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Dual Effect of the Anti-Allergic Astemizole on Ca^{2+} Fluxes in Rat Basophilic Leukemia (RBL-2H3) Cells: Release of Ca^{2+} from Intracellular Stores and Inhibition of Ca^{2+} Release-Activated Ca^{2+} Influx

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ABSTRACT. The antiallergic drugs astemizole and norastemizole inhibit exocytosis in mast cells, which might be relevant for their therapeutic action. From previous studies, it appeared that the drugs inhibited $^{45}Ca^{2+}$ influx. Here, we present a more detailed study on the effects of astemizole and norastemizole on Ca^{2+} fluxes. Fura-2-loaded rat basophilic leukemia (RBL-2H3) cells were activated through the high-affinity receptor for IgE (FcεRI) with antigen or by the endoplasmatic reticulum ATPase inhibitor thapsigargin, bypassing direct FcεRI-related events. It appeared that astemizole (>15 μM), in contrast to norastemizole, showed a dual effect on intracellular calcium concentration ($[Ca^{2+}]_i$): a rise in intracellular calcium concentration was induced, which originated in the release of intracellular Ca^{2+} stores, whereas Ca^{2+} influx via store-operated Ca^{2+} (SOC) channels was inhibited. Ca^{2+} influx was further characterized using Ba^{2+} influx, whereas processes in the absence of Ca^{2+} influx were studied using Ni^{2+} or EGTA. It was concluded that the drugs most likely affect the store-operated Ca^{2+} channels in RBL cells directly. The two effects of astemizole on Ca^{2+} fluxes had opposing influences on exocytosis, thereby accounting for the biphasic effect of increasing astemizole concentration on mediator release in RBL cells. BIOCHEM PHARMACOL **55**;8:1255–1262, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. astemizole; SOC; Ca^{2+} flux; RBL-2H3; exocytosis

Astemizole is a potent antiallergic and selective histamine (H_1) antagonist with a long duration of action [1]. It is quickly metabolized *in vivo*, with norastemizole as one of the major metabolites. These metabolites also show activity against allergic reactions [2, 3]. In addition to H_1 antagonism, astemizole also inhibits mast cell mediator release, which might be relevant for its therapeutic effect.

The RBL-2H3† is a frequently used model for the mucosal type of mast cells, which play a role in allergic reactions [4]. As these cells do not contain H_1 receptors, inhibition of exocytosis thus proceeds via an alternative mechanism. The signal transduction processes leading to exocytosis in RBL cells have been well investigated [4, 5]. These processes involve activation of FcεRI [4, 6], protein tyrosine phosphorylation [7, 8], and an increase in IP_3 levels [9]. IP_3 binds to its receptor on the endoplasmic reticulum, thereby activating emptying of endoplasmic Ca^{2+} stores. Regulation of Ca^{2+} entry into the cytosol is linked to the filling state of the intracellular Ca^{2+} stores [10] and is controlled by an inward I_{CRAC} [11, 12]. $[Ca^{2+}]_i$ is raised for a prolonged period by a sustained influx, which depends on the presence of extracellular Ca^{2+} [13].

Recently, it was demonstrated that antiallergic drugs can influence the mechanism of FcεRI-induced Ca^{2+} entry into mast cells. Compounds such as loratadine, AL3264 and oxatomide inhibit Ca^{2+} influx, which is important for their inhibiting effect on mast cell degranulation [14, 15]. We have demonstrated that astemizole and norastemizole inhibit exocytosis after FcεRI cross-linking in RBL-2H3 cells by affecting Ca^{2+} fluxes via SOC channels [16]. Based on these results, we further investigated actions of astemizole and norastemizole on $[Ca^{2+}]_i$ and Ca^{2+} fluxes in RBL-2H3 cells using FcεRI- or TG [17] activation to characterize the mechanism by which the drugs affect Ca^{2+} homeostasis. The divalent cation Ba^{2+}, which passes SOC channels, was used to study the influx mechanism [18]. With Ni^{2+}, a blockade of SOC influx could be established. Furthermore, EGTA was used in studies excluding Ca^{2+} influx. It is concluded that the antiallergic drug astemizole induces release of Ca^{2+} from internal Ca^{2+} stores and inhibits Ca^{2+}

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† *Abbreviations:* $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DNP_{30}-HSA, human dinitrophenyl albumin; FcεRI, the high-affinity receptor for IgE; I_{CRAC}, Ca^{2+} release-activated extracellular Ca^{2+} current; IP_3, inositol 1,4,5-trisphosphate; K_d, dissociation constant; RBL-2H3, rat basophilic leukemia cells; SOC, store-operated Ca^{2+} channel; TG, thapsigargin.

