1999). In addition, exocytosis in mast cells can be stimulated by a variety of chemically heterogeneous secretagogues via FcεRI-independent mechanisms; these secretagogues comprise growth factors (e.g. nerve growth factor (NGF)) acting via receptor tyrosine kinases (e.g. the *TrkA* high-affinity receptor for NGF), various endogenous (substance P, bradykinin, complement C3a) or exogenous basic peptides (e.g. mastoparan), and synthetic non-peptide molecules (e.g. compound 48/80) (Metcalfe et al., 1997). Several studies indicate that all of the above-mentioned basic peptides and compound 48/80 activate mast cells in a receptor-independent manner by direct activation of certain heterotrimeric G proteins (Aridor et al., 1993; Ross and Higashijima, 1994). Exocytosis induced by activation of these G proteins does not require the presence of extracellular Ca^{2+}, but involves the release G protein βγ-subunits (Pinxteren et al., 1998; Wilson et al., 1989). Via activation of G proteins functionally coupled to a phosphatidylinositol-specific phospholipase C, mastoparan and compound 48/80 have also been shown to induce the release Ca^{2+} from intracellular stores (Mousli et al., 1989). However, the functional impact of this Ca^{2+} release with regard to the exocytotic effect of both secretagogues is uncertain, since activation of G proteins in rat peritoneal mast cells by stable GTP-analogues (e.g. GTPγS) leads to exocytosis even when $[Ca^{2+}]_i$ is buffered to very low values (Fernandez et al., 1984; Neher, 1988).

In contrast, the exocytotic effect induced by antigen/IgE or NGF requires the presence of millimolar concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and the entry of extracellu-

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lar Ca^{2+} into the intracellular space (Foreman et al., 1973; Pearce and Thompson, 1986; Reischl et al., 1999). For different reasons, it is assumed that Ca^{2+} enters the cytoplasm via specialized Ca^{2+}-permeable ion channels in the plasma membrane. Activation of mast cells via FcεRI receptors is accompanied by the opening of store-operated plasmalemmal Ca^{2+} channels, mediating a sustained influx of Ca^{2+} (Zhang and McCloskey, 1995). These voltage-independent Ca^{2+} channels were initially characterized in rat peritoneal mast cells and the corresponding Ca^{2+} current was designated a Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}) (Hoth and Penner, 1992). CRAC currents can be blocked by tri- and bivalent cations in a characteristic rank order of potency ($Ba^{2+} \approx Sr^{2+} < Ni^{2+} < Mn^{2+} \approx Co^{2+} \approx Be^{2+} < Cd^{2+} < Zn^{2+} < La^{3+}$) (Hoth and Penner, 1992). With a similar pattern, these cations have also been demonstrated to inhibit the uptake of $^{45}Ca^{2+}$ and the antigen/IgE-induced exocytotic response in rat peritoneal mast cells and rat basophilic leukemia cells (RBL 2H3)(Choi et al., 1993; Hide and Beaven, 1991). In rat peritoneal mast cells, a Ca^{2+}-permeable nonspecific cation channel has also been described; however, the contribution of this type of channel to the total Ca^{2+} influx in agonist-induced exocytosis seems to be of minor importance (Fasolato et al., 1993). In summary, these findings strongly indicate that the influx of Ca^{2+} through store-operated Ca^{2+} channels is essential for antigen/IgE-induced exocytosis in rat peritoneal mast cells.

As predicted by the Nernst equation, the size of $I_{Ca^{2+}}$ through open Ca^{2+} channels, under physiological Ca^{2+} gradients, depends markedly on the cell membrane potential (V_m), such that $I_{Ca^{2+}}$ increases as V_m becomes negative (Matthews et al., 1989; Penner et al., 1988). Patch-clamp measurements performed in rat peritoneal mast cells suggest a strong dependence of V_m on the bath temperature. At room temperature, the resting membrane potential is unstable and ranges between 0 and 30 mV (Lindau and Fernandez, 1986). The influx of Ca^{2+} under these conditions has been proposed to rapidly depolarize cells and therefore to be a self-restricting process (Dietrich and Lindau, 1994). Hill et al. (1996) have recently shown that an increase of the bath temperature to 37 °C dramatically increases the fraction of rat peritoneal mast cells showing a resting conductance for Cl^-. This Cl^- conductance is due to activated outwardly rectifying Cl^- channels expressed in these cells. An influx of extracellular Cl^- ($[Cl^-]_o$) through this type of Cl^- channel has previously been demonstrated to cause a stable negative membrane potential in rat peritoneal mast cells of ≈ -40 mV (Matthews et al., 1989; Penner et al., 1988). These data indicate that the membrane potential in rat peritoneal mast cells, at 37 °C, may be more negative than deduced earlier from measurements performed at room temperature. Outwardly rectifying Cl^- channels can also be activated by other measures, e.g. intracellular application of cAMP, GTPγS, Ca^{2+} or extracellular application of basic peptides or compound 48/80. In store-depleted rat peritoneal mast cells, activation of outwardly rectifying Cl^- channels via intracellular application of cAMP leads to hyperpolarization accompanied by an increased influx of Ca^{2+} (Matthews et al., 1989; Penner et al., 1988). The finding that the outwardly rectifying Cl^- current in rat peritoneal mast cells drastically enhances $I_{Ca^{2+}}$ has raised the possibility that the opening of outwardly rectifying Cl^- channels may be important for agonist-induced exocytosis. By means of the stilbene-Cl^- channel blocker DIDS (4,4′-diisothiocyanatostilbene 2,2′-disulfonic acid) and by exchange of Cl^- against channel-impermeable anions (aspartate, glutamate), Dietrich and Lindau recently showed that the outwardly rectifying Cl^- channel is not essential for compound 48/80-induced exocytosis in rat peritoneal mast cells (Dietrich and Lindau, 1994). This finding is not unexpected, since compound 48/80-induced exocytotic responses are largely independent of the presence or influx of extracellular Ca^{2+} (Metcalfe et al., 1997). Using a similar experimental approach, the aim of the present study was to assess the importance of outwardly rectifying Cl^- channels for agonist-induced exocytosis in rat peritoneal mast cells activated by NGF, a secretagogue whose action completely depends on the presence of millimolar $[Ca^{2+}]_o$.

