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Structure–activity relationships of astemizole derivatives for inhibition of store operated Ca^{2+} channels and exocytosis

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Abstract

The effects of a series of analogues of the antiallergic drug astemizole on the exocytosis of the enzyme β-hexosaminidase were studied in a mast cell model, the rat basophilic leukemia (RBL-2H3) cell. Besides differences in the effects on FcεRI receptor-stimulated exocytosis, changes were also observed in Ca^{2+} influx and in the perturbation of the cell membrane. A strong correlation was found between the effects on antigen- and thapsigargin-stimulated $^{45}Ca^{2+}$ influx. Furthermore, the inhibition of $^{45}Ca^{2+}$ influx was correlated with the inhibition of β-hexosaminidase release and membrane stabilization. It is concluded that the astemizole analogues are capable of inhibiting mast cell β-hexosaminidase release through inhibition of Ca^{2+}-store-operated Ca^{2+} channels (SOC). Compounds with high lipophilicity also released Ca^{2+} from intracellular stores. Lowering of the hydrophobicity by introduction of nitrogens or truncation at different sites in the astemizole structure decreased inhibitory activity on SOC channels. The inhibition of SOC channels cannot completely be ascribed to non-specific membrane effects. The piperidinyl–benzimidazole moiety was found to be important for inhibition of SOC channels. The observed differences in activity possibly depend on the way the compounds penetrate the membrane bilayer. Astemizole is an interesting new tool to study SOC channels and can be a lead for the design of mast cell-stabilizing antiallergic drugs.

Keywords: Astemizole; RBL-2H3; Ca^{2+} channel; Store-operated; Antiallergic; Exocytosis

1. Introduction

After the discovery of the antihistamine activity of compounds that possessed a *N*-(4-piperidinyl)-1*H*-benzimidazol-2-amine moiety (central part of structure 1, Table 1), a series of related structures were synthesized and investigated (Janssens et al., 1985a,b,c). From these studies, astemizole (compound 2, Table 1) emerged as an excellent inhibitor of immediate type hypersensitivity reactions (Tasaka et al., 1990). The compound does not affect the central nervous system even at high doses (Awouters et al., 1983) and acts on histamine H_1 receptors (Van Wauwe et al., 1981). More detailed pharmacological studies have shown that astemizole is quickly transformed in substantial amounts to active antiallergic metabolites like desmethylastemizole, 5- and 6-hydroxyastemizole and norastemizole (compound 16) (Kamei et al., 1991). These compounds have similar activity on histamine H_1 receptors as astemizole (Richards et al., 1984). Although histamine H_1 receptor antagonism has been suggested to be the main mechanism of action of these compounds in allergic reactions, the overall effects of astemizole or comparable histamine H_1-antagonists on cellular processes cannot fully explain the therapeutic effect (Estelle and Simons, 1992). Astemizole also inhibits allergy by reducing the release of mediators in allergic reactions, such as histamine, leukotrienes and prostaglandins, from mast cells (Tasaka et al., 1990). We have suggested that this non-histamine H_1 receptor dependent effect on allergic reactions is due to membrane distortion (Fischer et al., 1995). In previous studies, we have investigated astemizole (compound 2) and norastemizole (compound 16) for their effect on the rat basophilic leukemia (RBL-2H3) cell as a model for the mast cell (Fischer et al., 1997, 1998). There, it was shown that astemizole acts in a dual manner on β-hexosaminidase

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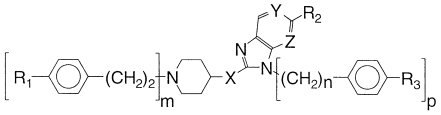
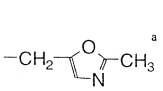
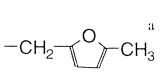
release by influencing Ca^{2+} fluxes in the cell, on the one hand by stimulating Ca^{2+} release from intracellular Ca^{2+} stores, and on the other by inhibiting Ca^{2+} influx. Ca^{2+} influx into mast cells is mediated by a Ca^{2+} channel that is activated upon emptying of intracellular Ca^{2+} stores, the so called store-operated Ca^{2+} channels (SOC channels) (Berridge, 1995; Felder et al., 1994; Hoth, 1995). The concomitant current of Ca^{2+} ions through this channel is activated by stimulation of FcϵRI receptors (Zhang and McCloskey, 1995). Astemizole induces emptying of Ca^{2+} stores in resting mast cells, leading to a rise in $[Ca^{2+}]_i$. In itself this stimulates the influx of extracellular Ca^{2+} after opening of SOC channels; however, the situation is more complicated because astemizole also inhibits this Ca^{2+} influx (Fischer et al., 1998). SOC channels can also be activated by means of thapsigargin, which inhibits the Ca^{2+}-ATPase that pumps Ca^{2+} back into the stores (Thastrup et al., 1990). In contrast to astemizole, norastem-