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influx most likely by a direct effect on SOC channels. The metabolite norastemizole only inhibits Ca^{2+} influx. This inhibition of SOC channels constitutes the major cause of inhibition of exocytosis, while the release from the intracellular stores is responsible for exocytosis in resting cells exposed to higher astemizole concentrations (>30 μM).

MATERIALS AND METHODS

Materials

Astemizole and norastemizole (for structures see [16]) were generously provided by the Janssen Research Foundation. Stock solutions of these compounds, Fura-2-acetoxymethylester (Fura-2-AM, Sigma), and thapsigargin (Calbiochem) were prepared in dimethylsulfoxide. Antidinitrophenyl IgE (Sigma), human dinitrophenyl albumin (DNP_{30}-HSA, Sigma) and $^{45}CaCl_2$ ($^{45}Ca^{2+}$, Amersham) were prepared in buffer solution, as indicated.

Cell Culture

RBL-2H3 cells were cultured in flasks and used as previously described [19]. After harvesting, the cells were plated in 24-well tissue culture plates at a density of 4×10^5 cells/well and incubated overnight to be used in the $^{45}Ca^{2+}$ influx experiments. The cells used in $[Ca^{2+}]_i$ measurements were treated as described in the section concerned. The cells were sensitized with IgE (1 μg/mL) for one hr in Eagle's minimal essential medium, after which the medium was replaced by a Tyrode's salt buffer (137 mM of NaCl, 2.7 mM of KCl, 0.31 mM of NaH_2PO_4, 12 mM of $NaHCO_3$, 1.8 mM of $CaCl_2$, 0.5 mM of $MgCl_2$, 10 mM of HEPES, 5.6 mM of glucose, 0.1% bovine serum albumin (BSA), pH 7.4). After a resting period of 10 min, indicated concentrations of the drug were added and coincubated for 10 min. The cells were subsequently activated with either antigen (DNP_{30}-HSA, 40 ng/mL) or TG (0.2 μM). In experiments where $NiCl_2$ was used as a Ca^{2+} channel inhibitor, this was added prior to the drug.

Measurement of $^{45}Ca^{2+}$ Influx

RBL cells cultured overnight in well plates were activated at 37° in the presence of $^{45}CaCl_2$ (30 μCi/mL and 10 μCi/mL for antigen and TG activation, respectively). The duration of activation with antigen or TG was 5 or 10 min, respectively. Thereafter, cell stimulation was terminated by cooling on ice-water, and the cells were immediately washed four times with ice-cold buffer. The incorporated $^{45}Ca^{2+}$ was measured after lysis with 1% Triton X-100 in a liquid scintillation counter. $^{45}Ca^{2+}$ influx was determined as a percentage of control influx (no drug present), and samples and controls were corrected for either natural leakage into the cell or residual cell-bound $^{45}Ca^{2+}$ (±5% of control).

Measurement of $[Ca^{2+}]_i$

The time-course of $[Ca^{2+}]_i$ was determined both in cell suspensions and in monolayers of cells. For the measurements 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 of sulfinpyrazone, and loaded with Fura-2-AM (10 μM) for 15 min at 37°, and thereafter washed three times with Tyrode's buffer containing gelatin (0.05%) instead of BSA and sulfinpyrazone (0.1 mg/mL). The Fura-loaded cells were resuspended in 400 μL 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 gelatin (0.05%), and Ca^{2+} was assayed in stirred cuvettes at 37° as indicated below.