2. Materials and methods

4,4′-Diisothiocyanatostilbene2,2′-disulfonic acid (DIDS), diethylstilbestrol, nystatin, L-glutamic acid, L-aspartic acid, compound 48/80, and HEPES were obtained from Sigma (Deisenhofen, Germany). Mastoparan (H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH2) was obtained from Calbiochem (Schwalbach, Germany) and Bachem (Heidelberg, Germany). [[1,2-3[*N*]]-5-hydroxytryptamine creatinine sulfate ([^{3}H]5-HT) was from NEN (Cologne) Germany. All other salts, including sodium methyl sulfate, were purchased from Merck, Darmstadt, Germany. For unknown reasons, the exocytotic effect of NGF is markedly enhanced by coapplication of lyso-phosphatidylserine. In the present study, all experiments with NGF were performed in the presence of 1 μM lyso-phosphatidylserine.

2.1. Preparation of peritoneal mast cells

Rat peritoneal mast cells were obtained from male Wistar rats (Charles River, Sulzfeld, Germany) weighing 200–400 g, by peritoneal lavage, as previously described (Lindau and Fernandez, 1986; Mousli et al., 1989). In brief, rats were deeply anesthetized with ether and decapitated. Consecutively, 20 ml of "extracellular solution" [containing (mM): NaCl 137.0, KCl 2.7, $CaCl_2$ 2.0, $MgCl_2$ 5.0, HEPES 10.0, ascorbic acid 1.0, glucose 5.6; pH 7.3]

was injected into the peritoneal cavity. After a short abdominal massage, the lavage fluid was aspirated by means of a wide-bore pipette through a small incision in the abdominal wall. Aliquots (50 μl) of the resulting cell suspension were plated on glass coverslips and kept in the dark, at 22 °C, until used (within 120 min). Before the start of an experiment, non-adherent cells and particles were removed by briefly dipping the coverslips into extracellular solution. The coverslips were transferred to a perfusion chamber, prefilled with 0.5 ml extracellular solution, and mounted on the stage of an inverted light microscope (Axiovert 135, Zeiss, Göttingen, Germany). The peritoneal mast cells could be easily identified under the microscope by their characteristic morphological features (round shape, granular appearance, small prominent nucleus).

2.2. Electrophysiological recordings

For nystatin-perforated patch recordings, the "pipette solution" or "internal solution" (IS) contained (mM) KCl 137.0, NaCl 2.7, $MgCl_2$ 3.0, $CaCl_2$ 1, HEPES 10.0; pH 7.3. Immediately before the recordings were started, nystatin was dissolved in dimethylsulfoxide (DMSO) at a concentration of 250 000 U/ml. Aliquots of this concentrated solution were mixed with internal solution, yielding a final nystatin concentration of 1250 U/ml. Whole-cell currents were measured in voltage-clamp experiments using the nystatin-perforated patch approach of the whole-cell patch-clamp technique (Hamill et al., 1981; Horn and Marty, 1988). All electrophysiological experiments were performed at room temperature (20–24 °C). Patch pipettes were pulled from borosilicate glass (Hilgenberg, Malsfeld, Germany). The patch pipettes were heat-polished to a resistance between 2.0 and 3.5 MΩ, when filled with nystatin-containing internal solution. The tips of the patch pipettes were filled with nystatin-free buffer solution. As previously described, when challenged with step depolarizations, perforation of the membrane patch could be revealed by characteristic changes of the capacitance transients and by a continuous decline of the access resistance. In order to standardize experiments, recordings were started when the access resistance fell to a value of < 25 MΩ; this point was usually reached 3–10 min after giga-seal formation. Whole-cell perforated-patch recordings were acquired by a computer-based patch-clamp amplifier system (EPC-9, HEKA Electronics, Germany). Capacitance transients were cancelled before each series of test pulses, using the automatic compensation of the EPC-9. Currents were filtered using an eight-pole Bessel filter at 2.9 Hz and digitized at 100 μs. Series of incremental voltage pulses of 100 ms duration were applied every second from -80 to $+80$ mV in 20-mV steps from a holding potential of 0 mV (standard pulse protocol). A complete series of test pulses was repeated at 80-s intervals, except when otherwise indicated. All shown currents were leak subtracted. Leak currents were determined by applying six short voltage steps of alternating polarity (leak holding potential -100 mV) at the beginning of each test pulse. Steady-state currents were determined by calculating the mean of current signals obtained in the last 10 ms of single sweeps. Cl^- channel blockers were applied by means of a hydrostatic superfusion system; the bath solution (500 μl) could be completely exchanged by the superfusion solution within 1 min.

