ELSEVIER

European Journal of Pharmacology 322 (1997) 97–105

ejp

Effects of the anti-allergics astemizole and norastemizole on Fc RI receptor-mediated signal transduction processes

Marcel J.E. Fischer *, Jeannette J.C. Paulussen, Jolien A.E. Kok-Van Esterik, Vincent S. Van der Heijden, Nico J. De Mol, Lambert H.M. Janssen

Department of Pharmaceutical Chemistry, Utrecht Institute for Pharmaceutical Sciences, Faculty of Pharmacy, Utrecht University, P.O. Box 80082, 3508 TB Utrecht, Netherlands

Received 5 August 1996; revised 11 December 1996; accepted 13 December 1996

Abstract

The non-sedating anti-allergic drug astemizole, apart from its potential to antagonise H_1 receptors, inhibits the release of inflammation mediators from mast cells. To study the mechanism of this inhibition, we investigated the effects of astemizole and one of its active metabolites, norastemizole, on different phases of Fc RI (the high affinity receptor for the immunoglobulin IgE) receptor-activated signal transduction in rat basophilic leukemia cells (RBL-2H3), leading to exocytosis. Cells were stimulated either through antigen, or thapsigargin, or synergistic combinations of Fc RI receptor activation with either adenosine A_3 receptors or integrins, activated by fibronectin adherence. The effects of the drugs on mediator release, inositol 1,4,5-trisphosphate formation, tyrosine phosphorylation of cellular proteins and Ca^{2+} fluxes were investigated. Inositol 1,4,5-trisphosphate levels are not affected. Astemizole increased tyrosine phosphorylation in resting cells, especially a 96-kDa protein band. Particularly tyrosine phosphorylation related to post Ca^{2+} processes is changed after cell triggering in the presence of astemizole. Both drugs inhibit the influx of $^{45}Ca^{2+}$, with similar dose response curves as for the inhibition of exocytosis. Astemizole but not norastemizole, when used in resting cells, released Ca^{2+} from intracellular stores. Astemizole (> 15 μM) also induced exocytosis in resting cells. It did not induce additional changes in its inhibiting effect when cells were triggered with synergistic combinations of Fc RI receptor activation with either adenosine A_3 receptors or integrins. Effects on haemolysis of erythrocytes and differential scanning calorimetry in liposomes showed clear differences in membrane perturbation between astemizole and norastemizole. The observed differences, and the role of membrane perturbation in the action on Ca^{2+} fluxes, are discussed.

1. Introduction

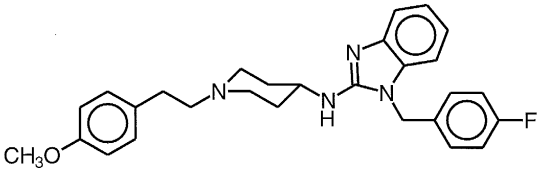
Astemizole (Fig. 1) has been found to be a useful histamine H_1 receptor antagonist with little or no effect on the central nervous system (Awouters et al., 1983). The anti-allergic activity of this compound has been evaluated in different species and tissues. Astemizole has been reported to inhibit immediate type hypersensitivity reactions in vivo, in rats and guinea pigs, and in vitro in guinea pig lungs (Niemegeers et al., 1986; Tasaka et al., 1990). Furthermore, astemizole showed a long duration of receptor binding and action due to slow dissociation from histamine H_1 receptors (Laduron et al., 1981), and a very low toxicity (Niemegeers et al., 1986). The drug is rapidly distributed and metabolised (Waterkeyn et al., 1987). It is a very lipophilic weak base (log P_{oct} is 5.5, pK_a values are 5.6 and 8.5) and therefore tends to concentrate in tissues and cellular organelles by non-specific binding (Waterkeyn et al., 1987). The major metabolites are desmethylastemizole, 5- and 6-hydroxyastemizole and norastemizole (Richards et al., 1984). The metabolites are also effective against allergic reactions and contribute to the therapeutic effect (Richards et al., 1984; Kamei et al., 1991).

Along with histamine H_1 receptor antagonism, inhibition of mediator release from mast cells has been reported for astemizole (Howarth, 1990; Estelle and Simons, 1992; Faraj and Jackson, 1992). This contributes to the ultimate reduction of asthmatic and allergic responses.

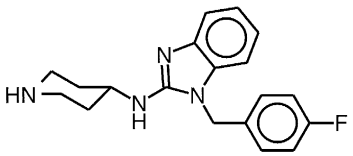
Mast cell activation is controlled by aggregation of the high affinity receptors for the immunoglobulin IgE (Fc RI receptors) and subsequent signal transduction processes (Adamczewski and Kinet, 1994). In order to study the

* Corresponding author. Fax: (31-30) 253-6655.

PII S0014-2999(96)00981-8



Astemizole (pK_a 5.6 and 8.5)



Norastemizole (pK_a 5.8 and 9.8)

Fig. 1. Chemical structures of astemizole and norastemizole.

mechanism with which astemizole inhibits mast cell exocytosis, we investigated its effect on some Fc RI receptor-mediated processes leading to exocytosis: tyrosine phosphorylation of cellular proteins (Scharenberg and Kinet, 1994), inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) formation (Beaven and Metzger, 1993) and Ca^{2+} fluxes (Hoth and Penner, 1993). The cells were triggered in various ways: by aggregation of Fc RI receptors (Beaven and Metzger, 1993) and by methods that bypass early steps after Fc RI receptor aggregation. We used the rat basophilic leukemia (RBL-2H3) cell line, which is a well characterized model for mucosal mast cells (Adamczewski and Kinet, 1994).

It has been suggested that the inhibiting effect of astemizole on mast cell mediator release is caused by effects of the drug on cell membranes (Lau and Pearce, 1989; Tasaka et al., 1990; Estelle and Simons, 1992). Therefore, we included the metabolite norastemizole in these studies. Both drugs appear to be equally active in reducing allergic responses (Kamei et al., 1991). Norastemizole like astemizole is a base, however, with considerably lower lipophilicity (log P_{oct} is 3.1, pK_a values are 5.8 and 9.8) (Waterkeyn et al., 1987). We compared in this study the membrane related effects of astemizole and norastemizole on the hypotonic induced haemolysis of erythrocytes (Lau and Pearce, 1990), and on phase transitions in liposomes (Fischer et al., 1995).

