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Pharmacological inhibition of outwardly rectifying Cl^- currents in rat peritoneal mast cells: a comparison of different stilbene derivatives

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Abstract

Diethylstilbestrol and other stilbene derivatives can provide some inhibition of the outwardly rectifying Cl^- current ($I_{(Cl^-,OR)}$) in rat peritoneal mast cells. In order to elucidate structure–activity relationships of diethylstilbestrol, 12 stilbenes as well as 17β-estradiol and hexestrol were tested in rat peritoneal mast cells using the nystatin-perforated patch approach of the whole-cell patch-clamp technique. Since *trans*-stilbene showed no effect, the substituents of diethylstilbestrol must be of importance. The introduction of only one hydroxy group in *trans*-stilbene produced potent inhibition of the $I_{(Cl^-,OR)}$ (IC_{50}: 3.3 μM). But in contrast, resveratrol with hydroxy groups at positions 4, 3′, and 5′ as well as methoxy substituted stilbene derivatives and 17β-estradiol were ineffective. On the other hand, hexestrol potently inhibited $I_{(Cl^-,OR)}$ indicating that the aromatic ring systems can also be connected by an ethyl bridge. In summary, a hydroxy group at position 4 (or 4′) is a prerequisite for diethylstilbestrol-mediated inhibition of $I_{(Cl^-,OR)}$.

Keywords: Stilbene; Mast cell; Cl^- channel; Exocytosis; Diethylstilbestrol; Hexestrol

1. Introduction

Rat peritoneal mast cells express a non-voltage gated outwardly rectifying Cl^- channel (Matthews et al., 1989; Penner et al., 1988). In the open state, this channel allows Cl^- to enter the cell interior and thereby to achieve a negative membrane potential (V_m). The membrane potential in rat peritoneal mast cells is of physiological importance since it influences the electrochemical driving force for transmembranous Ca^{2+} currents (Matthews et al., 1989; Penner et al., 1988). The molecular identity of the outwardly rectifying Cl^- channel (ORCC) expressed in rat peritoneal mast cells is currently unclear. A recent study has provided evidence that type 7 Cl^- channel (ClC-7)-mRNA is present in rat peritoneal mast cells (Kulka et al., 2002). However, the presence of ClC-7-protein within the plasma membrane of these cells remains to be shown.

A characteristic feature of outwardly rectifying channel-mediated Cl^- currents ($I_{(Cl^-,OR)}$) is a potent inhibition by certain stilbene derivatives, e.g. DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), e.g. SITS (4′-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid), and diethylstilbestrol (E-α,β-diethyl-4,4′-stilbenediol) (Matthews et al., 1989; Roloff et al., 2001). The structural properties which enable stilbene molecules to inhibit $I_{(Cl^-,OR)}$ are unknown. In order to address this question, a panel of 14 different stilbene and structurally related non-stilbene molecules were tested in the present study regarding their ability to inhibit $I_{(Cl^-,OR)}$ in rat peritoneal mast cells. Because diethylstilbestrol acts as an exceptionally potent inhibitor of $I_{(Cl^-,OR)}$, this molecule was taken as a starting point and lead compound for structural variation (Roloff et al., 2001). Chemically, diethylstilbestrol is a *trans*-stilbene whose aliphatic ethene part is substituted by two ethyl groups. The phenyl rings of diethylstilbestrol carry each a hydroxy group at (para)-positions 4 and 4′. The stilbene derivatives assessed in the present study can be grouped into molecules either modified at their aromatic rings (DIDS, resveratrol, SITS, *trans*-4-hydroxystilbene, *trans*-4-hydroxy-4′-methoxystilbene), or at their aliphatic ethene part (tamoxifen, *trans*-diethylstilbene, *cis*-diethylstilbene), or both (clomiphene, chlorotrianisene, diethylstilbestrol). In addition, *trans*-stilbene, hexestrol (a diethylstilbestrol related non-stilbene)

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and 17β-estradiol (a steroid estrogen receptor agonist) were tested.