For current-clamp measurements, patches were permeabilized with nystatin as described above. The current-clamp recordings were performed by using the current-clamp mode of the pulse program (HEKA Electronics). Secretagogues (NGF, compound 48/80) were applied in bolus form to the bath solution. At the end of each measurement, the seal quality was checked under voltage-clamp conditions; only cells with intact giga-seals were analyzed.

2.3. Secretion assay

For secretion assays, a modified Krebs–Ringer–Henseleit buffer containing 0.2% bovine serum albumin and [mM] 137 NaCl, 2.7 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 0.4 NaH_2PO_4, 10 HEPES and 5.6 glucose (pH 7.3) was used. Experiments were performed as previously described (Mousli et al., 1989). Briefly, peritoneal lavage fluids collected from three rats were combined and the mast cells were purified by using a bovine serum albumin gradient centrifugation to at least 90% purity. Consecutively, purified mast cells were incubated for 60 min, at 37 °C, in the presence of 1 μCi/ml 5-[1,2-^{3}H[N]]hydroxytryptamine creatine sulfate. After a wash step, the cells were resuspended in buffer and stimulated in the absence or presence of different test compounds in a shaking water bath (37 °C), as indicated. The proportion of the total cellular radioactivity released into the supernatant was determined and, expressed in [% of total], taken as a measure of secretion. Spontaneous release of radioactivity was always below 5%. All experimental conditions were tested in duplicate.

2.4. Statistics and curve fitting

Data are means ± S.E.M. For statistical analysis of differences between experimental groups (current-clamp experiments), a one-sided Kruskal–Wallis test in combination with Dunnett's post-test was used. A P-value equal or below 0.05 was defined to indicate a significant difference. Concentration–response curves were fitted to a four-parameter logistic equation by computer-assisted curve fitting (Prism 2, GraphPad software, San Diego, USA). The equation fitted was: Response $= I_{min} + (I_{max} - I_{min})/(1 + 10^{(\log IC50 - X)nH})$, where I_{min} is the control re-

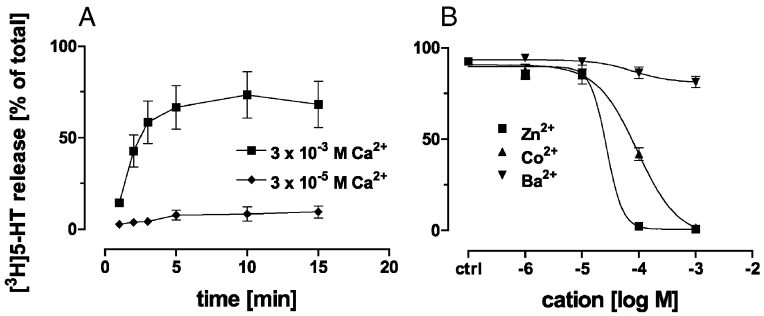

Fig. 1. Effect of co-stimulation with 1 μg/ml nerve growth factor and 1 μM lyso-phosphatidylserine on [^{3}H]5-HT release from [^{3}H]5 HT-loaded rat peritoneal mast cells [% of total] under different experimental conditions: (A) time-dependent stimulation of [^{3}H]5-HT release in the presence of 3 or 0.03 mM extracellular Ca^{2+} ($n = 3$). (B) Dose-dependent effect of different bivalent cations on [^{3}H]5-HT release from rat peritoneal mast cells stimulated by NGF/lyso-phosphatidylserine for 15 min ($n = 3$). Results are expressed as means ± S.E.M.

sponse, I_{max} is the maximal inhibition, X is the antagonist concentration and nH is the Hill coefficient.