Activation of adenosine A_3 receptors (Ramkumar et al., 1993) or integrins, by way of cell adherence to fibronectin (Yasuda et al., 1995), are both synergistic with Fc RI receptor activation and are physiologically relevant. Therefore, the inhibiting effect of astemizole on exocytosis induced by these synergistic triggers was also investigated.

2. Materials and methods

2.1. Reagents

Astemizole and norastemizole were gifts from Janssen Pharmaceutica. Stock solutions of these compounds, the fluorescent probe fura-2-acetoxymethylester (fura-2-AM, Sigma) and thapsigargin (Calbiochem) were prepared in dimethylsulphoxide. Anti-dinitrophenyl monoclonal IgE (IgE, Sigma), human dinitrophenyl albumin antigen (DNP_{30}-HSA, Sigma), monoclonal anti-phosphotyrosine IgG_1 coupled to horse radish peroxidase (PY20, Transduction Laboratories), bovine plasma fibronectin (Gibco), D-*myo*-[^{3}H]inositol 1,4,5-trisphosphate potassium salt and [^{45}Ca]Cl_2 ($^{45}Ca^{2+}$, Amersham) were prepared in culture medium or buffer. Culture medium and other reagents were of highest analytical grade.

2.2. Cell culture and stimulation of exocytosis

RBL-2H3 cells were cultured and used in the assay for β-hexosaminidase as previously described (Fischer et al., 1995). For the $^{45}Ca^{2+}$ influx and phosphorylation experiments overnight cultures of RBL cells were used in 24-well tissue culture plates (4×10^5 cells/well). After sensitization with IgE (1 μg/ml) or vehicle (in case of stimuli other than antigen) for 1 h, the growth 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). Before stimulation cells were exposed to astemizole or norastemizole for 10 min at the indicated concentrations. Exocytosis was induced either by the antigen DNP_{30}-HSA, by thapsigargin, or by the combination *N*-ethyl-carboxamido-adenosine (NECA) and DNP_{30}-HSA at 37°C. Duration of stimulations and concentrations were as indicated. After incubation supernatants and/or lysates were collected for assay of β-hexosaminidase or radioactivity, or cells were lysed for assay of protein tyrosine phosphorylation.

2.3. Fibronectin-adhered RBL cells

High binding ELISA plates (Costar) were coated for 3.5 h with fibronectin (10 μg/ml in phosphate-buffered saline (PBS, according to Gibco) pH 7.3) at 37°C. Finally, the remaining non-specific binding sites were blocked by washing the plates with bovine serum albumin (2% in PBS). Control wells were incubated with bovine serum albumin (2% in PBS). Suspended RBL-2H3 cells were sensitized with IgE (10 ng/ml) for 1 h with careful agitation. After washing, the cells were resuspended in Tyrode's buffer and plated in the fibronectin or bovine serum albumin treated wells (4×10^4 cells/well). Cells were allowed to adhere during 20 min. Compounds were added and incubated for 10 min. The cells were stimulated for 30 min with DNP_{30}-HSA (20 ng/ml). Samples of the supernatant (50 μl) were taken and the volume withdrawn was replaced with Triton-X-100 (1%). After 2 min samples of the lysate were taken. Supernatant and lysate were assayed for β-hexosaminidase as described (Fischer et al., 1995). Results are expressed as percentages of total enzyme content. The effect of the compounds is presented as a percentage of control (no drug present, fibronectin and

bovine serum albumin experiments have their own controls).

2.4. Determination of protein tyrosine phosphorylation

The cells were activated with antigen or thapsigargin for 30 min. After lysis the supernatant was immediately exchanged with 100 μl of ice-cold lysis buffer/10^6 cells (Tris buffer containing 50 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, 150 mM NaCl, 0.4 mM sodium orthovanadate, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml antipain). Proteins were solubilized for 30 min at 4°C. Insoluble material was removed by centrifugation and the supernatant was treated under reducing conditions with Laemmli's buffer containing dithiothreitol (95°C, 5 min). After electrophoresis on 7.5 or 10% polyacrylamide gels the proteins (10 μg/lane) were blotted onto a polyvinylidene difluoride membrane with an electroblotter (Bio-Rad, Mini Trans-Blot Cell). The membranes were blocked with blocking buffer (Tris-buffered saline (pH 7.6) with 5% bovine serum albumin and 0.05% Tween-20) and treated with 0.25 μg/ml of anti-phosphotyrosine antibody PY20 coupled to horseradish peroxidase in Tris-buffered saline containing 0.5% bovine serum albumin and 0.05% Tween-20. After extensive washing with a large volume of Tris-buffered saline containing 0.05% Tween-20, the phosphorylated proteins were visualized using the Enhanced ChemiLuminescence kit (Amersham) and subsequently exposed to Kodak X-AR film. To compare quantities of proteins in individual lanes the blots were silver-stained (Kovarik, 1987).

2.5. Determination of inositol 1,4,5-trisphosphate formation

Monolayer cells (2×10^6 cells/well) were sensitized for 1 h with IgE (1 μg/ml) and triggered with antigen (40 ng/ml). After stimulation for 5 min the cells were solubilized in perchloric acid (3.5%). Ins(1,4,5)P_3 formation was determined according to Bominaar and Van Haastert (1994). In short, the acidic samples were neutralized with $KHCO_3$ (50%). After centrifugation ($14\,000 \times g$) the supernatant was mixed with [^{3}H]Ins(1,4,5)P_3-tracer and Ins(1,4,5)P_3 binding protein and incubated at 0°C for 10 min. The pellet, that remained after centrifugation and aspiration of the supernatant, was dissolved in water by vortexing and a subsequent addition of scintillator. Radioactivity was counted and compared to a standard curve to establish Ins(1,4,5)P_3 concentrations.

2.6. Measurement of influx of extracellular $^{45}Ca^{2+}$

After incubation at 37°C the cells were triggered with antigen or thapsigargin in the presence of $^{45}CaCl_2$ (30 μCi/ml or 10 μCi/ml, respectively). Preincubation with compound and cell triggering were as indicated under protein tyrosine phosphorylation. The supernatants were removed after 5 min in the case of antigen activation, and after 10 min with thapsigargin activation. Immediate and thorough washing with ice-cold Tyrode's buffer removed extracellular $^{45}Ca^{2+}$, after which the cells were lysed in 1% Triton X-100. Incorporated $^{45}Ca^{2+}$ was measured using a liquid scintillation counter (Wallac) and the results in the presence of drug were expressed as a percentage relative to the control (no drug present) and were corrected for adsorption of $^{45}Ca^{2+}$ to the cells (5–10% of control).