Previous studies have shown that some stilbene derivatives (e.g. DIDS, diethylstilbestrol) at higher concentrations (>10 μM) are capable to impair agonist-induced exocytotic responses in rat peritoneal mast cells via $I_{(Cl^-,OR)}$-independent mechanisms (Roloff et al., 2001; Vliagoftis et al., 1992). Interestingly, this secretostatic effect depends on the type of stimulus used. Secretagogues whose action depends on the presence of extracellular $[Ca^{2+}]_e$ (nerve growth factor [NGF] in the presence of lysophosphatidylserine [lysoPS], antigen/immunoglobulin E [Ag/IgE]) are inhibited more potently than $(Ca^{2+})_e$-independent acting stimuli (compound 48/80, mastoparan) (Hill et al., 1996; Pearce and Thompson, 1986; Roloff et al., 2001). Therefore, the stilbene derivatives chosen were also assessed regarding their ability to inhibit NGF/lysophosphatidylserine- and mastoparan-stimulated exocytotic responses. As a measure of exocytosis the release of [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) from [^{3}H]5-HT-loaded rat peritoneal mast cells was used in the present study. Furthermore, the effect of the stilbene derivatives on unstimulated [^{3}H]5-HT-release was tested, in order to monitor for putative toxic/membranolytic actions.

2. Materials and methods

The compounds used were purchased from different manufacturers (as indicated) and of the highest available degree of purity: chlorotrianisene, clomiphene, DIDS, diethylstilbestrol, 17β-estradiol, HEPES, hexestrol, lysophosphatidylserine, nerve growth factor 7S (mouse submandibular gland), nystatin, SITS, tamoxifen, *trans*-resveratrol, *trans*-stilbene were all received from Sigma-Aldrich Chemie (Deisenhofen, Germany). Mastoparan was obtained from Bachem (Heidelberg, Germany). 5-[1,2-^{3}H[*N*]]-hydroxytryptamine creatine sulfate ([^{3}H]5-HT) was purchased from NEN (Cologne) Germany. The synthesis of the E- and Z-isomeric forms of diethylstilbene was accomplished by reductive coupling of propiophenone using titanium chloride/zinc/pyridine; the mixture of the isomers was separated and purified on a silica gel column (Leimner and Weyerstahl, 1982). *Trans*-4-hydroxybenzaldehyde was obtained by the *Wittig* reaction of benzyltriphenylphosphonium chloride and 4-hydroxybenzaldehyde in the presence of sodium ethanolate and *trans*-4-hydroxy-4′-methoxystilbene prepared using the same method with corresponding educts (Friedrich and Henning, 1959). All products were characterized by ^{1}H and IR spectra and also by comparison of the melting points with literature data.

2.1. Preparation of rat peritoneal mast cells

Rat peritoneal mast cells were obtained by peritoneal lavage from male Wistar rats (Charles River, Sulzfeld, Germany) as previously described (Mousli et al., 1989; Roloff et al., 2001). In brief, rats in deep ether anesthesia were decapitated. Then, 20 ml of extracellular solution containing NaCl 137, KCl 2.7, $CaCl_2$ 2.0, $MgCl_2$ 5.0, HEPES 10.0, ascorbic acid 1.0, and glucose 5.6 mM (pH 7.3) were injected into the peritoneal cavities. After a short abdominal massage, the lavage fluid was reaspirated using a wide-bore pipette through a small incision in the abdominal wall. Aliquots (50 μl) of the resulting cell suspension were plated on glass cover-slips and kept in the dark at 22 °C until use (within 120 min). Cover slips were placed below an inset in 35-mm culture dishes (fluid volume ≈ 500 μl) and mounted on the stage of an inverted microscope (Axiovert 135, Zeiss, Göttingen, Germany). Before starting an experiment, cover-slips were superfused with extracellular solution in order to remove non-adherent cells and particles. The peritoneal mast cells were identified under the light microscope by their characteristic morphological features (round shape, granular appearance, small prominent nucleus).

2.2. Electrophysiological recordings

For nystatin-perforated patch recordings, the pipette or internal solution always contained KCl 137, NaCl 2.7, $MgCl_2$ 3.0, $CaCl_2$ 1, HEPES 10.0 mM (pH 7.3). Immediately before starting the recordings, nystatin was dissolved in dimethylsulfoxide (DMSO) at 250,000 U ml^{-1}. Aliquots of this stock solution were mixed with internal solution to a yield a final nystatin concentration of 1250 U ml^{-1}. 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; Hill et al., 1996; 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) and fire-polished to a resistance of 2.0–3.5 MΩ when filled with the nystatin-containing internal solution. The tips of the patch-pipettes were filled with nystatin-free buffer solution. As previously described (Hill et al., 1996), when challenged with step depolarisations, perforation of the membrane patch was indicated by characteristic changes of the capacitance transient and by a continuos decline of the input resistance. To standardise experiments, recordings were started when the input resistance fell to a value of 25 MΩ or less; this point was reached usually 5–10 min after gigaseal 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 8-pole Bessel filter at 2.9 Hz and digitized at 100 μs. Series of incremental voltage pulses (from −80 to +80 mV in 20-mV steps, duration 100 ms) were applied every

second from holding potential 0 mV (standard pulse protocol). A complete series of test pulses was repeated at 60- or 30-s intervals. All currents shown were corrected by leak subtraction. Leak currents were determined by applying six short voltage steps of alternating polarity (leak holding potential −100 mV) at the start of each test pulse. Steady-state currents were determined by calculating the mean of current signals obtained over the last 10 ms of single sweeps. For analysis of drug effects, the current responses at ±80 mV over time were used. Test compounds were applied by superfusion after the current maximum has been reached. Per experiment only a single concentration of each test compound was assessed.