3. Results

3.1. Effects of $[Ca^{2+}]_o$ and bivalent cations on agonist-induced [^{3}H]5-HT release

Purified rat peritoneal mast cells were incubated for different time periods (1–15 min) in the presence of 3 or 0.03 mM Ca^{2+} with NGF (1 μg/ml) and 1.0 μM lyso-phosphatidylserine ($n = 3$, Fig. 1A). The maximum exocytotic effect observed with NGF/lyso-phosphatidylserine in the presence of 3 or 0.03 mM Ca^{2+} amounted to 73 ± 13 and 9.5 ± 3 ([^{3}H]5-HT release [% of total]), respectively. In the presence of 3 mM Ca^{2+}, the exocytotic effect was half maximal ($t_{0.5}$) after an incubation time of 2.2 min (t_{max}: 10 min). In contrast, within the same series of experiments, the exocytotic effect induced by 2×10^{-5} M mastoparan was comparably larger at 0.03 mM Ca^{2+} (3 mM $[Ca^{2+}]_o$: 21.9 ± 3.8, 0.03 mM $[Ca^{2+}]_o$: 53.7 ± 15 [^{3}H]5-HT release (% of total); data not shown). Different bivalent cations inhibited the NGF/lyso-phosphatidylserine-induced exocytotic response in a dose-dependent

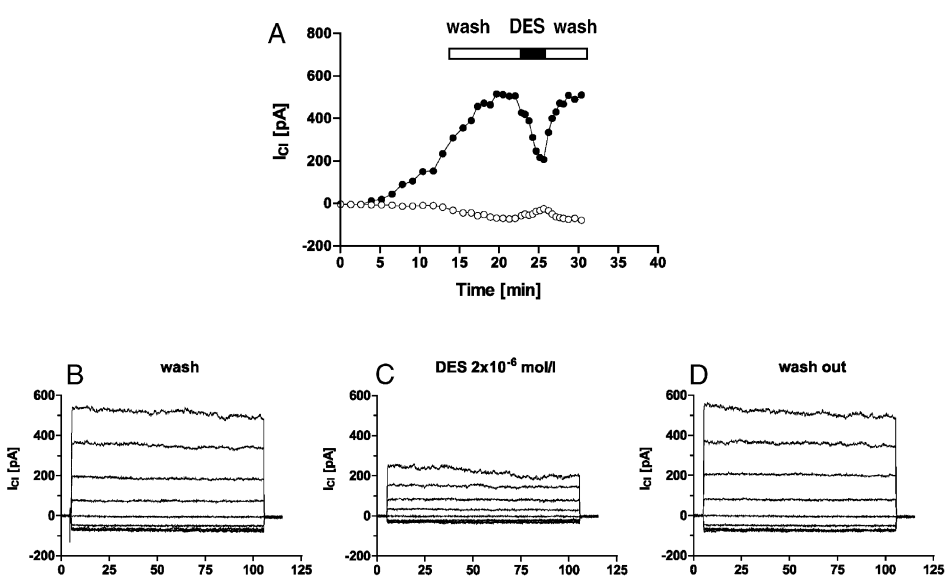

Fig. 2. Representative whole-cell patch-clamp experiment (nystatin-perforated patch) showing the time-dependent development of an outward rectifying current in rat peritoneal mast cells challenged by step hyper- and depolarizations, and its reversible block by 2 μM diethylstilbestrol (DES). (A) Time course (holding potential: 0 mV); test potentials (100 ms): +80 mV (closed circles), −80 mV (open circles). (B–D) Original current traces ((−80)–(+80) mV, step size +20 mV): (B) before superfusion with 2 μM DES, (C) at the end of the DES superfusion period, (D) after washout of DES.

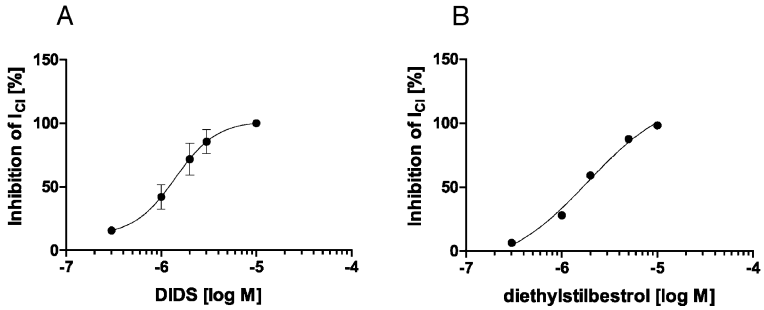

Fig. 3. Dose-dependent effect of (A) DIDS and (B) diethystilbestrol on normalized steady-state whole-cell outward currents in rat peritoneal mast cells investigated by means of the patch-clamp technique (nystatin-perforated patch), challenged by step depolarizations (test potential: +80 mV, holding potential: 0 mV). Results are expressed as means ± S.E.M. The error bars in (B) are unresolvably small.

manner (IC_{50}: Zn^{2+}: 3×10^{-5}, Co^{2+}: 10^{-4} M, Ba^{2+}: $> 10^{-3}$ M; $n = 3$; Fig. 1B).