2.7. Measurement of intracellular Ca^{2+} concentration

The time course of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined in cells in suspension. The isolated cells were resuspended at 2×10^6 RBL cells/ml in minimal essential medium containing Hepes (10 mM) and sensitized with IgE (1 μg/ml, 60 min) under careful agitation in a polypropylene tube to prevent adherence. Cell batches of 1 ml were concentrated to 200 μl in Tyrode's buffer containing 0.1 mg/ml sulfinpyrazon, and loaded with the fluorescent probe fura-2-AM (10 μM) for 15 min at 37°C and thereafter washed three times with Tyrode's buffer containing gelatine (0.05%) instead of bovine serum albumin and sulfinpyrazon (0.1 mg/ml). The fura-loaded cells were resuspended in 400 μl of the latter buffer and slowly agitated at room temperature. Aliquots of 100 μl were diluted to 1 ml (final cell titer 5×10^5/ml) with warm Tyrode's buffer containing gelatine (0.05%) and Ca^{2+} was assayed fluorimetrically in stirred cuvettes at 37°C at excitation of 340/380 nm and emission of 510 nm.

2.8. Hypotonic haemolysis of erythrocytes

Determination of erythrocyte haemolysis was performed and calculated according to Lau and Pearce (1990). In short, bovine erythrocytes in Alsever solution were washed three times in a phosphate buffer (10 mM phosphate and 154 mM NaCl, pH 7.4) and resuspended at 4×10^8 cells/ml in phosphate buffer. Haemolysis was induced with a hypotonic phosphate buffer (68 mM NaCl) to produce 50% haemolysis in 5 min. This represents the control condition. The haemoglobin content in the supernatant was measured spectrophotometrically at 550 nm. The effect of the compounds was expressed as percentage inhibition of haemolysis relative to the control situation.

2.9. Statistics

Results are expressed as means ± standard deviation (S.D.) of triplicate determinations in each of two or more independent experiments. A Student's *t*-test was used with a significance reliability of 95%.

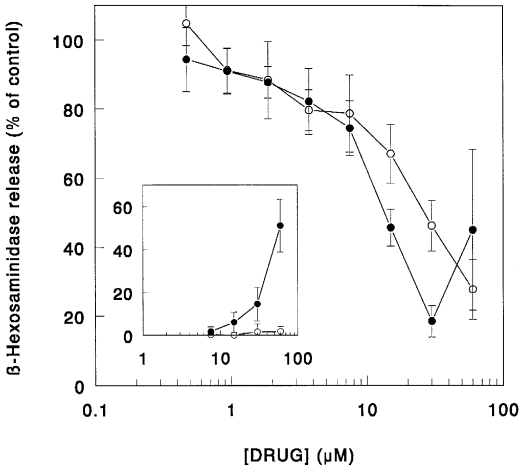


Fig. 2. Concentration dependent inhibition of β-hexosaminidase release in antigen activated RBL-2H3 cells by astemizole (●) and norastemizole (○). The data represent the percentages of total enzyme secreted compared to a control without drug. Results are means ± S.D. of at least six experiments. Inset: effect of the drugs on β-hexosaminidase release in non-activated cells.

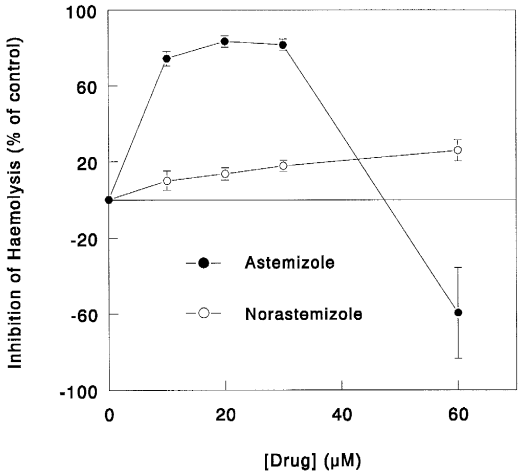


Fig. 3. The effect of astemizole and norastemizole on hypotonic haemolysis of erythrocytes (4×10^8 cells/ml). Haemolysis was assayed 5 min after exposure to the drugs. Negative values represent elevation of haemolysis. The bars indicate S.D. from four individual experiments.

3. Results

3.1. Effects on exocytosis

Astemizole and norastemizole are able to inhibit the Fc RI receptor-induced degranulation of β-hexosaminidase concentration dependently with IC_{50} values of 14.4 ± 1.5 and 27.6 ± 5.6 μM, respectively (Fig. 2). Previously, we have shown that if RBL cells were triggered with the Ca^{2+} ionophore A23187 the inhibition was substantially reduced resulting in IC_{50}s for astemizole and norastemizole of 45 ± 5 and 65 ± 5 μM, respectively (Fischer et al., 1995). Triggering with the synergistic combination of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (Paulussen et al., 1996a) and A23187 (0.25 μM) increases the IC_{50}s even further: 80 ± 10 and > 100 μM, respectively, for astemizole and norastemizole (Fischer et al., 1995). These results indicate that the compounds affect the exocytosis process at stages before the elevation of the intracellular Ca^{2+} concentration.

Interestingly, astemizole is able to induce exocytosis without stimulation at concentrations higher than 15 μM, while norastemizole lacks this property (Fig. 2 inset). This behaviour of astemizole is prominent above 30 μM and explains the increased release at 60 μM astemizole (Fig. 2).

3.2. Effects on haemolysis

In order to evaluate the effects of the drugs on cellular membranes, erythrocyte cells that are in a condition of hypotonic haemolysis are exposed to astemizole and norastemizole. The lipid composition of the membranes of erythrocytes and RBL cells is comparable (Chang et al., 1995). Stabilization of the membrane is observed as an inhibition of haemolysis (Lau and Pearce, 1990). From Fig. 3 it can be seen that norastemizole only slightly stabilizes the membrane, while astemizole has large stabilizing abilities at concentrations where it inhibits exocytosis. In contrast, at higher concentration (≥ 60 μM) the stabilization of the membrane reverses to stimulation of haemolysis.