2.3. Secretion assay

For secretion assays, a modified Krebs–Ringer–Henseleit buffer (MKRH) containing 0.2% bovine serum albumin and NaCl 137, KCl 2,7, $CaCl_2$ 0,3, $MgCl_2$ 1, NaH_2PO_4 0,4, HEPES 10, and glucose 5,6 mM (pH 7.3) was used. Experiments were performed as previously described (Mousli et al., 1989). Briefly, peritoneal lavages of three rats were combined and the containing mast cells were enriched using a bovine serum albumin-gradient centrifugation procedure to at least 90% purity. Consecutively, the enriched mast cells solution was incubated for 60 min, at 37 °C, in the presence of 1 μCi/ml 5-[1,2-^{3}H[*N*]]-hydroxytryptamine creatine sulfate. After washing, 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 amount 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 increased with incubation time; within the first 20 min, in the absence of test compounds, spontaneous release of [^{3}H]5-HT was always below 5%. All experimental conditions were determined in duplicate.

2.4. Log P-values

As a measure of lipophilicity, Log *P*-values of the compounds were determined. The Log *P*-values were calculated by using the online version of the program LogKow/KowWin (http://esc.syrres.com/interkow/kowdemo.htm).

2.5. Statistics and curve fitting

Data are means ± S.E.M. Concentration–response curves were fitted to a four parameter logistic equation through computer-assisted curve fitting (Prism 2, GraphPad software, San Diego, USA). The equation fitted was: $\text{Response} = I_{min} + (I_{max} - I_{min})/(1 + 10^{(\log \text{IC50} - X)nH})$, where I_{min} is the control response, I_{max} is the maximal inhibition, X is the test compounds concentration and n_H the Hill coefficient.

3. Results

3.1. Inhibition of $I_{(Cl^-,OR)}$ (see also Table 1)

In a few whole-cell recording experiments, transient openings of large conductance ion channels were observed (see Fig. 2). However, the contribution of this currents to the whole cell current was negligible since no shift of the reversal potential (≈ 0 mV) could be observed after inhibition of $I_{(Cl^-,OR)}$ (Table 1).

3.1.1. Trans-stilbene (Fig. 1A)

Trans-stilbene represents the basic stilbene motif present in all stilbene derivatives tested in the present study

Table 1
The table summarises the results obtained for different stilbene derivatives and hexestrol regarding their ability to inhibit outwardly rectifying chloride currents ($I_{(Cl^-,OR)}$) and agonist-induced exocytosis in rat peritoneal mast cells

Substance	Log *P*	$I_{(Cl^-,OR)}$		[^{3}H]5-HT secretion (IC_{50})	
		IC_{50} [μM]	Inhibition at 10 μM [% of control]	NGF/lyso-phosphatidyl-serine (μM)	Mastoparan (μM)
Clomiphene	6.7	5.1	85	>10	>10
Trans-diethylstilbene	6.7	4.1	82	>10	>10
Tamoxifen	6.3	3.5	89	>10	>10
Chlorotrianisene	6.2	>10	5	>10	>10
Diethylstilbestrol	5.6	1.6	98	>10	>10
Hexestrol	5.6	1.1	92	>10	>10
Trans-stilbene	4.5	>10	26	>10	>10
Trans-4-hydroxy-4′-methoxystilbene	4.1	>10	9	>10	>10
Trans-4-hydroxystilbene	4.0	3.2	79	>10	>10
DIDS	3.1	1.4	100	96	>100
Resveratrol	3.1	>10	2.3	>10	>10
SITS	0.9	9.2	58	72	>100

The compounds were ordered by their respective Log *P*-values.

(with the exception of *cis*-diethylstilbene). Superfusion of rat peritoneal mast cells with 10 μM of *trans*-stilbene reduced steady-state Cl^- currents elicited by a test potential of +80 mV by 25.9 ± 9.1 [(% of control); $n = 3$]. This small inhibitory effect could be fully reversed by superfusion with stilbene-free buffer solution.