3.2. Effects of DIDS and diethylstilbestrol on $I_{Cl^-(OR)}$

In whole-cell voltage-clamp experiments, 26 out of 26 rat peritoneal mast cells challenged by means of a series of step depolarizations developed, in a time-dependent manner, an outwardly rectifying current (half maximal activation of $I_{Cl^-(OR)}$: diethylstilbestrol group 14.9 ± 0.7 min ($n = 15$), DIDS group 14.3 ± 0.9 min ($n = 11$). After the current maximum (I_{max}) was reached, the cells were superfused with the respective Cl^- channel blocker ([I_{max}] diethylstilbestrol group: 210 ± 40 pA, [I_{max}] DIDS group 255 ± 62 pA); per experiment only one single test concentration was assessed (Fig. 2). DIDS and diethylstilbestrol reduced the normalized steady-state maximum currents (I_{max}) in a concentration-dependent manner, IC_{50}(DIDS): $1.4 \pm 0.1 \times 10^{-6}$ M, IC_{50}(diethylstilbestrol): $1.9 \pm 0.1 \times 10^{-6}$ M (Fig. 3). The inhibitory effect of both Cl^- channel blockers could be partly reversed by superfusion with blocker-free solution.

3.3. Effect of DIDS and diethylstilbestrol on the membrane potential

The resting membrane potential V_m of unstimulated rat peritoneal mast cells amounted to 5.1 ± 1.1 mV ($n = 34$), as investigated by means of the patch-clamp technique (current-clamp) using the nystatin-perforated patch ap-

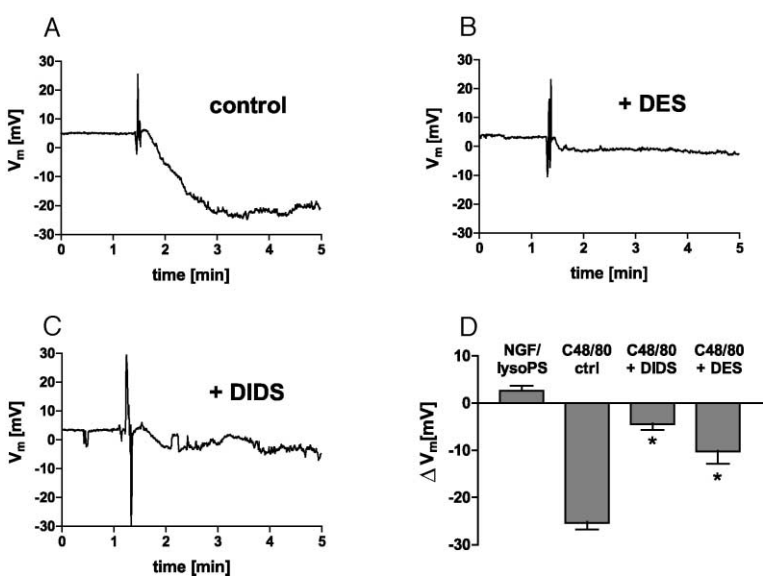

Fig. 4. Effect of 10 μM DIDS and DES (diethystilbestrol) on compound 48/80-induced membrane potential (V_m) hyperpolarization in rat peritoneal mast cells. The current-clamp recordings were performed by means of the whole-cell patch-clamp technique (nystatin-perforated patch). (A–C) Original traces (the large artifact indicates the time of application of compound 48/80; final concentration: 17 μg/ml): (A) without DIDS or diethylstilbestrol; (B) in the presence of diethylstilbestrol; (C) in the presence of DIDS. (D) Summary of maximum hyperpolarization achieved by application of compound 48/80 in the absence ($n = 8$) or presence of DIDS ($n = 7$) or diethylstilbestrol ($n = 9$) and by application of 1 μg/ml NGF/1 μM lyso-phosphatidylserine alone ($n = 9$). Results are expressed as means ± S.E.M.($^* \triangleq < 0.05$).