3.3. Effect on inositol 1,4,5-trisphosphate generation

As mentioned above, the main site of action of the drugs seems to be located at processes prior to or at the point where cellular Ca^{2+} levels increase during cell activation. With Fc RI receptor aggregation, the emptying of $Ins(1,4,5)P_3$-sensitive endoplasmic Ca^{2+} stores precedes Ca^{2+} influx (Beaven and Metzger, 1993). Therefore, the effect of astemizole on $Ins(1,4,5)P_3$ levels in the cell is determined (Table 1). $Ins(1,4,5)P_3$ levels do not significantly change after the addition of 30 μM astemizole, either with or without antigen activation of the cells. This shows that the immediate Fc RI receptor-related processes are not affected by the drugs and that inhibiting activity must be situated later than phospholipase Cγ1 activation (Beaven and Metzger, 1993).

Table 1
Antigen-induced elevation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) levels in RBL-2H3 cells (2×10^6 cells) and the effect of 30 μM astemizole. Values are means ± S.D. from four experiments

	$Ins(1,4,5)P_3$ (pmol/2×10^6 cells)	
	+ antigen	− antigen
Control	4.33 ± 0.34	1.16 ± 0.74
Astemizole	4.77 ± 1.38	0.58 ± 0.21

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

The effect of the drugs upon various ways of cell stimulation and the absence of effect on Ins(1,4,5)P_3 levels indicates that the drugs induce inhibition of exocytosis by activity at the level of Ca^{2+} fluxes. The influx of $^{45}Ca^{2+}$ is investigated with two methods of cell activation: (1) Fc RI receptor aggregation and (2) triggering of the cells with thapsigargin. Thapsigargin is a specific inhibitor of the Ca^{2+}-ATPase in the endoplasmic reticulum keeping the Ca^{2+} stores in an emptied state (Thastrup et al., 1990). This generates capacitative Ca^{2+} entry and induces signal transduction processes leading ultimately to exocytosis (Ali et al., 1994) bypassing primary events that are coupled to Fc RI receptor aggregation. With Fc RI receptor aggregation the $^{45}Ca^{2+}$ influx shows a drug concentration dependence (Fig. 4), which is similar to that seen for β-hexosaminidase release (Fig. 2). With thapsigargin activation similar results were obtained (data not shown). In resting cells (Table 2, −antigen) 30 μM astemizole slightly increases basal $^{45}Ca^{2+}$ influx and stimulates release of β-hexosaminidase from the cells. In contrast, norastemizole shows no increase of basal control values.

3.5. Effect on intracellular Ca^{2+} concentration

To gain insight into the mechanism of Ca^{2+} influx inhibition by astemizole or norastemizole, the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored using fura-2 loaded cells. In resting cells astemizole (30 μM) enhances $[Ca^{2+}]_i$ (Fig. 5). This rise is substantially lower when influx of extracellular Ca^{2+} is prohibited in the presence of EGTA. The induced rise in $[Ca^{2+}]_i$ after the addition of antigen in Fig. 5A is due to the release of Ca^{2+}

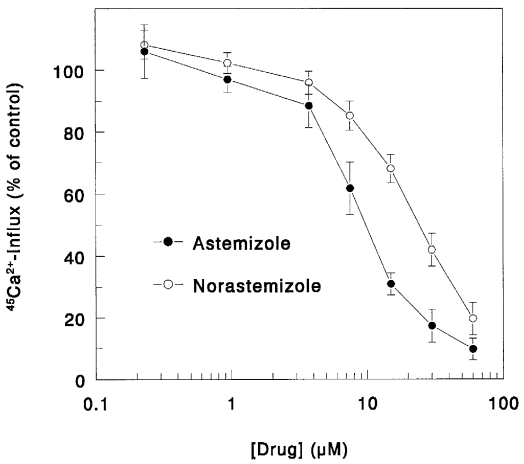


Fig. 4. Effects of astemizole (●) and norastemizole (○) on antigen-stimulated $^{45}Ca^{2+}$ influx in RBL-2H3 cells. Cells (4×10^5 cells/well) were sensitized with IgE (1 μg/ml) for 1 h. Activation was initiated with DNP_{30}-HSA (40 ng/ml). Release and influx are expressed as percentage relative to control experiments without compound. Results are means ± S.D. of at least six experiments.

Table 2
The influence of astemizole and norastemizole on $^{45}Ca^{2+}$ influx and exocytosis in RBL-2H3 cells after antigen activation. Exocytosis was measured as release of β-hexosaminidase. The effect of 30 μM of compound is expressed relative to the control values from activated cells in the absence of drug (+antigen) and is corrected for basal influx or release (±4% of control)

Compound (30 μM)	$^{45}Ca^{2+}$ influx (%, $n=6$)		Exocytosis (%, $n=8$)	
	+antigen	−antigen	+antigen	−antigen
Control	100	0	100	0
Astemizole	26.5 ± 4.1	12.0 ± 2.8	18.5 ± 4.6	14.6 ± 7.9
Norastemizole	48.7 ± 9.2	0.74 ± 0.6	46.2 ± 7.3	1.6 ± 3.7