Trans-stilbene derivatives substituted at their phenyl nucleus.

3.1.2. Trans-4-hydroxystilbene (Fig. 1B)

Trans-4-hydroxystilbene concentration-dependently inhibited $I_{(Cl^-,OR)}$ in rat peritoneal mast cells (IC_{50}: 3.2 μM). When applied at a concentration of 3 μM, the inhibitory effect of *trans*-4-hydroxystilbene was partly reversible ($n = 3$); at a concentration of 10 μM ($n = 9$) suppression of $I_{(Cl^-,OR)}$ could not be reversed, even by extensive superfusion with stilbene-free buffer solution.

3.1.3. Trans-4-hydroxy-4′-methoxystilbene (Fig. 1C)

In comparison with *trans*-4-hydroxystilbene, *trans*-4-hydroxy-4′-methoxystilbene is characterised by an additional methoxy substituent at position 4′ of the other phenyl ring systems. At a concentration of 10 μM, *trans*-4-hydroxy-4′-methoxystilbene induced a reduction of $I_{(Cl^-,OR)}$ elicited by standard test pulses (±80 mV) by about 8.7 ± 8.3 [(% of control); $n = 3$]. This effect was reversible.

3.1.4. Resveratrol (Fig. 1D)

Resveratrol represents a phytostilbene with two differently hydroxylated aromatic rings. At a concentration of 10 μM, resveratrol only slightly reduced $I_{(Cl^-,OR)}$ by about 2.3 ± 2.3% ($n = 3$). Superfusion of rat peritoneal mast cells with 100 μM of resveratrol caused a suppression of the $I_{(Cl^-,OR)}$ by about 46.6 ± 7.0 [(% of control); $n = 6$]. This inhibitory effect was completely reversible.

3.1.5. DIDS (Fig. 1E) and SITS (Fig. 1G)

The anions of the sulfonic acid derivatives DIDS and SITS were used. In contrast to all other stilbene derivatives tested the extent of the inhibitory effect of DIDS and SITS on $I_{(Cl^-,OR)}$ varied with the test pulse-duration, i.e., current inhibition was minimal at the beginning and increased up to a plateau during the course of a 100 ms-test pulse. With DIDS, a complete inhibition of $I_{(Cl^-,OR)}$ was achieved at 10 μM ($n = 15$; IC_{50} 1.4 μM). After short periods of incubation with DIDS, its inhibitory effect was partly reversible, whereas the inhibitory effect of SITS could be fully reversed even after minutes of incubation when the superfusion medium was changed to a SITS-free buffer solution. SITS was less potent in inhibiting $I_{(Cl^-,OR)}$ (IC_{50}: 9.2 μM; $n = 3$).

3.1.6. Trans-stilbene derivatives substituted at their vinyl group

3.1.6.1. Trans-diethylstilbene (Fig. 1H). Superfusion of rat peritoneal mast cells with 10 μM of *trans*-diethylstilbene effectively reduced steady state $I_{(Cl^-,OR)}$ elicited by test potentials of ±80 mV to 81.8 ± 4.3 [(% of control); IC_{50}: 4.1 μM; $n = 3$]. Upon the start of superfusion, *trans*-diethylstilbene-mediated inhibition of $I_{(Cl^-,OR)}$ occurred