Table 1
Structures of *N*-heterocyclic 4-piperidinamine compounds



compd	R_1	R_2	R_3	X	Y	Z	n	m	p
2	CH_3O	H	F	NH	CH	CH	1	1	1
3	CH_3O	H	F	NH	CF	CH	1	1	1
4	CH_3O	H	F	NH	CH	N	1	1	1
5	CH_3O	H	F	NH	N	N	1	1	1
6	CH_3O	H	F	O	CH	CH	1	1	1
7	CH_3O	H	F	S	CH	CH	1	1	1
8	CH_3O	H	F	CH_2	N	CH	1	1	1
9	CH_3O	H	H	NH	CH	N	1	1	1
10	CH_3O	H	H	NH	CH	CH	2	1	1
11	CH_3O	F	F	NH	CH	CH	1	1	1
12	CH_3O	CH_3O	F	NH	CH	CH	1	1	1
13	CH_3O	H	-	NH	CH	CH	-	1	H[a]
14	NH_2	H	F	NH	CH	CH	1	1	1
15	NO_2	H	F	NH	CH	CH	1	1	1
16	-	H	F	NH	CH	CH	1	H[a]	1
17	-	H	-	NH	CH	CH	-	CH_3[a]	
18	-	H	-	NH	CH	N	-	H[a]	

[a] Replaces functional group as indicated.

izole does not stimulate Ca^{2+} release from stores and only inhibits SOC channels, which causes inhibition of exocytosis.

The mechanism by which these compounds inhibit SOC channels is unclear. A recent study with some Ca^{2+} entry blockers (econazole, SKF 96365) suggested that the effect on Ca^{2+} influx in mast cells is related to effects on the membrane structure (Franzius et al., 1994). To evaluate the mechanism of astemizole-and norastemizole-induced inhibition of β-hexosaminidase release in more detail, a series of structurally related compounds was investigated. The structures differ in hydrophobic regions, and some side chains have been truncated (Table 1). RBL-2H3 cells were used as an in vitro mast cell model to study β-hexosaminidase release and Ca^{2+} fluxes in the presence of these compounds. As a parameter for membrane distortion induced by the drugs, we assayed membrane stabilization in erythrocytes (Lau and Pearce, 1990).

2. Materials and methods

2.1. Reagents

Astemizole, norastemizole and their analogues were gifts from Janssen Pharmaceutica Research Laboratories (Beerse, Belgium). Their synthesis has been described earlier (Janssens et al., 1985a,b,c). The purity of all compounds, as checked with high-performance liquid chromatography, was $>96\%$. Stock solutions of compounds in dimethylsulfoxide (10 mM) were used within one month and were stored at $-20°C$ in the dark. No detectable loss in activity was noted within that time period. Thapsigargin was obtained from Calbiochem. Fura-2-acetoxymethylester (Fura-2-AM), anti-dinitrophenyl immunoglobulin E (IgE) and human dinitrophenyl albumin were from Sigma. $^{45}CaCl_2$ was purchased from Amersham. Bovine blood was obtained from Biotrading (Mijdrecht, The Netherlands). Culture media were from Gibco.

Fluorescence was measured with a Perkin-Elmer LS50B Fluorometer. Intracellular free Ca^{2+} measurements were performed with a dual wavelength filter-fluorometer from Photon Technology International.

2.2. Cell culture conditions and FcεRI-receptor stimulation

RBL-2H3 cells were cultured in flasks in Eagle's minimal essential medium in a water saturated atmosphere of 95% air and 5% CO_2. The medium was supplemented with 15% heat-inactivated fetal calf serum, glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and tylosin (60 μg/ml). Cells were detached with trypsin (0.05%) and EDTA (0.02%) for 10 min. After the cells were washed, they were resuspended in medium and cultured overnight in 24- and 96-well plates at densities of 4×10^5 and 1×10^4 cells/well, respectively. The FcεRI-receptor was sensitized for 1 h with IgE-specific for dinitrophenyl at the indicated concentration. The medium was replaced by a Tyrode's salt buffer (137 mM NaCl, 2.7 mM KCl, 0.31 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, 0.1% bovine serum albumin, pH 7.4). After a 10-min incubation, compounds were added at the indicated concentrations and further incubated for 10 min. The cells were either stimulated with an antigen-specific for dinitrophenyl (dinitrophenyl coupled to human serum albumin) or with thapsigargin, at concentrations as stated in the appropriate sections.

2.3. Assay of β-hexosaminidase after activation of exocytosis

The assay was performed as described previously (Fischer et al., 1995). In short, after 30 min of stimulation with antigen the exocytosis reaction was stopped by putting the 96-well plates on ice. Samples were collected before and after lysis of the cells with 1% Triton-X-100. The amount of enzyme present in these samples was detected in a fluorescence assay that used 4-methyl umbelliferyl-*N*-acetyl-β-D-glucosaminide (160 μM) as a substrate in citrate buffer (0.1 M, pH 4.5). After 1 h at 37°C, ice-cold glycine buffer was added (0.2 M, pH 10.7). Fluorescence of methylumbelliferone was determined against a standard at an excitation wavelength of 360 nm and an emission wavelength of 452 nm. Release of β-hexosaminidase in the absence of compounds was calculated as a percentage of the total enzyme present in the cell. The data are relative to the effect in antigen- or thapsigargin-activated cells without drug. Corrections were made for the leakage of enzyme ($\pm 4\%$ of total), which was measured in the absence of stimulation and drugs.