For the measurements in monolayers, sterile glass coverslips were incubated overnight in 12-well plates with 5×10^5 RBL cells/well, after which the medium was replaced with fresh medium containing IgE (0.4 μg/mL). The coverslips were then incubated for at least 30 min. This medium was subsequently replaced by Tyrode's buffer. This buffer and all other buffers contained sulfinpyrazone (0.1 mg/mL). The cells were loaded with 2 μM of Fura-2-AM for 30 min at 37°, washed twice with Tyrode's buffer, and allowed to rest for 10 min at room temperature. The buffer was replaced with a gelatin (0.05%) containing Tyrode's buffer without BSA. The coverslips were transferred to a stirred quartz cuvette with 1.6 mL of Tyrode's buffer.

For the measurement of $[Ca^{2+}]_i$, fluorescence was monitored with a dual wavelength filter-fluorometer (Photon Technology International) at 37° (excitation 340/380 nm, emission 510 nm). The $[Ca^{2+}]_i$ was calculated by calibration using ionomycin (3 μM) and EGTA (20 mM). With cells in suspension, Triton X-100 (0.1%) was added after ionomycin to quickly obtain basal levels. For calculations of $[Ca^{2+})]_i$, a K_d (dissociation constant) of 224 nM was used [20]. During the experiment, EGTA, $NiCl_2$, $BaCl_2$, TG, DNP_{30}-HSA and drugs were added as indicated.

RESULTS

Effect of Astemizole on $[Ca^{2+}]_i$ after Cell Stimulation

The effect of astemizole on $[Ca^{2+}]_i$ was investigated with cells in suspension and in monolayer. Cells in suspension reacted comparably to those in monolayers (not shown), but the assay of $[Ca^{2+}]_i$ was more reproducible. Therefore, it was decided to primarily study $[Ca^{2+}]_i$ in cells in suspension, with occasional controls using adhered cells. The rate and extent of the drug-induced increase in $[Ca^{2+}]_i$ in the resting cells increased with the concentration of astemizole given (Fig. 1A). In the presence of 30 μM of astemizole, which is high enough to fully inhibit the antigen-induced $[Ca^{2+}]_i$ increase, a drug-induced rise in

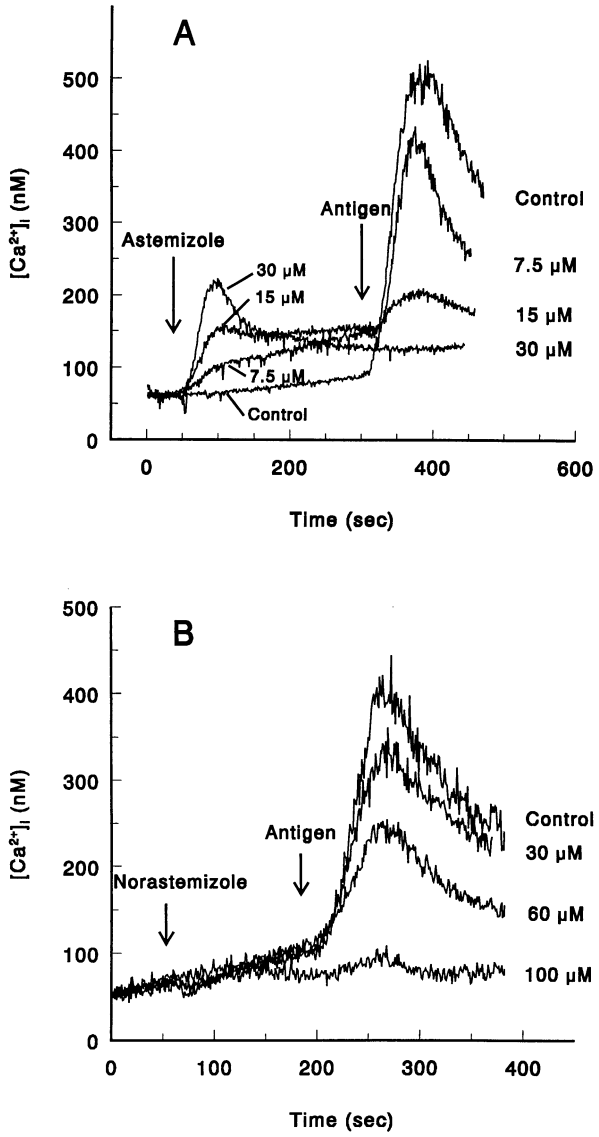

FIG. 1. The effect of astemizole (A) and norastemizole (B) on $[Ca^{2+}]_i$ in RBL-2H3 cells in suspension. Freshly isolated cells were sensitized with IgE (1 μg/mL), loaded with Fura-2-AM (10 μM), and exposed to vehicle (control) or drug at the indicated concentrations. Cells were activated with DNP_{30}-HSA (40 ng/mL) as indicated by the arrow.