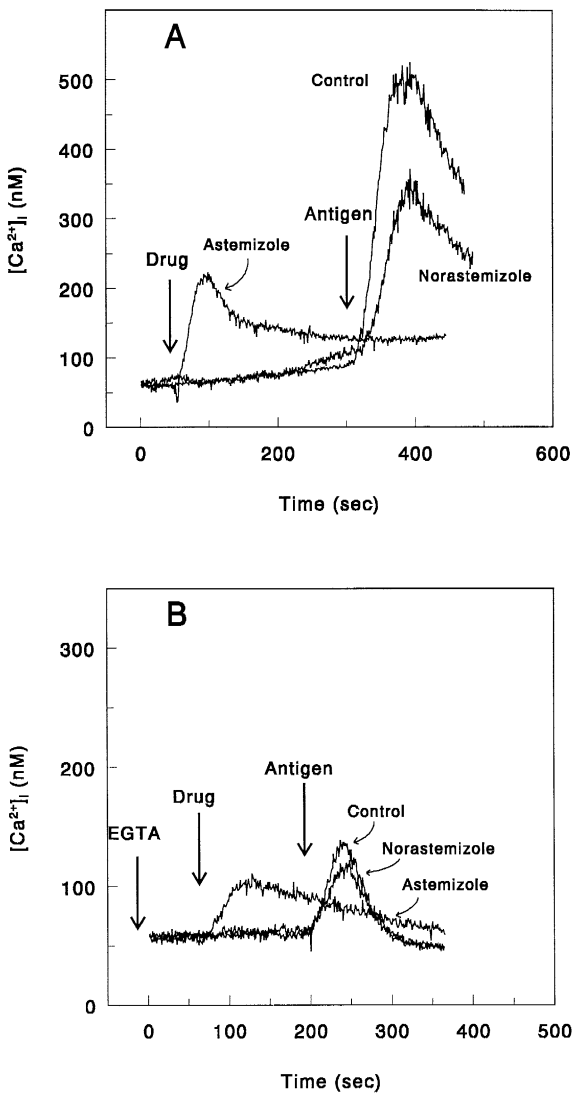


Fig. 5. The effect of astemizole and norastemizole (30 μM) on $[Ca^{2+}]_i$ as measured in fura-2-loaded RBL-2H3 cells. The cells were sensitized with IgE. Panel A shows $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, while panel B is without free extracellular Ca^{2+} due to the presence of 3 mM EGTA. At the time points indicated by the large arrows, the drug and antigen were added to the cell suspension. Results are representative of at least three independent experiments.

from intracellular stores and influx of extracellular Ca^{2+} (Ali et al., 1994). In the presence of EGTA this rise is much smaller (Fig. 5B) and is only due to release from intracellular stores. The antigen-induced rise in $[Ca^{2+}]_i$ is not observed in the presence of astemizole. Norastemizole has no effect on resting cells, but only decreases the antigen-induced rise in $[Ca^{2+}]_i$. These results demonstrate that astemizole is able to release Ca^{2+} from intracellular stores. Emptying of the stores activates the influx of Ca^{2+} into the cells, which explains the higher astemizole-induced rise in $[Ca^{2+}]_i$ when free extracellular Ca^{2+} is present. Obviously, antigen activation of the cells has no further effect on Ca^{2+} influx.

3.6. Protein tyrosine phosphorylation

Tyrosine phosphorylation activates enzymes involved in several stages leading to exocytosis. Therefore, the effect of astemizole and norastemizole on tyrosine phosphorylation of cellular proteins in RBL cells is investigated. Enhanced phosphorylation is observed after Fc RI receptor activation (Fig. 6, lanes 1 and 2). These phosphorylations include protein bands with molecular weights of 40–45, 72 and around 110–115 kDa. The 40–45 kDa region has been found to contain cytosolic mitogen-activated protein kinases (Offermans et al., 1994), while the 72 kDa band consists at least of a pp72 protein (syk) that activates phospholipase Cγ1 (Jouvin et al., 1994; Minoguchi et al., 1994). The increase in phosphorylation of the 110 kDa and the 40–45 kDa bands appear only in the presence of extracellular Ca^{2+} and these proteins are assumed to be phosphorylated after elevation of $[Ca^{2+}]_i$ (Benhamou et al., 1992). Remarkably, astemizole influences the tyrosine phosphorylation of cellular proteins in resting cells (Fig. 6, lane 3), while norastemizole does not (not shown). Astemizole prominently increases the phosphorylation of a 96-kDa band. This band is not phosphorylated upon antigen activation (lane 2). Furthermore, some increase in the region of 40–44 kDa is observed. Upon antigen activation in the presence of astemizole the increased phosphorylation of the 96-kDa band remains (lane 4). Further decrease in phosphorylation of the band at 110–115 kDa is prominent. The phosphorylation of bands in the region 40–44 kDa is somewhat increased further. As mentioned above these phosphorylations are related to post Ca^{2+} processes (Benhamou et al., 1992).

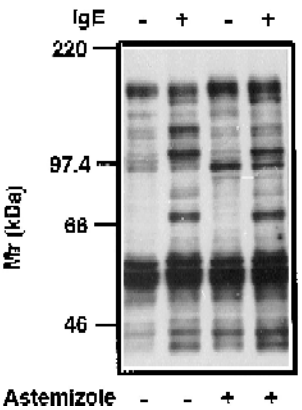


Fig. 6. Effect of 30 μM astemizole on protein tyrosine phosphorylation in resting (−IgE) and antigen activated (+IgE) RBL-2H3 cells. The cells were sensitized with IgE (1 μg/ml) and triggered for 30 min with DNP_{30}-HSA (40 ng/ml). Cells were lysed and extracted proteins were separated on 7.5% gel by electrophoresis and immunoblotted as described in Section 2.

Besides having a strong effect on Ca^{2+} fluxes, thapsigargin activation bypasses directly Fc RI receptor-related events and thus generates fewer phosphorylation processes. The effect of thapsigargin stimulation on tyrosine phosphorylation in the presence of astemizole again strongly increases the phosphorylation of the 96 kDa band and astemizole once more decreases phosphorylation of 42 and 110–115 kDa bands, although only slightly for the 42 kDa band (data not shown). These changes are in agreement with inhibition of Ca^{2+} influx.

Norastemizole has no significant effects on phosphorylation and also no drug-induced phosphorylation at 96 kDa is observed (data not shown).

3.7. Effects on the synergistic adenosine A_3 receptor and Fc RI receptor activation

The effectiveness of the signal transduction processes under physiological conditions may be much higher by the cooperative use of different receptors to control exocytosis. In RBL cells adenosine receptors of the A_3 subtype are coupled via Gi_3 to phospholipase Cβ (Palmer et al., 1995), leading to synergistic responses after combined activation with Fc RI receptors (Ramkumar et al., 1993). We investigated the effect of astemizole on the synergistic cell triggering with low antigen and NECA concentration (0.5 ng/ml and 1 μg/ml, respectively). Individually they are not capable of inducing degranulation. The effect of astemizole on the synergistic trigger is compared to the effect on exocytosis obtained with a standard antigen concentration (20 ng/ml). It is found that there is no difference in astemizole dependent inhibition of mediator release after cell activation with either method (data not shown). This suggests that astemizole influences the common part of both activation routes and indicates that it inhibits processes beyond activation of phospholipase C.