2.4. Determination of $^{45}Ca^{2+}$ influx

Overnight cultures in 24-well plates were sensitized with IgE (1 μg/ml) as indicated above. After the medium was changed to Tyrode's buffer containing test compound, the cells were activated with antigen or thapsigargin in the presence of $^{45}CaCl_2$ (30 or 10 μCi/ml, respectively). Stimulation was continued for 5 or 10 min for antigen- or thapsigargin-induced activation, respectively. The cells were washed thoroughly with ice-cold Tyrode's buffer and subsequently lysed with 1% Triton-X-100. Radioactivity was assayed in the lysates by liquid scintillation counting. The total absolute $^{45}Ca^{2+}$ influx found for stimulation with either thapsigargin or antigen was approximately 3000 dpm. The influx of $^{45}Ca^{2+}$ was expressed as a percentage relative to a control (no compound present) stimulated with either antigen or thapsigargin. Corrections were made for $^{45}Ca^{2+}$ binding to cells or leakage of isotope into the cells (± 5–10% of total), by substracting values for a blank without stimulation.

2.5. Intracellular free Ca^{2+} measurements

Changes in $[Ca^{2+}]_i$ were recorded by using the fluorescence indicator Fura-2. For this cells in suspension (2×10^6 cells/ml) were sensitized with dinitrophenyl-specific IgE (1 μg/ml), washed with Tyrode's buffer containing sulfinpyrazone (0.1 mg/ml) and loaded with Fura-2-acetoxymethylester. The loading with 10 μM Fura-2-AM was accomplished at a cell density of 2×10^7 cells/ml in 15 min at 37°C. Extracellular Fura-2 was removed by washing with Tyrode's buffer without bovine serum albumin and supplemented with sulfinpyrazone and gelatin (0.05%). Aliquots of Fura-2-loaded cells were diluted to 5×10^5 cells/ml and fluorescence was monitored at 37°C at excitation wavelengths of 340 and 380 nm and emission at 510 nm with continued stirring. After the indicated time intervals, the compounds and antigen trigger were added. The $[Ca^{2+}]_i$ was calculated from the 340/380 fluorescence ratio after each experiment by calibration with ionomycin (3 μM) and EGTA (20 mM) according to Grynkiewicz et al. (1985).

2.6. Hypotonic haemolysis of erythrocytes

The assay was based on the method described by Lau and Pearce (1990). Bovine blood in Alsever solution was washed twice in a phosphate buffer (10 mM NaH_2PO_4 and 154 mM NaCl, pH 7.4) and the erythrocytes were resuspended at a stock density of 1.2×10^9 cells/ml. To induce hypotonic haemolysis, 0.1 ml stock solution was diluted with 1.4 ml of hypotonic phosphate-buffered saline containing 68 mM NaCl, whether or not supplemented with compound. The resulting NaCl concentration of 68 mM produced 50% haemolysis. The final concentration of the compounds was 20 mM. After incubation for 5 min the cells were centrifuged (2 min, $1500 \times g$) and absorbance of the haemoglobin-containing supernatant was monitored at 543 nm ($A_{control}$ and A_{compd}, respectively). Spontaneous leakage of haemoglobin was measured in the presence of buffer with 154 mM NaCl (A_{154}) and represented $\pm 5\%$ of the total haemoglobin present. The inhibition of lysis in the presence of a compound was calculated by using the following formula:

$$\%\ \text{inhibition of haemolysis} = 100 - \left(\frac{A_{compd} - A_{154}}{A_{control} - A_{154}} \times 100 \right)$$

2.7. Determination of partition coefficient and ionization constant

The logarithm of the partition coefficient ($\log P_{oct}$) of several compounds was determined by using the double extraction method between an aqueous and octanol phase. The octanol phases were analysed by ultraviolet spectroscopy.

The ionization constants (pK_a) were determined by potentiometric titration with 0.1 M NaOH of either an aqueous solution (low pK_a) or solutions in water–methanol mixtures (high pK_a). The latter pK_a is a result of a linear extrapolation of the results for a 50, 60 and 70% methanol mixture in water.

2.8. Statistics

Results are expressed as means $\pm$ standard deviation (S.D.) of triplicate determinations in each of two or more independent experiments. Data were linearly correlated and investigated by using NCSS software (Number Cruncher Statistical Systems, Version 5.01 Hintze, 1987). Results were considered significant at $P < 0.05$.