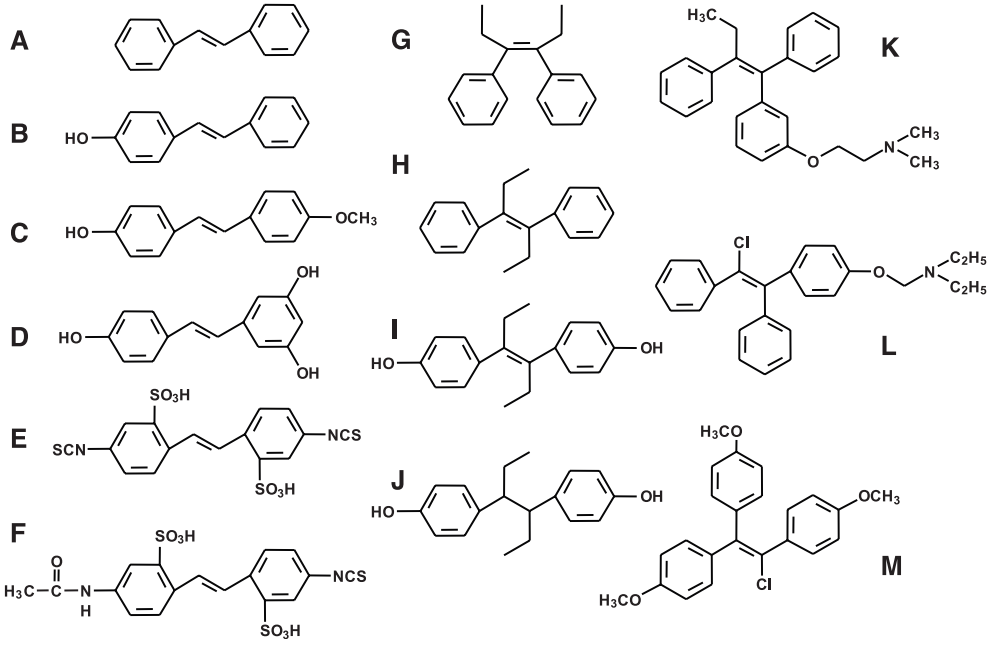

Fig. 1. Depiction of structural formulas of the test compounds used in the present study. Left column (*trans*-stilbene derivatives substituted at the aromatic ring system): (A) *trans*-stilbene, (B) *trans*-4-hydroxystilbene, (C) *trans*-4-hydroxy-4′-methoxystilbene, (D) resveratrol, (E) DIDS, (F) SITS; middle column (stilbene derivatives substituted at their ethene moiety): (G) *cis*-diethylstilbene, (H) *trans*-diethylstilbene, (I) diethylstilbestrol, (J) hexestrol; right column (triphenylethene derivatives): (K) clomiphene, (L) tamoxifen, (M) chlorotrianisene.

after a delay of 1–2 min. The suppression of $I_{(Cl^-,OR)}$ by *trans*-diethylstilbene could not be reversed by superfusion with stilbene-free buffer solution. *Cis*-diethylstilbene (Fig. 1G) at a concentration of 10 μM was completely ineffective (data not shown).

3.1.7. Diethylstilbestrol (Fig. 1I)

In comparison with *trans*-diethylstilbene, the estrogen receptor agonist diethylstilbestrol possesses two additional hydroxy groups at positions 4 and 4′ of both phenyl rings. Superfusion of mast cells with diethylstilbestrol led to a rapid and potent suppression of $I_{(Cl^-,OR)}$. At a concentration of 10 μM, a complete inhibition of $I_{(Cl^-,OR)}$ could be observed (IC_{50}: 1.6 μM; $n=3$). Suppression of $I_{(Cl^-,OR)}$ achieved by diethylstilbestrol at a concentration of 2 μM was completely reversible.

3.1.8. Hexestrol (Fig. 1J)

The only structural difference between hexestrol and diethylstilbestrol is the ethane instead of the ethene bridge between the phenyl nuclei. Thus, hexestrol does not belong to the stilbenes.

Superfusion of rat peritoneal mast cells with hexestrol led to rapid, potent, and partially reversible inhibition of $I_{(Cl^-,OR)}$ (IC_{50}: 1.1 μM; $n=3$; Fig. 2).

3.1.9. Triphenylethene derivatives

3.1.9.1. Tamoxifen (Fig. 1K). Like *trans*-diethylstilbene, the triphenylethene derivative tamoxifen can be regarded as a stilbene substituted at the ethene bridge (see Fig. 1K). Tamoxifen concentration-dependently inhibited the $I_{(Cl^-,OR)}$ in rat peritoneal mast cells (IC_{50}: 3.5 μM; $n=3$). At a concentration of 10 μM, tamoxifen effectively inhibited $I_{(Cl^-,OR)}$ by about 89 ± 6.2 [(% of control); $n=3$]. Inhibition of $I_{(Cl^-,OR)}$ was only partially reversible. The onset of current inhibition after start of the tamoxifen superfusion occurred with a delay of about 1–2 min.

3.1.10. Clomiphene (Fig. 1L)

Despite being a stilbene substituted at the ethene bridge, clomiphene also exhibits a modification at one of its two phenyl rings. Similarly to tamoxifen, upon superfusion, the onset of the inhibitory effect of clomiphene on $I_{(Cl^-,OR)}$