3.8. Effect on exocytosis of fibronectin-adhered cells

In recent work cell adherence to fibronectin involving binding of cellular integrins has been found to act synergistically with Fc RI receptor activation (Hamawy et al., 1993). Here we compare RBL cells attached to a fibronectin coating to cells adhered to a bovine serum albumin coating. This results in strong differences in mor-

phological appearance. Fibronectin-adhered cells show cell spreading and are firmly connected to the matrix, as was also demonstrated by Hamawy et al. (1992). The cells attached to bovine serum albumin are very loosely bound to the matrix and maintain a round form as observed in cell suspensions. The optimal condition for synergistic Fc RI receptor and integrin activation is found with concentrations for IgE and antigen of 10 and 20 ng/ml, respectively. The resulting β-hexosaminidase release for fibronectin and bovine serum albumin-adhered cells is 54.2 and 28.0% in the absence of the drugs, respectively. This increased efficiency of exocytosis by fibronectin-adhered cells has been reported before (Hamawy et al., 1993).

Astemizole at concentrations lower than 15 μM shows no difference in effect on exocytosis induced by the two triggers (Fig. 7). However, an increase in the astemizole concentration above 15 μM induces large increases in mediator release in bovine serum albumin-adhered cells. This stimulation of release is also apparent in non-activated cells, which indicates that the effect is a non-Fc RI receptor-related event. Remarkably, fibronectin-adhered RBL cells are not susceptible for this drug-induced release (Fig. 7). At 60 μM astemizole completely blocks the mediator release in fibronectin-adhered cells (Fig. 7). The IC_{50} of astemizole and fibronectin-adhered RBL cells is similar to that under standard conditions (Fig. 2). These results indicate that astemizole does not influence the integrin-mediated route to exocytosis.

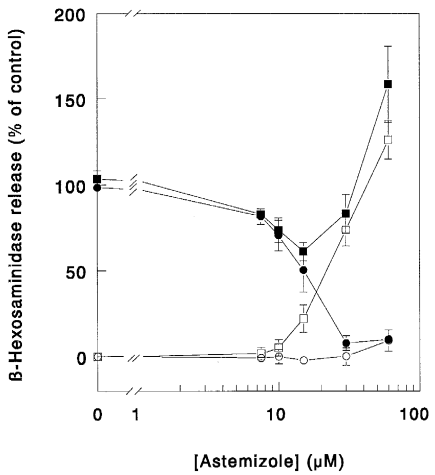


Fig. 7. Effect of astemizole on β-hexosaminidase release upon Fc RI receptor-induced activation of RBL-2H3 cells that were adhered to fibronectin coated surfaces (○ and ●) or to bovine serum albumin (□ and ■). The cells were sensitized with a low IgE concentration (10 ng/ml, closed symbols) or the effect of the drug on non-sensitized cells was measured (open symbols). Cells were stimulated with DNP_{30}-HSA (20 ng/ml) for 30 min. Release is the percentage against a control without drug. The control values are 54.2 and 28.0% of total β-hexosaminidase for fibronectin and bovine serum albumin adhered cells, respectively. The bars represent standard deviations from at least eight experiments.

4. Discussion

The compounds astemizole and its metabolite norastemizole concentration dependently inhibit the release of mediators from RBL-2H3 cells. The effects of the drugs is investigated at different phases of processes leading to exocytosis, to establish the possible site(s) of action. The lack of effect of astemizole on $Ins(1,4,5)P_3$ levels suggests an action on processes beyond phospholipase C activation. It appears that the compounds inhibit $^{45}Ca^{2+}$ influx. The IC_{50} values of astemizole and norastemizole for antigen- and thapsigargin-induced $^{45}Ca^{2+}$ influx are similar to those for antigen-induced exocytosis. The effects of astemizole on $[Ca^{2+}]_i$ demonstrate that the drug, along with inhibition of Ca^{2+} influx, empties intracellular Ca^{2+} stores. Furthermore, it appeared that especially the phosphorylation of proteins which change in phosphorylation after Ca^{2+} influx, is decreased by astemizole. All these experiments are in agreement with the conclusion that the main site of action of astemizole is at the level of Ca^{2+} fluxes. It should be indicated that comparable suggestions have been made for compounds like econazole and SKF 96365 (Mason et al., 1993).

Recently Paulussen et al. (1996b) found that the IC_{50} of the histamine H_1 receptor antagonist oxatomide for inhibition of $^{45}Ca^{2+}$ influx was lower than that for exocytosis. From this it was concluded that the inhibition of $^{45}Ca^{2+}$ influx had to pass a threshold value of approximately 40% before the exocytosis becomes inhibited. For astemizole and norastemizole this threshold value apparently does not apply as the IC_{50} values are similar (Figs. 2 and 4).

Changes in phosphorylation are induced by astemizole, of which the increased phosphorylation of a band at 96 kDa in resting cells is most pronounced. The identity of this protein(s) is not known. Obviously the phosphorylation of this band is not changed upon antigen or thapsigargin activation of the cells. Norastemizole has no effect on the phosphorylation of the 96 kDa band.

Norastemizole, which also inhibits exocytosis, has hardly any influence on the phosphorylation state of cellular proteins. As astemizole and norastemizole inhibit $^{45}Ca^{2+}$ influx, a plausible mechanism may be inhibition of the recently described Ca^{2+} release-activated Ca^{2+} (CRAC) channels, that occur a.o. in RBL-, mast- and T-cells (Berridge, 1995). Recently the name SOC (store-operated channels) has been suggested for this type of channels (Clapham, 1996). The role of phosphorylation in the activation of SOC channels is still controversial (Berridge, 1995). Our results do not support a role for phosphorylation in the regulation of SOC channels.

The lack of additional effects of the drugs seen with the synergistic activation of adenosine A_3 and Fc RI receptors can be understood in view of the fact that the main site of action of these drugs is at the level of Ca^{2+} fluxes. The Ca^{2+} fluxes occur in a later phase than phospholipase C activation, either by Fc RI or adenosine A_3 receptor

activation (Palmer et al., 1995), after which both routes converge (Atkinson et al., 1992). The same explanation probably also applies for the lack of additional effect of astemizole on exocytosis of fibronectin-adhered cells, although signalling involving integrins is more complicated than that via A_3 receptors, as it leads to changes in cytoskeletal organization and morphology (Swieter et al., 1993).