3. Results

3.1. Effect on β-hexosaminidase release

In order to study the effect of the compounds on exocytosis, the release of β-hexosaminidase from RBL-2H3 cells was assayed after stimulation of the FcϵRI-receptor. The effects of 30 μM of test compound were determined from concentration–effect curves and are summarized in Table 2. At 30 μM, the effect of the compounds varied from 10 to 85% inhibition of β-hexosa-

Table 2
Relative effect of *N*-heterocyclic 4-piperidinamine compounds at a concentration of 30 μM on β-hexosaminidase release triggered with antigen specific for dinitrophenyl (5 ng/ml) and the effect on $^{45}Ca^{2+}$ influx in RBL-2H3 cells after stimulation with antigen-specific for dinitrophenyl (40 ng/ml) or thapsigargin (0.2 μM)

Compd	β-Hexosaminidase release	$^{45}Ca^{2+}$ influx		Inhibition of haemolysis
	Antigen	Antigen	Thapsigargin	
2	18.5 ± 4.6	26.5 ± 4.1	11.8 ± 1.9	83.4 ± 2.1
3	41.8 ± 8.3	25.9 ± 2.5	5.1 ± 0.6	82.7 ± 2.1
4	39.6 ± 6.5	53.2 ± 7.6	54.0 ± 5.1	68.1 ± 2.4
5	53.3 ± 9.9	83.7 ± 7.7	77.3 ± 1.9	40.0 ± 4.3
6	40.8 ± 10.7	5.0 ± 1.2	−3.1 ± 2.0	76.5 ± 1.3
7	28.0 ± 7.8	18.0 ± 1.7	−0.3 ± 1.7	67.6 ± 0.2
8	33.0 ± 4.6	53.9 ± 5.6	53.4 ± 3.5	27.8 ± 9.1
9	65.9 ± 12.3	89.3 ± 6.8	59.0 ± 1.5	37.8 ± 4.3
10	13.5 ± 6.7	25.7 ± 0.6	8.6 ± 0.7	67.7 ± 2.9
11	39.1 ± 8.3	19.8 ± 3.5	5.8 ± 1.6	80.7 ± 2.8
12	37.8 ± 8.7	23.9 ± 0.9	22.9 ± 3.5	81.5 ± 2.0
13	52.3 ± 8.4	54.8 ± 4.9	21.4 ± 2.1	15.0 ± 6.1
14	21.4 ± 3.1	40.4 ± 2.7	39.1 ± 2.3	56.8 ± 1.2
15	50.2 ± 5.3	45.0 ± 6.5	37.8 ± 2.7	73.3 ± 6.4
16	46.2 ± 7.3	48.7 ± 9.2	49.8 ± 3.3	13.6 ± 3.2
17	87.4 ± 6.5	67.3 ± 8.2	86.8 ± 1.8	14.8 ± 3.0
18	90.5 ± 7.1	75.3 ± 4.4	66.7 ± 2.0	7.4 ± 4.5

Values are expressed as percentages, taking the response without drugs as 100, and are corrected for cell leakage.
Additionally, the compounds at a concentration of 20 μM were examined for their ability to inhibit hypotonic haemolysis of erythrocytes.
Data are relative to a control value of 50% haemolysis.
Values are means ± S.D. from at least four independent experiments.

minidase release. The effect of these compounds on β-hexosaminidase release elicited with other triggers was also investigated. With the calcium ionophore A23187, or with the synergistic combination of a phorbol ester and A23187 (Paulussen et al., 1996), these compounds were less effective in inhibiting enzyme release (data not shown). As these triggers activate post-Ca^{2+} processes in signal transduction, these findings suggest that the compounds act on steps prior to the elevation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$).

3.2. Effect on influx of $^{45}Ca^{2+}$

Recently, we found, for astemizole and norastemizole, that inhibition of Ca^{2+} fluxes is the primary event causing inhibition of mediator release. Therefore, the compounds from Table 1 were investigated for their effect on Ca^{2+} fluxes. The results for the compounds on $^{45}Ca^{2+}$ influx upon FcεRI stimulation are summarized in Table 2.

For astemizole (compound 2) and norastemizole (compound 16), it was demonstrated that Ca^{2+} influx proceeded via SOC channels, occurring after the depletion of intracellular Ca^{2+} pools (Fischer et al., 1997). Emptying of these Ca^{2+} stores is a trigger for the opening of the SOC channels (Berridge, 1995). This can be achieved by using thapsigargin, which is a sesquiterpene lactone that specifically inhibits the Ca^{2+}-ATPase in the endoplasmatic reticulum (Thastrup et al., 1990). This inhibition blocks regular Ca^{2+} reuptake into the stores, evoking large increases in $[Ca^{2+}]_i$ as a result of leakage of Ca^{2+} out of the stores. SOC channels are then activated because of the continued depletion of the Ca^{2+} stores, thus by passing direct FcεRI-related effects. From our previous investigations with astemizole and norastemizole, it was concluded that the efflux of intracellular Ca^{2+} into either stores or the extracellular space was not influenced by these compounds (Fischer et al., 1997, 1998). The effects of the series of