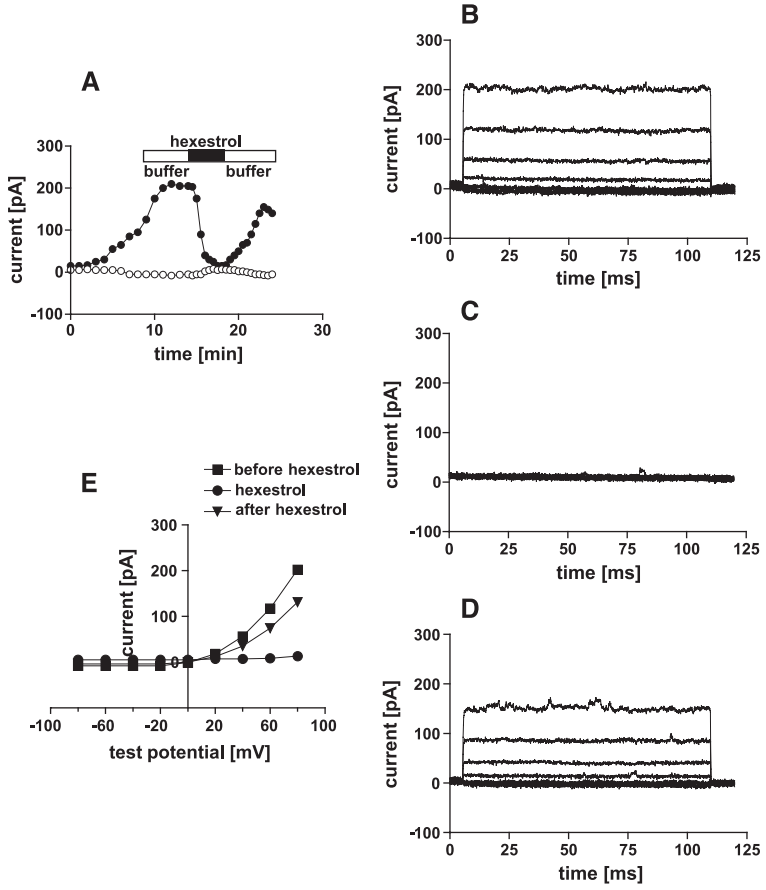

Fig. 2. Representative whole-cell, patch-clamp experiment (nystatin perforated patch), showing the time-dependent development of an outwardly rectifying current in rat peritoneal mast cell challenged by step hyper- and depolarisations and its partly reversible block by 3 μM hexestrol. (A) Time course (holding potential 0 mV); test potentials (100 ms) +80 mV (solid circles), −80 mV (open circles). (B–D) Original current traces from step protocol (−80 ±80 mV, in 20 mV increments), (B) before addition of 3 μM hexestrol; (C) at the start, and (D) end of hexestrol wash-out; the corresponding current–voltage relationships are represented in (E).

occurred with a delay of about 1–2 min. Clomiphene concentration-dependently inhibited $I_{(Cl^-,OR)}$ (IC_{50}: 5.1 μM; $n=3$); at a concentration of 10 μM, the inhibitory effect amounted to 85.2 ± 8.9 [% of control].

3.1.11. Chlorotrianisene (Fig. 1M)

In comparison with tamoxifen and clomiphene, the more hydrophilic triphenylethene derivative chlorotrianisene, at the maximal test concentration of 10 μM ($n=3$), only minimally inhibited $I_{(Cl^-,OR)}$ by about 4.6 ± 12.6 [(% of control), $n=3$].

3.1.12. β-Estradiol (Fig. 1N)

At a concentration of 10 μM ($n=3$), the steroid estrogen receptor agonist 17β-estradiol inhibited $I_{(Cl^-,OR)}$ by about 21.8 ± 8.3 [(% of control), $n=3$].

3.2. Secretion studies

3.2.1. Spontaneous [^{3}H]5-HT release

Incubation with either 10 μM tamoxifen or clomiphene increased the spontaneous release of [^{3}H]5-HT from [^{3}H]5-HT-loaded rat peritoneal mast cells in a time-dependent manner; after a maximum incubation time of 60 min, the respective values amounted to: tamoxifen/control 23.4 ± 8.0/12.7 ± 8.1 ($n=3$), clomiphene/control 17.0 ± 3.8/3.2 ± 1.0 ($n=3$) ([^{3}H]5-HT release [% of total]). The other stilbene derivatives studied in the present investigation did not increase the spontaneous release of [^{3}H]5-HT, when present at a concentration of 10 μM for up to 60 min (data not shown).