Comparing the activity of astemizole and norastemizole some differences are observed: norastemizole is less active on inhibition of exocytosis and inhibition of thapsigargin- and antigen-induced $^{45}Ca^{2+}$ influx (Figs. 2 and 4, and Table 2). Astemizole has a large effect on membrane structures, as appears from the strong inhibition of haemolysis relative to norastemizole. Furthermore, astemizole distorts the lipophilic core of the membrane bilayer structure, as demonstrated by its effect on phase transitions in an artificial membrane bilayer composed of L-α-phospatidylcholine dipalmitoyl liposomes (Fischer et al., 1995). For norastemizole this change in phase transition was not observed (data not shown). Norastemizole, in contrast to astemizole, possesses a structure where a lipophilic 4-methoxyphenyl-ethyl substituent is missing. This change lowers the log P with approximately two log units. Astemizole has a lower pK_a than norastemizole, which might even be lower in a lipophilic environment (Miyazaki et al., 1992). Due to its higher pK_a value norastemizole will be more protonated around physiological pH at the terminally standing pipiridinyl nitrogen. These differences in physicochemical properties are responsible for the observed differences in effects on membranes.

The induced changes on the membrane level might have consequences for cellular processes. Tyrosine phosphorylation of cellular proteins often involves aggregation of membrane bound proteins (Metzger, 1992). Therefore, the effect of astemizole on tyrosine phosphorylation in resting cells may be explained by its effect on membrane structures and aggregation of membrane-bound protein tyrosine kinases. For norastemizole, in contrast to astemizole, neither drug-induced release, nor a large effect on erythrocyte membranes is observed. The drug does, however, inhibit $^{45}Ca^{2+}$ influx and exocytosis. This suggests that membrane disturbance is not essential for the primary effect of the drugs on $^{45}Ca^{2+}$ influx, but that more specific interactions of the drug and a target related to Ca^{2+} fluxes are involved.

Astemizole induces exocytosis at concentrations higher than 15 μM. This is not a cytotoxic effect due to lysis of the cell membrane as lactate dehydrogenase leakage occurs only at concentrations ≥ 100 μM (Fischer et al., 1995). It is assumed that this drug-induced exocytosis is caused by the release of Ca^{2+} from the intracellular stores. This release activates the SOC channels and ultimately leads to exocytosis. The effect on the stores might be caused by disturbance of the endoplasmic membrane structures. Fibronectin adherence is apparently capable of reducing drug-induced release. As this release seems to be related to effects of astemizole on emptying of Ca^{2+} stores, we assume that the Ca^{2+} stores in fibronectin-adhered cells are better stabilized, which might be related to the morphological and cytoskeletal changes evoked by fibronectin adherence (Swieter et al., 1993).

In conclusion, astemizole and norastemizole inhibit exocytosis in RBL cells mainly by the action on the level of Ca^{2+} fluxes. A more detailed study of the effect on Ca^{2+} fluxes is underway as is a study of a wider range of astemizole and norastemizole derivatives to elucidate the possible role of drug-membrane interactions in the inhibition of exocytosis. The effects described herein might be relevant for the anti-allergic effect of clinically used astemizole. Furthermore, astemizole is one of the first drugs for which a specific inhibition of capacitative Ca^{2+} entry is described, which makes it a potential tool for studying this entry mechanism.

Acknowledgements

We thank Prof. Dr J.P. Tollenaere for stimulating discussions. We would like to thank Drs P. van Dijken (Department of Biochemistry, University of Groningen, Groningen, Netherlands) for assistance with the $Ins(1,4,5)P_3$ measurements. The Utrecht Institute for Pharmaceutical Sciences is the Utrecht part of the Research School Groningen Utrecht Institute for Drug Exploration (GUIDE).

References

Adamczewski, M. and J.P. Kinet, 1994, The high-affinity receptor for immunoglobulin E, Chem. Immunol. 59, 173.

Ali, H., K. Maeyama, R. Sagi-Eisenberg and M.A. Beaven, 1994, Antigen and thapsigargin promote influx of Ca2+ in rat basophilic RBL-2H3 cells by ostensibly similar mechanisms that allow filling of inositol 1,4,5-trisphosphate-sensitive and mitochondrial Ca2+ stores, Biochem. J. 304, 431.

Atkinson, T.P., M.A. Kaliner and R.J. Hohman, 1992, Phospholipase Cγ1 is translocated to the membrane of leukemia cells in response to aggregation of IgE receptors, J. Immunol. 148, 2194.

Awouters, F.H.L., C.J.E. Niemegeers and P.A.J. Janssen, 1983, Pharmacology of the specific histamine H_1-antagonist astemizole, Arzneim. Forsch.-Drug Res. 33, 381.

Beaven, M.A. and H. Metzger, 1993, Signal transduction by Fc receptors: the Fc RI case, Immunol. Today 14, 222.

Benhamou, M., V. Stephan, K.C. Robbins and R.P. Siraganian, 1992, High-affinity IgE receptor-mediated stimulation of rat basophilic leukemia (RBL-2H3) cells induces early and late protein-tyrosine phosphorylations, J. Biol. Chem. 267, 7310.

Berridge, M.J., 1995, Capacitative calcium entry, Biochem. J. 312, 1.

Bominaar, A.A. and P.J.M. Van Haastert, 1994, Phospholipase C activity in *Dictyostelium discoideum* using endogenous nonradioactive phosphatidylinositol 4,5-bisphosphate as substrate, Methods Enzymol. 238, 207.

Chang, E.Y., Y. Cheng, D. Holowka and B. Baird, 1995, Alteration of lipid composition modulates Fc RI signaling in RBL-2H3 cells, Biochemistry 34, 4376.

Clapham, D.E., 1996, TRP is cracked but is CRAC TRP, Neuron. 16, 1069.

Estelle, F. and R. Simons, 1992, The antiallergic effects of antihistamines (H_1-receptor antagonists), J. Allergy Clin. Immunol. 90, 705.

Faraj, B.A. and R.T. Jackson, 1992, Effect of astemizole on antigen-mediated histamine release from the blood of patients with allergic rhinitis, Allergy 47, 630.

Fischer, M.J.E., J.J.C. Paulussen, D.A. Horbach, E.P.W. Roelofsen, J.C. Van Miltenburg, N.J. De Mol and L.H.M. Janssen, 1995, Inhibition of mediator release in RBL-2H3 cells by some H_1-antagonist derived anti-allergic drugs: relation to lipophilicity and membrane effects, Inflamm. Res. 44, 92.