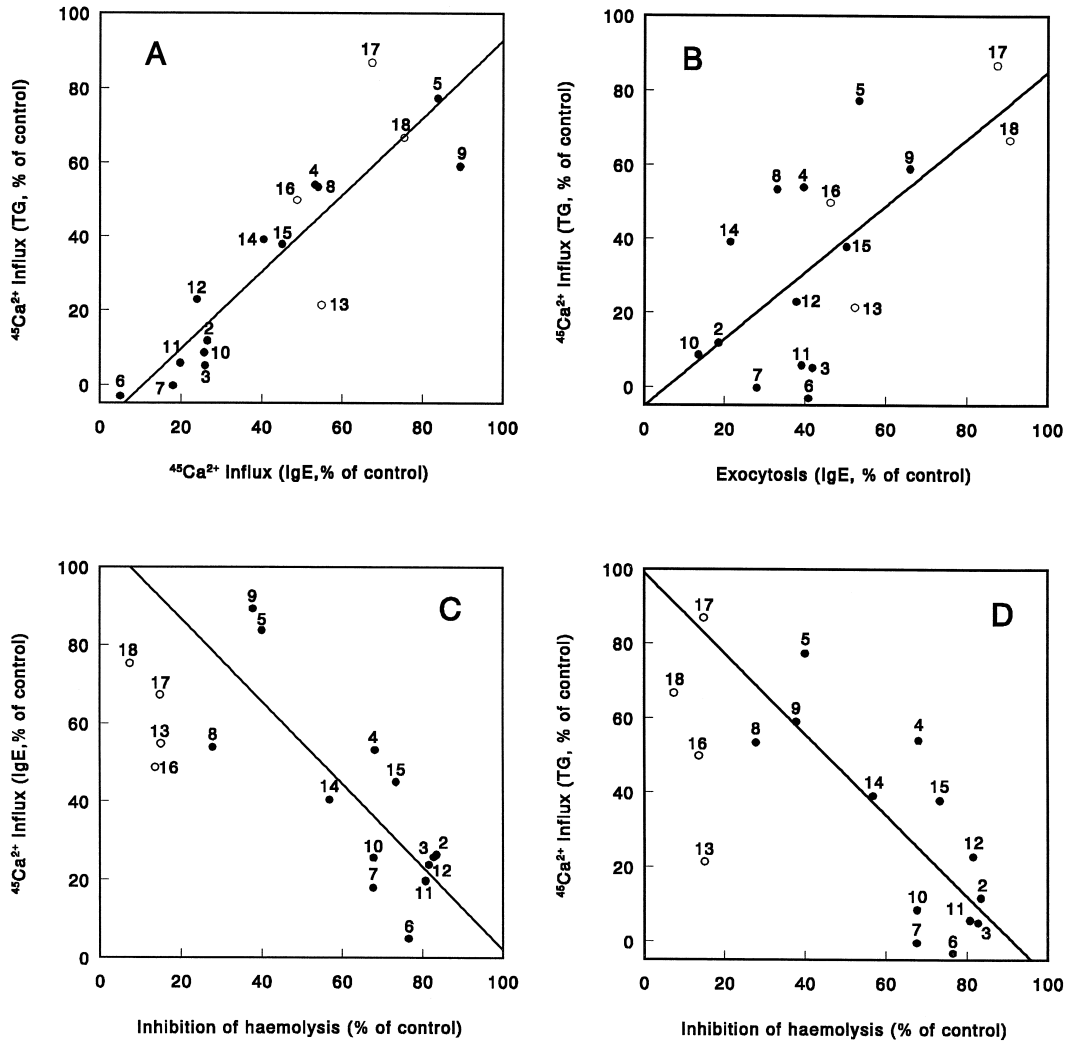


Fig. 1. Relation between several activity parameters of a series of astemizole derivatives: (A) $^{45}Ca^{2+}$ influx in RBL-2H3 cells after antigen- or thapsigargin stimulation; (B) antigen-induced β-hexosaminidase release vs. $^{45}Ca^{2+}$ influx after thapsigargin stimulation; (C) inhibition of haemolysis vs. antigen-induced $^{45}Ca^{2+}$ influx; and (D) inhibition of haemolysis vs. thapsigargin-induced $^{45}Ca^{2+}$ influx. Open symbols (○) are for truncated compounds. Lines indicate the calculated regression as mentioned in the text. Data are from Table 2.

compounds on $^{45}Ca^{2+}$ influx after thapsigargin stimulation are shown in Table 2.

The effects of the compounds on the two methods of activation of Ca^{2+} influx were strongly correlated (see Fig. 1A, $r^2 = 0.80$, $F = 60.1$, $P < 0.0001$, $n = 17$). These results confirm our previous conclusion that astemizole and norastemizole do not affect processes at the FcϵRI level, but influence processes occurring later in the signal transduction cascade, after Ca^{2+} stores have been emptied (Fischer et al., 1997). These conclusions can now be extended to a wider range of astemizole analogues.