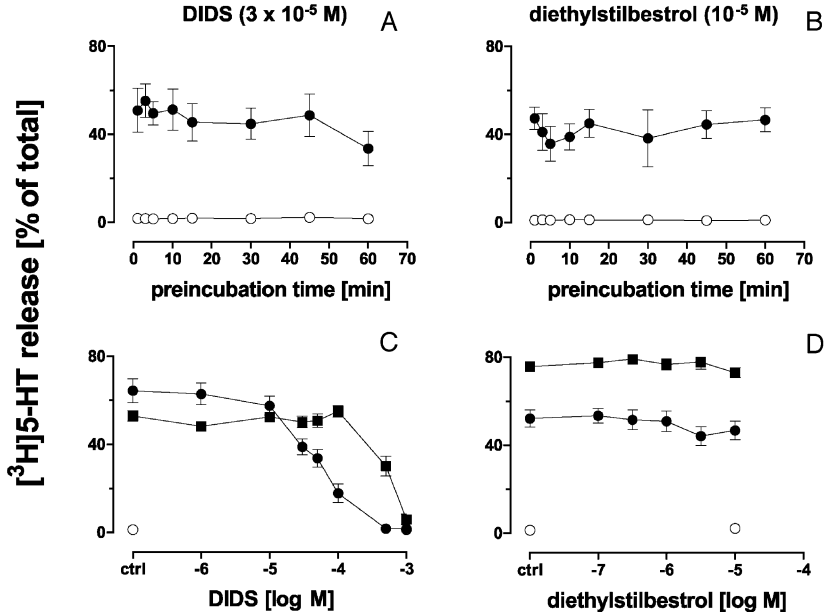

Fig. 5. Effect of DIDS and diethystilbestrol on [^{3}H]5-HT release from [^{3}H]5-HT-loaded rat peritoneal mast cells [% of total], in the absence or presence 1 μg/ml NGF/1 μM lyso-phosphatidylserine or 20 μM mastoparan. (A,B) Time-dependent effect of DIDS (2×10^{-5} M) and diethylstilbestrol (10^{-5} M) on [^{3}H]5-HT release from rat peritoneal mast cells stimulated by NGF/lyso-phosphatidylserine (closed symbols) or not (open symbols). (C,D) Concentration-dependent effects of DIDS (C) and diethylstilbestrol (D) on unstimulated- (open symbols), NGF/lyso-phosphatidylserine- (circles) and mastoparan-stimulated (squares) [^{3}H]5-HT secretion from rat peritoneal mast cells. Results are expressed as means ± S.E.M.

proach. Preliminary dose-finding experiments (not shown) revealed that, under the given experimental conditions (room temperature), a relatively high concentration of compound 48/80 had to be used in order to achieve membrane hyperpolarization within a time interval appropriately short to assume a causal relation with the application of compound 48/80. Bolus application of compound 48/80 (final concentration: 17 μg/ml) hyperpolarized the membrane potential of single rat peritoneal mast cells to a new steady state -25 ± 2 mV within < 2 min ($n = 8$, Fig. 4). The presence 10 μM DIDS ($\Delta V_m - 4.5 \pm 1.2$ mV; $n = 7$; $P < 0.05$) or diethylstilbestrol ($\Delta V_m - 10.6 \pm 2.4$ mV; $n = 9$; $P < 0.05$) in the bath solution significantly reduced the hyperpolarizing effect of compound 48/80. Addition of 50 μl NGF (200 μg/ml) to the bath solution, which contained 1 μM lyso-phosphatidylserine, induced no hyperpolarization (ΔV_m 3 ± 1 mV; $n = 9$), but instead a small depolarization.

3.4. Effects of Cl^- channel inhibitors and substitution

Preincubation of rat peritoneal mast cells for different time periods (1–60 min) with the Cl^- channel inhibitors DIDS (3×10^{-5} M; $n = 4$) or diethylstilbestrol (10^{-5} M; $n = 4$) only marginally reduced the NGF/lyso-phosphatidylserine-induced exocytotic response (incubation time 15 min; Fig. 5A,B). In addition, dose–response experiments with DIDS (10^{-6}–10^{-3} M) and diethylstilbestrol (10^{-7}–10^{-5} M) were performed (Fig. 5C,D): in concentrations below or equal to 10^{-5} M, neither DIDS (preincubation

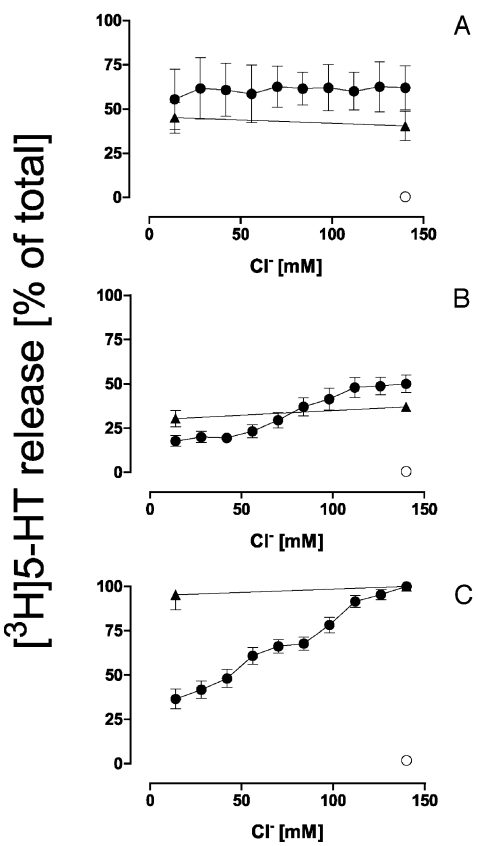

Fig. 6. Effect of replacement of Cl^- in the extracellular medium ($[Cl^-]_o$) by methylsulfate (A), aspartate (B) or glutamate (C) on NGF/lyso-phosphatidylserine- (circles) or mastoparan-induced (triangles) [^{3}H]5-HT release from [^{3}H]5-HT-loaded rat peritoneal mast cells [% of total]. Results are expressed as means ± S.E.M.