$[Ca^{2+}]_i$ which was approximately 35% of that due to antigen in the absence of drug was observed. The drug-induced response did not return to basal values within experimental time limits. The rise in $[Ca^{2+}]_i$ caused by astemizole was quite remarkable and was not observed with other antiallergic drugs we recently investigated [15, 21]. Norastemizole up to 100 μM did not show a drug-induced rise in $[Ca^{2+}]_i$ in resting cells (Fig. 1B). After FcεRI activation, norastemizole decreased the rise in the $[Ca^{2+}]_i$ concentration dependently. However, the effect was less than that of astemizole. These results demonstrate that astemizole has a dual effect on $[Ca^{2+}]_i$\: a drug-induced rise immediately after addition of the drug and an inhibition of the antigen-activated increase in $[Ca^{2+}]_i$.

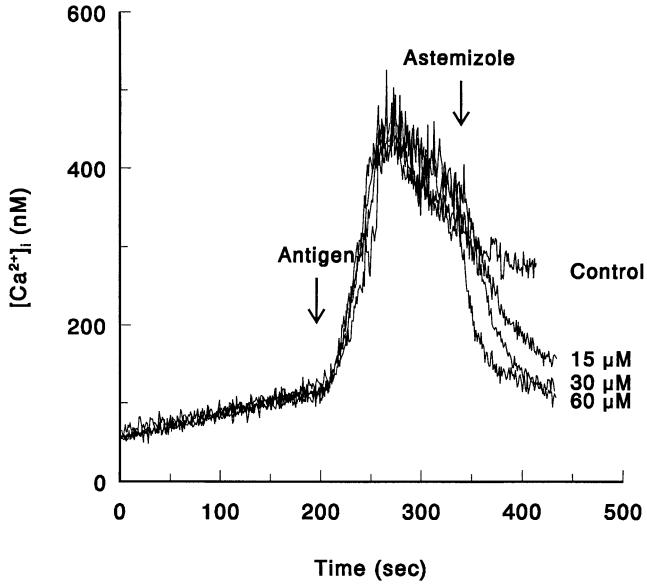

FIG. 2. The effect of astemizole on the sustained phase of $[Ca^{2+}]_i$ elevation in RBL-2H3 cells in suspension. Sensitized and Fura-2-loaded cells were triggered with antigen (40 ng/mL). Astemizole was added at the time indicated with the arrow and at the indicated concentration.

To study the effect on Ca^{2+} influx alone, astemizole was added to the cells after antigen activation had initiated emptying of the stores and opened plasma membrane Ca^{2+} channels, resulting in a sustained increase in $[Ca^{2+}]_i$ (Fig. 2). Compared to the control, astemizole immediately decreased the sustained $[Ca^{2+}]_i$ to base line values in a concentration-dependent manner. In contrast to the resting cells in Fig. 1A, no increase in $[Ca^{2+}]_i$ following addition of the drug was observed, suggesting that the astemizole-induced rise in $[Ca^{2+}]_i$ did not originate from influx from the extracellular medium.

A more direct way to activate emptying of intracellular Ca^{2+} stores and promote influx of extracellular Ca^{2+}, bypassing immediate FcεRI-related events, is stimulation with TG [17, 22]. TG specifically blocks the Ca^{2+} ATPase in the endoplasmic reticulum, and this prevention of Ca^{2+} reuptake into the intracellular stores induces an increase in $[Ca^{2+}]_i$ due to store leakage and the subsequent sustained emptied state of the Ca^{2+} stores. The results of experiments with TG activation are shown in Fig. 3. Obviously, the astemizole-induced rise in $[Ca^{2+}]_i$ observed in resting cells was similar to that in Fig. 1. TG activation induced a larger increase in $[Ca^{2+}]_i$ than activation with antigen; however, the overall form of the sustained phase was similar to that observed with antigen stimulation. Astemizole inhibited the TG-activated increase in $[Ca^{2+}]_i$ concentration dependently, whereas norastemizole reduced the $[Ca^{2+}]_i$ at 30 μM (Fig. 3). For both drugs, the relative decrease in the maximum rise in $[Ca^{2+}]_i$ was somewhat lower than in Fig. 1. Considering the more direct activation of Ca^{2+} influx with TG, the similarities in Figs. 1 and 3 indicate that the effect of the drugs after FcεRI activation was mainly due to inhibition of Ca^{2+} fluxes.