Hamawy, M.M., C. Oliver, S.E. Mereenhagen and R.P. Siraganian, 1992, Adherence of rat basophilic leukemia (RBL-2H3) cells to fibronectin-coated surfaces enhances secretion, J. Immunol. 149, 615.

Hamawy, M.M., S.E. Mergenhagen and R.P. Siraganian, 1993, Cell adherence to fibronectin and the aggregation of the high affinity immunoglobulin-E receptor synergistically regulate tyrosine phosphorylation of 105-115-kDa proteins, J. Biol. Chem. 268, 5227.

Hoth, M. and R. Penner, 1993, Calcium release-activated calcium current in rat mast cells, J. Physiol. 465, 359.

Howarth, P.H., 1990, Histamine and asthma – an appraisal based on specific H_1-receptor antagonism, Clin. Exp. Allergy 20, 31.

Jouvin, M.H.E., M. Adamczewski, R. Numerof, O. Letourneur, A. Valle and J.P. Kinet, 1994, Differential control of the tyrosine kinases lyn and syk by the two signaling chains of the high affinity immunoglobulin E receptor, J. Biol. Chem. 269, 5918.

Kamei, C., M. Mio, K. Izushi, K. Kitazumi, S. Tsujimoto, K. Fujisawa, Y. Adachi and K. Tasaka, 1991, Antiallergic effects of major metabolites of astemizole in rats and guinea pigs, Arzneim. Forsch.-Drug Res. 41, 932.

Kovarik, A., 1987, An improved colloidal silver staining method of protein blots on nitrocellulose membranes, J. Folia Biol. (Prague) 33, 253.

Laduron, P.M., P.F.M. Janssen, W. Gommeren and J.E. Leysen, 1981, In vitro and in vivo bonding characteristics of a new long-acting histamine H_1 antagonist, astemizole, Mol. Pharmacol. 21, 294.

Lau, H.Y.A. and F.L. Pearce, 1989, Effects of antihistamines on isolated human lung mast cells, basophil leukocytes and erythrocytes, Agents Actions 27, 83.

Lau, H.Y.A. and F.L. Pearce, 1990, Effects of antihistamines on isolated rat peritoneal mast cells and on model membrane systems, Agents Actions 29, 151.

Mason, M.J., B. Mayer and L.J. Hymel, 1993, Inhibition of Ca^{2+} transport pathways in thymic lymphocytes by econazole, miconazole and SKF 96365, Am. J. Physiol. 264, C654.

Metzger, H., 1992, Transmembrane signaling: The joy of aggregation, J. Immunol. 149, 1477.

Minoguchi, K., M. Benhamou, W.D. Swaim, Y. Kawakami, T. Kawakami and R.P. Siraganian, 1994, Activation of protein tyrosine kinase $p72^{syk}$ by Fc RI aggregation in rat basophilic leukemia cells, J. Biol. Chem. 269, 16902.

Miyazaki, J., K. Hideg and D. Marsh, 1992, Interfacial ionization and partitioning of membrane-bound local anaesthetics, Biochim. Biophys. Acta 1103, 62.

Niemegeers, C.J.E., F. Awouters and P.A.J. Janssen, 1986, The pharmacological profile of a specific, safe effective and non-sedative anti-allergic, astemizole, Agents Actions 18, 141.

Offermans, S., S.V.P. Jones, E. Bombien and G. Schultz, 1994, Stimulation of mitogen-activated protein kinase activity by different secretory stimuli in rat basophilic leukemia cells, J. Immunol. 152, 250.

Palmer, T.M., T.W. Gettys and G.L. Stiles, 1995, Differential interaction with and regulation of multiple G-proteins by the rat A_3 adenosine receptor, J. Biol. Chem. 270, 16895.

Paulussen, J.J.C., M.J.E. Fischer, E.P.W. Roelofsen, D.A. Horbach, N.J. de Mol and L.H.M. Janssen, 1996a, Oxatomide and derivatives as inhibitors of mediator release from a mast cell model; structure activity relationships, Arzneim. Forsch.-Drug Res. 46, 496.

Paulussen, J.J.C., M.J.E. Fischer, J.A.E. Kok-Van Esterik, R.C. Tiemessen, N.J. De Mol and L.H.M. Janssen, 1996b, Influence of the anti-allergic drug oxatomide on the signal transduction mechanism in a mast cell model, Eur. J. Pharmacol. 312, 121.

Ramkumar, V., G.L. Stiles, M.A. Beaven and H. Ali, 1993, The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells, J. Biol. Chem. 268, 16887.

Richards, D.M., R.N. Brogden, R.C. Heel, T.M. Speight and G.S. Avery, 1984, Astemizole, a review of its pharmacodynamic properties and therapeutic efficacy, Drugs 28, 38.

Scharenberg, A.M. and J.P. Kinet, 1994, Initial events in Fc RI signal transduction, J. Allergy Clin. Immunol. 94, 1142.

Swieter, M., M.M. Hamawy, R.P. Siraganian and S.E. Mergenhagen, 1993, Mast cells and their microenvironment: the influence of fibronectin and fibroblasts on the functional repertoire of rat basophilic leukemia cells, J. Periodontol. 64, 492.

Tasaka, K., M. Akagi, K. Izushi and M. Mio, 1990, Antiallergic effects of astemizole on immediate type hypersensitivity reactions, Methods Find. Exp. Clin. Pharmacol. 12, 531.

Thastrup, O., P.J. Cullen, B.K. Drøbak, M.R. Hanley and A.P. Dawson, 1990, Thapsigargin, a tumor promotor, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase, Proc. Natl. Acad. Sci. USA 87, 2466.

Waterkeyn, C., P. Laduron, W. Meuldermans, A. Trouet and Y. Schneider, 1987, Uptake, subcellular distribution and biotransformation of [^{3}H]-labelled astemizole in cultured rat hepatocytes, Biochem. Pharmacol. 36, 4129.

Yasuda, M., Y. Hasunuma, H. Adachi, C. Sekine, T. Sakanishi, H. Hashimoto, C. Ra, H. Yagita and K. Okumura, 1995, Expression and function of fibronectin binding integrins on rat mast cells, Int. Immunol. 7, 251.