3.3. Agonist-induced [^{3}H]5-HT-release (Table 1)

The ability of different stilbene derivatives to impair the NGF/lysophosphatidylserine- or mastoparan-induced exocytotic release of [^{3}H]5-HT from [^{3}H]5-HT-loaded rat peritoneal mast cells was tested in a series of time course experiments (incubation time 1–60 min). Mastoparan was always used at a final concentration of 2×10^{-5} M; the final concentrations for NGF and lysophosphatidylserine amounted to 1 μg/ml and 1 μM, respectively. At a test concentration of 10 μM, the following compounds did not inhibit the agonist-induced [^{3}H]5-HT release: *trans*-stilbene, *trans*-diethylstilbene, *trans*-4-hydroxystilbene, *trans*-4-hydroxy-4′-methoxystilbene, and chlorotrianisene. After incubation of cells for 5 min, which is a maximal-effective time-period, diethylstilbestrol reduced the NGF/lysophosphatidylserine-, but not the mastoparan-induced [^{3}H]5-HT release by 29.2 ± 5.3 [(% of control); $n=3$]. Agonist-induced exocytotic responses were not impaired by incubation of cells with 10 μM DIDS or SITS for up to 30 min. After an incubation period of 60 min, a reduction of the NGF/lysophosphatidylserine-induced release of [^{3}H]5-HT by DIDS (43.8 ± 9.3) and SITS [31.9 ± 7.7 (% of control); $n=3$] became apparent. By incubating rat peritoneal mast cells for 60 min with 1–300 μM DIDS (IC_{50}: 96 μM) or SITS (IC_{50}: 72 μM), a complete inhibition of the NGF/lysophosphatidylserine-induced exocytotic response could be achieved ($n=3$). Under identical experimental conditions, DIDS and SITS also reduced the exocytotic response induced by mastoparan, but with a ≈ 10 fold lower potency (data not shown).

Tamoxifen and clomiphene (10 μM) partially reduced the NGF/lysophosphatidylserine induced exocytotic response in rat peritoneal mast cells in a time-dependent manner. After a maximal incubation time of 60 min, exocytosis was reduced by about 55.5 ± 1.6 and 17.1 ± 3.8 ([^{3}H]5-HT release [% of control]; $n=3$), respectively.

4. Discussion

The stilbene derivative diethylstilbestrol has previously been shown to act as a potent inhibitor of the outwardly rectifying chloride current ($I_{(Cl^-,OR)}$) in rat peritoneal mast cells (Roloff et al., 2001). To characterise structural properties enabling diethylstilbestrol to inhibit $I_{(Cl^-,OR)}$, different diethylstilbestrol-related stilbene and non-stilbene derivatives were tested in the present study in rat peritoneal mast cells. In comparison with diethylstilbestrol, the unsubstituted stilbene, *trans*-stilbene, did not inhibit chloride currents in concentrations up to 10 μM. Thus, the structural moiety discerning diethylstilbestrol from *trans*-stilbene must be of importance. Diethylstilbestrol is substituted at the aromatic phenyl rings by two hydroxy groups in positions 4 and 4′, and, at the aliphatic ethene bridge, by two ethyl groups. Accordingly, in order to analyse the structure–activity relationships of diethylstilbestrol different stilbene derivatives, either modified at their aromatic and/or their aliphatic part, were tested in the present study. In addition, the non-stilbene molecule hexestrol was assessed.

Hexestrol differs from diethylstilbestrol only by lacking the double-bond within its aliphatic part. Interestingly, hexestrol inhibited $I_{(Cl^-,OR)}$ as potent as diethylstilbestrol (IC_{50}-value 1.1 vs. 1.6 μM). Thus, the conjugated double bond within the aliphatic part of the *trans*-stilbene scaffold of the diethylstilbestrol molecule probably is not essential for inhibition of $I_{(Cl^-,OR)}$.

Diethylstilbene differs from diethylstilbestrol by the absence of the hydroxy groups at both phenyl rings. *Cis*-diethylstilbene at a concentration of 10 μM showed no effect on $I_{(Cl^-,OR)}$ (data not shown). In contrast, *trans*-diethylstilbene inhibited the $I_{(Cl^-,OR)}$ nearly as potent as diethylstilbestrol (IC_{50}: 4.1 vs. 1.6 μM). Consequently, the presence of this special phenolic moiety seems not to be essential for diethylstilbestrol mediated inhibition of $I_{(Cl^-,OR)}$. In accordance with these findings, *trans*-4-hydroxystilbene, characterized by only a single hydroxy group at position 4 potently inhibited the chloride current (IC_{50}: 3.3 μM).

Stilbene derivatives modified at positions 4 and/or 4′ with methoxy substituents, as in the case of *trans*-4-hydroxy-4′-methoxystilbene or chlorotrianisene, did not inhibit $I_{(Cl^-,OR)}$. This finding indicates that methoxy-substituents, in contrast to hydroxy groups, might sterically prevent an interaction of these molecules with a specific binding site at the ORCC-protein. But also other mechanisms could underlie this failure of action (see below).