In order to compare inhibition of $^{45}Ca^{2+}$ influx with inhibition of antigen-stimulated β-hexosaminidase release, we determined whether the effects on thapsigargin-induced $^{45}Ca^{2+}$ influx and β-hexosaminidase release due to activation of FcϵRI were correlated (Fig. 1B). The regression analysis showed a fair-to-positive correlation, taking into account that Ca^{2+} influx is only one step in the complicated signal transduction processes leading to exocytosis ($r^2 = 0.46$, $F = 12.5$, $P = 0.003$, $n = 17$). This correlation demonstrates that the inhibition of SOC channels is a major mechanism by which the astemizole derivatives inhibit mast cell β-hexosaminidase release, but that the contribution of other processes cannot be excluded.

The effect of the astemizole derivatives on $^{45}Ca^{2+}$ influx depended on their chemical structure. The truncated compounds 13, 16, 17, and 18 (indicated with an open circle in Fig. 1) did not behave like outliers because their effects were also correlated (Fig. 1B). Replacement of the conjugated aliphatic nitrogen in astemizole by an oxygen or sulphur in the compounds 6 and 7, respectively, created more flexibility in the molecule and changed the planar conformation around the N–C2(benzimidazole) bond (structure 1). This change in conformation did not seem to be of importance for inhibition of thapsigargin-stimulated $^{45}Ca^{2+}$ influx as compounds 6 and 7 had activities comparable to that of astemizole (Table 2). Compounds 6 and 7 fully inhibited Ca^{2+} influx at 30 μM. Concentration–effect curves were made for thapsigargin-induced Ca^{2+} influx for compounds 6 and 7. The IC_{50}s were 5.27 ± 0.28 and 5.36 ± 0.49 μM for compound 6 and 7, respectively (Fig. 2), which is lower ($P = 0.05$) than that of astemizole (12.6 ± 2.4 μM, data not shown). For antigen-induced Ca^{2+} influx identical curves were obtained, with somewhat higher IC_{50} values (10.3 ± 1.5 and 10.2 ± 0.47 μM for compound 6 and 7, respectively; Fig. 2).

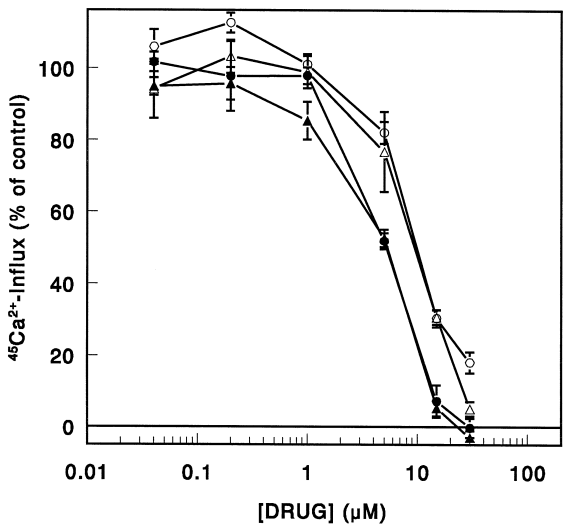


Fig. 2. Effect of compounds 6 (triangles) and 7 (circles) on thapsigargin (0.2 μM, closed symbols) and antigen-specific for dinitrophenyl (40 ng/ml, open symbols) stimulated $^{45}Ca^{2+}$ influx. Data are presented as percentages of control (with stimulation but without compounds).

Table 3
log P_{oct} and pK_a values of astemizole and related compounds

Compound	log P_{oct}	pK_a [a]
2	5.48	5.6 and 8.5
5	4.34	nd[b]
8	4.23	nd
9	4.18	nd
16	3.09	5.8 and 9.8

[a] The compounds have two ionizable groups.
[b] nd, not determined.

Introduction of one or two nitrogens in the benzimidazole ring lowered activity (compounds 4, 5, 8 and 9). The introduction of lone electron pairs in the structure reduced the lipophilicity (see Table 3), and this may be the cause of the decreased inhibition of β-hexosaminidase release and Ca^{2+} influx.

3.3. Influence on intracellular free Ca^{2+} concentration

Astemizole has a dual effect on Ca^{2+} fluxes (Fischer et al., 1998). First, the release of Ca^{2+} from intracellular stores is stimulated irrespective of cell stimulation with antigen. Secondly, the influx of Ca^{2+} via SOC channels is inhibited and this was found to be a major cause for inhibition of β-hexosaminidase release. As shown in Table 2, several astemizole derivatives were also able to inhibit $^{45}Ca^{2+}$ influx. For some of the compounds, we monitored changes in the $[Ca^{2+}]_i$ over time. Fig. 3 shows the changes in $[Ca^{2+}]_i$ in Fura-2 loaded RBL cells. In the controls basal Ca^{2+} levels of ± 50 nM rapidly increased to 500 nM after addition of antigen to IgE-sensitized cells. This increase reflects the combined effect of store depletion and Ca^{2+} influx (Fischer et al., 1998; Dar and Pecht, 1992). After the maximum was reached there was a sustained phase during which there was influx and efflux of Ca^{2+}. During this phase, the state of the SOC channels, which is controlled by the level of depletion of the stores, was reversed from active to inactive (Hoth and Penner, 1993). This inactivation returned the signal to baseline values. The effect of astemizole (compound 2) was in accordance with previous observations: immediately upon addition of astemizole, $[Ca^{2+}]_i$ increased due to Ca^{2+} release from