time: 60 min; $n = 7$) nor diethylstilbestrol (preincubation time: 10 min; $n = 6$) inhibited the exocytotic response in rat peritoneal mast cells stimulated by NGF/lyso-phosphatidylserine or mastoparan. In concentrations above 10^{-5} M, DIDS inhibited dose dependently the NGF/lyso-phosphatidylserine and mastoparan-induced release of [^{3}H]5-HT in rat peritoneal mast cells (IC_{50}: 5×10^{-5} M (NGF/lyso-phosphatidylserine), $\approx 5 \times 10^{-4}$ M (mastoparan)). Because of its low solubility, concentrations of diethylstilbestrol higher than 10^{-5} M were not tested in the present study in order to avoid concentrations of the solvent dimethyIsulfoxide (DMSO) higher than 0.1% [v/v]. In another set of experiments, the extracellular Cl^- concentration ($[Cl^-]_o$) was varied in a range between 14 and 140 mM, by equimolar replacement of NaCl in the modified Krebs–Ringer–Henseleit buffer with sodium glutamate, sodium aspartate or sodium methyl sulfate. The substitution of Cl^- by aspartate ($n = 6$) and glutamate ($n = 10$), but not by methyl sulfate ($n = 6$), dose dependently reduced the NGF/lyso-phosphatidylserine-induced release of [^{3}H]5-HT; mastoparan-incubated controls were not affected by lowering of the [Cl^-] (Fig. 6).

4. Discussion

In rat peritoneal mast cells, an outwardly rectifying Cl^- channel has been described which allows Cl^- to permeate into the cytosol and thereby to induce a negative resting membrane potential (V_m) of about -40 mV (Matthews et al., 1989; Penner et al., 1988). Because the electrochemical driving force for Ca^{2+} to cross the plasma membrane is increased by membrane hyperpolarization, opening of outwardly rectifying Cl^- channels in rat peritoneal mast cells should cause an increase of Ca^{2+} currents through membrane store-operated Ca^{2+} channels. This Ca^{2+} inward current should facilitate the exocytotic response to secretagogues depending on $[Ca^{2+}]_o$. By interacting with high-affinity NGF receptors of the TrkA-type, NGF, in the presence of lyso-phosphatidylserine, stimulates exocytosis in rat peritoneal mast cells in a $[Ca^{2+}]_o$-dependent manner. A similar dependence on millimolar concentrations of $[Ca^{2+}]_o$ has previously been described for FcεRI-mediated exocytosis in mast cells and the immortalized mast cell-line RBL-2H3. Both the antigen/IgE/FcεRI- and the NGF/lyso-phosphatidylserine/TrkA-mediated exocytotic response can be blocked by different bivalent cations in a rank order of potency characteristic for blockade of store-operated Ca^{2+} channels in rat peritoneal mast cells (Choi et al., 1993; Hide and Beaven, 1991; Pearce and Thompson, 1986). Thus, the NGF/lyso-phosphatidylserine-induced exocytotic response represents a relevant model to study the role of outwardly rectifying Cl^- channels in $[Ca^{2+}]_o$-dependent, agonist-induced exocytosis in rat peritoneal mast cells. Exocytosis in rat peritoneal mast cells can also be induced in a $[Ca^{2+}]_o$-independent and consequently $I_{Cl^-(OR)}$-independent manner by a number of basic peptides (e.g. mastoparan, substance P) or polyaromatic compounds (e.g. compound 48/80). These secretagogues elicit exocytosis via direct activation of certain heterotrimeric GTP-binding proteins, a process which involves the release of G protein $\beta\gamma$-subunits as an effector mechanism (Aridor et al., 1993; Pinxteren et al., 1998; Ross and Higashijima, 1994). Therefore, mastoparan and compound 48/80 were used in the present study as control stimuli to monitor the outwardly rectifying Cl^- channel-independent exocytotic effects of the Cl^- channel blockers used.

In accordance with previous findings by Pearce and Thompson (1986), experiments performed to characterize the dependence of the NGF/lyso-phosphatidylserine-induced exocytotic effect on $[Ca^{2+}]_o$ revealed a nearly complete prevention of exocytosis at 0.03 mM $[Ca^{2+}]_o$ (Fig. 1A). In contrast, the exocytotic effect of mastoaparan was enhanced by a reduction of $[Ca^{2+}]_o$, a finding made before with different basic peptide secretagogues (Ross and Higashijima, 1994). In a second set of experiments, the influence of three different bivalent cations (Zn^{2+}, Co^{2+}, Ba^{2+}) on NGF/lyso-phosphatidylserine-induced exocytosis was assessed. As expected, these cations dose dependently inhibited exocytosis in an identical rank order of potency ($Zn^{2+} > Co^{2+} > Ba^{2+}$) as previously described for inhibition of I_{CRAC} in rat peritoneal mast cells (Hoth and Penner, 1992). In summary, these findings confirm the necessity of extracellular Ca^{2+} for rat peritoneal mast cells to execute the exocytotic signal of NGF/lyso-phosphatidylserine. Of note, regarding the above-studied parameters ($[Ca^{2+}]_o$, bivalent cation blockade), the antigen/IgE- and the NGF/lyso-phosphatidylserine-induced exocytotic signaling pathways show similar features.