Resveratrol differs from *trans*-4-hydroxystilbene by two hydroxyl groups localised at positions 3′ and 5′ of the phenyl ring and by the absence of an OH-group at position 4′. In comparison with *trans*-4-hydroxystilbene, resveratrol is more hydrophilic (Log *P* 3.08 vs. 4.04). In concentrations up to 10 μM, resveratrol exerted no inhibitory effect on $I_{(Cl^-,OR)}$. One possible explanation for this observation may be that the hydroxy groups at positions 3′ and 5′ of the phenyl ring for steric reasons do not allow an interaction of resveratrol with a specific binding site at the ORCC-protein. Alternatively, the hydrophilicity of resveratrol may cause insufficient insertion into the mast cell plasma membrane, thus preventing the molecule from reaching its target site at the Cl^- channel. The finding that DIDS and SITS, two other hydrophilic stilbenes, potently inhibit $I_{(Cl^-,OR)}$ is not an argument against this hypothesis since DIDS and SITS, as shown before in previous studies, inhibit $I_{(Cl^-,OR)}$ by plugging the pore of the outwardly rectifying Cl^- channel (Dietrich and Lindau, 1994; Lewis et al., 1993). This mechanism of action is due to the fact that DIDS and SITS, like Cl^-, are anions and capable to enter the pore of the ion channel. Moreover, DIDS and SITS have been shown to inhibit $I_{(Cl^-,OR)}$ via allosteric mechanisms. Due to this complexity of action, data obtained with DIDS and SITS are not suitable for interpreting the structure–activity relationships of diethylstilbestrol.

A modification of the aliphatic part of *trans*-stilbene with large aromatic substituents, as in the case of the triphenyl derivatives tamoxifen and clomiphene, does not impair the ability of these molecules to inhibit $I_{(Cl^-,OR)}$. The putative reason for the ineffectiveness of chlorotrianisene, another triphenyl derivative, has been described above.

In the present study, with the exception of DIDS and SITS, none of the compounds capable to inhibit the $I_{(Cl^-,OR)}$ impaired the process of agonist-induced exocytosis. Exocytosis involves a highly regulated fusion of secretory granules with the plasma membrane (Pinxteren et al., 2000). Assumingly, compounds influencing the biophysical properties of plasma membranes (e.g. membrane fluidity, etc.) would also alter the process of agonist-induced exocytosis. According to this assumption, the inability of the stilbene derivatives to impair exocytosis argues against an unspecific alteration of membrane properties as a putative mechanism for inhibition of chloride currents. Complementary, this result favours a more specific mechanism of action, which probably may involve the interaction with a specific binding site at the ORCC-protein.

The data received for inhibition of $I_{(Cl^-,OR)}$ with different estrogen receptor agonists (e.g. 17β-estradiol, diethylstilbestrol) or antagonists (e.g. tamoxifen) did not correlate with their established ability to affect estrogen receptors (Riggs and Hartmann, 2003). Therefore, an involvement of estrogen receptors appears unlikely.

The profile of stilbene derivatives capable to inhibit $I_{(Cl^-,OR)}$ corresponds with previous structure–activity data regarding diethylstilbestrol-induced inhibition of certain ATPases. Future studies will reveal, if structural homologies between these ATPases and the mast cell ORCC-protein may be the reason for this analogy (Martinez-Azorin et al., 1992).

In summary, the aim of the present study was to assess the importance of different structural features of the diethylstilbestrol molecule for inhibition of an outwardly rectifying chloride current $I_{(Cl^-,OR)}$ in rat peritoneal mast cells. Diethylstilbestrol represents a modified stilbene derivative. Because *trans*-stilbene was ineffective to inhibit $I_{(Cl^-,OR)}$, the chemical substituents discerning diethylstilbestrol from the simple *trans*-stilbene must be of importance. An analysis of action of different test compounds revealed that a hydroxy group at position 4 or 4′ of the phenyl rings of diethylstilbestrol represents a prerequisite for inhibition of $I_{(Cl^-,OR)}$. In contrast, *trans*-stilbene molecules modified at position 4 or 4′ by methoxy substituents were incapable to inhibit $I_{(Cl^-,OR)}$. Additionally, the absence of the double bond within the aliphatic part of the stilbene structure as well as a modification of the aliphatic part by certain substituents did not impair the ability of molecules to inhibit $I_{(Cl^-,OR)}$ in rat peritoneal mast cells. After identification of the gene coding for the rat peritoneal mast cell outwardly rectifying Cl^- channel protein, the structure–activity data generated in the present study are hoped to facilitate the development of optimised outwardly rectifying Cl^- channel-inhibitors.

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