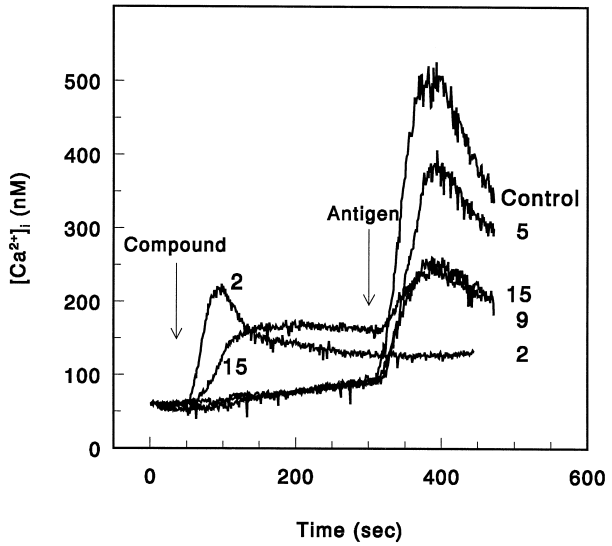


Fig. 3. Effect of compounds 2, 5, 9, and 15 on $[Ca^{2+}]_i$ in RBL-2H3 cells in suspension. Freshly isolated cells were sensitized with IgE (1 μg/ml), loaded with Fura-2-AM and monitored for changes in $[Ca^{2+}]_i$. Compounds (30 μM) or trigger (antigen-specific for dinitrophenyl, 40 ng/ml) was added at the indicated time points.

intracellular stores up to approximately 200 nM and then slowly decreased to basal Ca^{2+} levels (Fig. 3) (Fischer et al., 1998). The negative effect of astemizole on the increase in $[Ca^{2+}]_i$ elicited by antigen stimulation compared to the increase in the absence of compound is the result of inhibition of SOC channels (Fischer et al., 1998). Furthermore, additional opening of SOC channels after antigen stimulation may be limited, as they were already opened by store depletion after the addition of astemizole. These two effects are independent of each other, as can be seen by comparing compounds 9 and 15. Compound 15 also had a dual effect (Fig. 3), although the release from the stores and the inhibition of SOC channels were less pronounced than with astemizole. Norastemizole (compound 16) only inhibited SOC channels (Fischer et al., 1998). Compounds 5 and 9 behaved like norastemizole in that they did not cause Ca^{2+} release from Ca^{2+} stores. Moreover, they also inhibited Ca^{2+} influx. Compounds 5 and 9 are not truncated like norastemizole, but they possess extra nitrogen(s) in the benzimidazole ring and, as indicated before, this makes them less lipophilic (see Table 3). All three compounds were less effective than astemizole in inhibiting $^{45}Ca^{2+}$ influx (Fig. 3), and this is reflected by the decreased inhibition of β-hexosaminidase release (Table 2). The influence of compounds 2, 5, 9 and 15 on SOC channels, as indicated by the diminished increase in $[Ca^{2+}]_i$ after stimulation with antigen (Fig. 3), correlated with their inhibitory effect on thapsigargin-induced $^{45}Ca^{2+}$ influx.

3.4. Effect on hypotonic-induced haemolysis

For several antiallergic drugs a relation between their effect on exocytosis of mast cells and that on cellular membranes has been reported (Fischer et al., 1995; Franzius et al., 1994; Paulussen et al., submitted for publication). Such a relation might exist for the series of astemizole and norastemizole analogues because the less lipophilic compounds, such as the truncated compounds (e.g., 13, 17 and 18) or the compounds containing nitrogen in the benzimidazole group (5, 8 and 9), were less active than the parent compound astemizole (2) (Table 2). Therefore, we assayed haemolysis induced in erythrocytes under hypotonic conditions as a measure of their membrane-disturbing effects (Lau and Pearce, 1990). Astemizole and norastemizole have already been tested in this assay and in a thermotropic phase-transition assay with artificial bilayers (Fischer et al., 1995, 1997). In the haemolysis assay, biphasic behaviour was observed: with increasing concentrations of the drug the cell was first protected against lysis, but above a drug-specific concentration—mostly above 100 μM—the compounds induced lysis. For astemizole, this effect is already observed at 60 μM (Fischer et al., 1997). To avoid complications from drug-induced lysis, we investigated the effect of 20 μM of the compounds with approximately 10^8 erythrocytes. The results are presented as percent inhibition of haemolysis and are summarized in Table 2. The relation between inhibition of haemolysis and antigen- and thapsigargin-induced $^{45}Ca^{2+}$ influx is shown in Fig. 1C,D, respectively. As expected, the truncated compounds 13, 16, 17 and 18 (indicated with an open circle), which lack large hydrophobic groups, had little effect on haemolysis. After exclusion of these compounds, it was found that inhibition of haemolysis correlated fairly well with antigen-induced $^{45}Ca^{2+}$ influx ($r^2 = 0.61$, $F = 17.4$, $P = 0.002$, $n = 13$) and with the more short-routed activation of SOC channels by thapsigargin ($r^2 = 0.59$, $F = 15.9$, $P = 0.002$, $n = 13$). These correlations suggest that the inhibition of SOC channels is related to cell membrane distortion. It is also possible that the more lipophilic astemizole derivatives affected SOC channels to a greater extent. The more lipophilic compounds 2, 3, 6, 7, 10, 11 and 12 exerted a strong inhibition of SOC channels and haemolysis.