In the present study, the stilbene compound DIDS potently inhibited the steady state $I_{Cl^-(OR)}$, at $+80$ mV, in a dose-dependent manner (IC_{50}: 1.4 μM). The potency of this inhibitory effect of DIDS is in good agreement with previous findings of Dietrich and Lindau (1994). These authors described in detail the complex partially reversible and irreversible actions of DIDS on $I_{Cl^-(OR)}$ and obtained an IC_{50}-value for inhibition of the steady-state current at $+70$ mV of 2.3 μM. Like DIDS, another stilbene compound assessed in the present study, the estrogen receptor agonist diethylstilbestrol reduced $I_{Cl^-(OR)}$ in rat peritoneal mast cells in a dose-dependent manner and with a similar potency (IC_{50}: 1.9 μM; Fig. 3B). Complete inhibition of the outward Cl^- current was obtained at a concentration of 10 μM, with both stilbene compounds. Correspondingly, 10 μM DIDS and diethylstilbestrol largely prevented the compound 48/80-induced membrane-hyperpolarization in rat peritoneal mast cells. In contrast, at a concentration of 10 μM, neither DIDS nor diethylstilbestrol affected the NGF/lyso-phosphatidylserine- or mastoparan-induced exocytotic response in rat peritoneal mast cells. These findings indicate that the opening of outwardly rectifying Cl^-

channels is not essential for agonist-induced exocytosis in rat peritoneal mast cells. At concentrations above 10 μM, DIDS dose dependently inhibited agonist-induced exocytosis in rat peritoneal mast cells, but only when cells were preincubated with the compound for at least 15 min. This finding suggests, as previously concluded by others, that DIDS might affect the exocytotic signaling machinery via a Cl^- channel/transporter-independent, probably unspecific, mechanism (Dietrich and Lindau, 1994; Friis et al., 1994). Unlike DIDS, diethylstilbestrol, due to its lower solubility, could not be tested in concentrations above 10^{-5} M.

A reduction of the extracellular Cl^- concentrations $[Cl^-]_o$ from values of approximately 150 mM to values equal or below 50 mM by replacement with larger anions (glutamate, aspartate, methylsulfate) has previously been shown to drastically reduce the $I_{Cl^-(OR)}$ in rat peritoneal mast cells and rat bone marrow-derived mast cells (Hill et al., 1996; Matthews et al., 1989). In the present study, the replacement of $[Cl^-]_o$ down to 14 mM by methylsulfate did not impair the NGF/lyso-phosphatidylserine- or the mastoparan-induced exocytotic response (Fig. 5A). This finding is in line with our above-mentioned results obtained with DIDS and diethylstilbestrol and corroborates the conclusion that the agonist-induced exocytotic response in rat peritoneal mast cells is functionally independent of the opening of outwardly rectifying Cl^- channels. In contrast, replacement of methylsulfate by glutamate or aspartate dose dependently inhibited the NGF/lyso-phosphatidylserine-, but not the mastoparan-induced release of [^{3}H]5-HT. With regard to our above presented findings, the NGF/lyso-phosphatidylserine-selective inhibitory effects of glutamate and aspartate have to be interpreted as being independent of outwardly rectifying Cl^- channels. The nature of this inhibitory effect of both amino acids was not further investigated in the present study. Two alternative mechanisms could explain the obvious independence of the NGF/lyso-phosphatidylserine-induced exocytotic response from $I_{Cl^-(OR)}$: (a) hyperpolarization is not necessary for the influx of Ca^{2+} in amounts sufficient to trigger exocytosis; (b) a negative V_m is required for the entry of sufficient Ca^{2+}, but other hyperpolarizing effectors compensate for inhibition of $I_{Cl^-(OR)}$. With regard to the latter possibility, by means of $^{36}Cl^-$ uptake measurements, Friis et al. (1994) recently demonstrated that rat peritoneal mast cells take up $^{36}Cl^-$ also via a DIDS-insensitive, furosemide-sensitive $Na^+/K^+/2Cl^-$ cotransporter with slow kinetics, a process which could be accelerated by activation of FcεRI. In addition, the same group showed that, under certain experimental conditions, the electrogenic Na^+/K^+ATPase could hyperpolarize the membrane potential of rat peritoneal mast cells (Friis et al., 1997). Notably, the hyperpolarizing effect of compound 48/80 in the present study was only incompletely inhibited by 10 μM DIDS or diethylstilbestrol. In summary, these findings are compatible with the "model" of a second hyperpolarizing effector acting in a synergistic manner with $I_{Cl^-(OR)}$ in rat peritoneal mast cells. A number of studies of voltage-gated Ca^{2+} channels have shown that the entry of sufficient Ca^{2+} can occur even at a positive membrane potential, provided that the number and conductance of available Ca^{2+} channels are sufficient (Fenwick et al., 1982). Therefore, in the present study, the possibility cannot be excluded that the influx of Ca^{2+} putatively occurring at a $V_m \approx 0$ mV may be sufficient to permit exocytosis in the presence of NGF/lyso-phosphatidylserine. However, with regard to the relative small maximum of I_{CRAC} previously observed in rat peritoneal mast cells, this possibility seems to be less likely (Hoth and Penner, 1992). The present study demonstrates that exocytosis in rat peritoneal mast cells is not impaired by complete inhibition of outwardly rectifying Cl^- channels, even when secretagogues are used whose action completely depends on the influx of Ca^{2+} through store-operated Ca^{2+} channels.

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