Considering the importance of the lipophilicity of the compounds with regard to the effect on SOC channels, log P_{oct} values at pH 12 and pK_a values were determined (Table 3). The introduction of nitrogen into the benzimidazole ring (compounds 5, 8 and 9) decreased log P_{oct} by one log unit. The pK_a values of compounds 5, 8 and 9 were expected to be similar to that of astemizole, which implies that the distribution coefficient (log D) should show a similar pH dependence. As the effect of compounds 5, 8 and 9 on SOC channels was substantially less than that of astemizole, the effect on SOC channels seems to be related to the lipophilicity of the compounds.

4. Discussion

The observed differences in activity between astemizole and the related lipophilic derivatives, and norastemizole

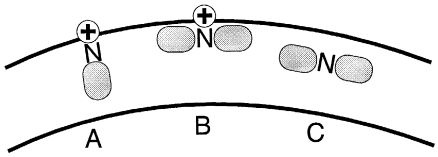


Fig. 4. Proposed models for insertion of norastemizole (A), astemizole charged (B) and uncharged (C) into a membrane bilayer.

may be due to different mechanisms of penetration into the membrane bilayer (see Fig. 4). Molecular dynamics simulations with nifedipine penetrating into 1-2 dimyristoyl phosphatidylcholine membranes supports such a suggestion (Kothekar and Gupta, 1994). The secondary nitrogen in the piperidinyl ring of norastemizole (compound 16) has a higher pK_a than in astemizole (compound 2), where it is a tertiary nitrogen (Table 3, pK_{a2}). In view of the shift to lower a pK_a value in a hydrophobic surrounding (Miyazaki et al., 1992), it is possible that the uncharged form of compound 2 is inserted into the membrane. The physicochemical properties make a model for drug insertion into membranes as depicted in Fig. 4 plausible. Similar membrane interactions have been suggested previously for the Ca^{2+} antagonist flunarizine and the anti-inflammatory drug phenylbutazone in liposomes (Thomas and Seelig, 1993; Sainz et al., 1993). Furthermore, the lipophilicity of compound 16 was reduced by two log units relative to that of compound 2. At physiological pH compound 16, due to its higher protonated fraction ($\log D \ll \log P_{oct}$), will be distributed less in hydrophobic regions of the membrane. This may also explain the effect of astemizole on intracellular Ca^{2+} stores. The involvement of lipophilic interactions in the release of Ca^{2+} from internal stores is further supported by the observation that the more lipophilic compounds 2 and 15 were able to induce Ca^{2+} release, in contrast to the less lipophilic compounds 5 and 9.

The relationship between inhibition of SOC channels and lipophilicity suggests that these drugs have non-specific effects. However, the inhibition of SOC channels was not entirely a non-specific effect, because very lipophilic compounds, such as meclozine, do distort membrane structures but do not affect SOC channels (Fischer et al., 1995). Furthermore, truncated compounds, such as 13 and 16, that had little effect on the membrane, were effective in inhibiting thapsigargin-induced Ca^{2+} influx (Fig. 1D). Comparison of the effect of the truncated compounds with that of the untruncated compounds showed that the piperidinyl–benzimidazole moiety is especially important for the inhibiting effect on SOC channels. We hypothesise that this moiety interacts with the transmembrane part of the SOC channel, affecting the activity of this channel. Little is known about the identity of the SOC channel, which complicates the accurate assignment of specific protein targets.

5. Conclusion

Astemizole and its analogues inhibit mast cell β-hexosaminidase release through inhibition of SOC channels, thereby reducing the influx of Ca^{2+}. The most lipophilic compounds have a dual effect on Ca^{2+} fluxes: in addition to inhibition of SOC channels they also induce Ca^{2+} release from Ca^{2+} stores. Inhibition of SOC channels is a major cause for the inhibition of β-hexosaminidase release from mast cells, which is thought to be relevant for the antiallergic effect of these agents. The correlation between membrane effects and inhibition of SOC channels suggest that effects at the membrane level are important, or that hydrophobic interactions of the drugs are involved. However, the effect of the drugs on SOC channels is not completely non-specific because the piperidinyl–benzimidazole moiety seems to be important for inhibition of SOC channels. The specific inhibition of SOC channels makes astemizole an interesting tool to study the role of SOC channels in mast cells and other cell types possessing SOC channels such as T-cells (Chung et al., 1994; Fanger et al., 1995). The structure-activity relations indicate that astemizole is a new lead for the design of mast cell-stabilizing antiallergic drugs.

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