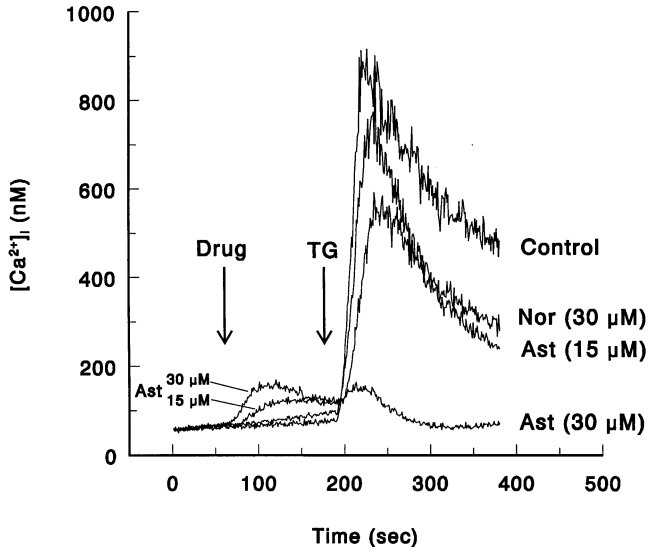

FIG. 3. Effects of norastemizole (Nor) and astemizole (Ast) on $[Ca^{2+}]_i$ in thapsigargin (0.2 μM)-stimulated RBL-2H3 cells. Drugs at the indicated concentration were added to the cells 2.5 min prior to stimulation.

Effects on $[Ca^{2+}]_i$ Excluding Extracellular Ca^{2+} Influx

Experiments excluding influx of extracellular Ca^{2+} by binding Ca^{2+} to EGTA can provide more insight into the effect of the drugs on the release of Ca^{2+} from intracellular stores (Fig. 4). The control experiment without drug shows that stimulation of the cells increased $[Ca^{2+}]_i$ to a peak height of approximately 80–100 nM above basal level, which was 350 nM lower than with FcεRI activation without EGTA (Figs. 1 and 4A), and 700 nM lower than with TG stimulation (Figs. 3 and 4B). The difference can be explained by the absence of Ca^{2+} influx in the presence of EGTA; the increase in signal solely reflects emptying of stores. The subsequent rapid rate of decline in the $[Ca^{2+}]_i$ of the controls was caused by efflux of Ca^{2+} to the extracellular space in the case of the TG stimulus and a combined effect of efflux and reuptake in stores in the case of antigen stimulation. Emptying of the intracellular Ca^{2+} stores by either antigen or TG activation opened SOC channels. This was demonstrated by the immediate rise in $[Ca^{2+}]_i$ upon addition of a concentration of Ca^{2+} which exceeded that of EGTA (Fig. 4).

Figure 4 also represents the influence of astemizole and norastemizole at 30 μM in the presence of EGTA. The astemizole-induced increase in $[Ca^{2+}]_i$ before cell stimulation was again observed, but the maximum signal was only approximately 30% of the original response in the presence of free extracellular Ca^{2+}. This demonstrates that the drug-induced rise in $[Ca^{2+}]_i$ in resting cells was initiated with release of Ca^{2+} from the stores and that this generated an influx of extracellular Ca^{2+}. In the presence of free extracellular Ca^{2+}, this influx caused the additional 70% of the astemizole-induced $[Ca^{2+}]_i$ response. After restoring the free extracellular $[Ca^{2+}]$, astemizole inhibited the influx of Ca^{2+} (Figs. 4A and 4B), which once more shows

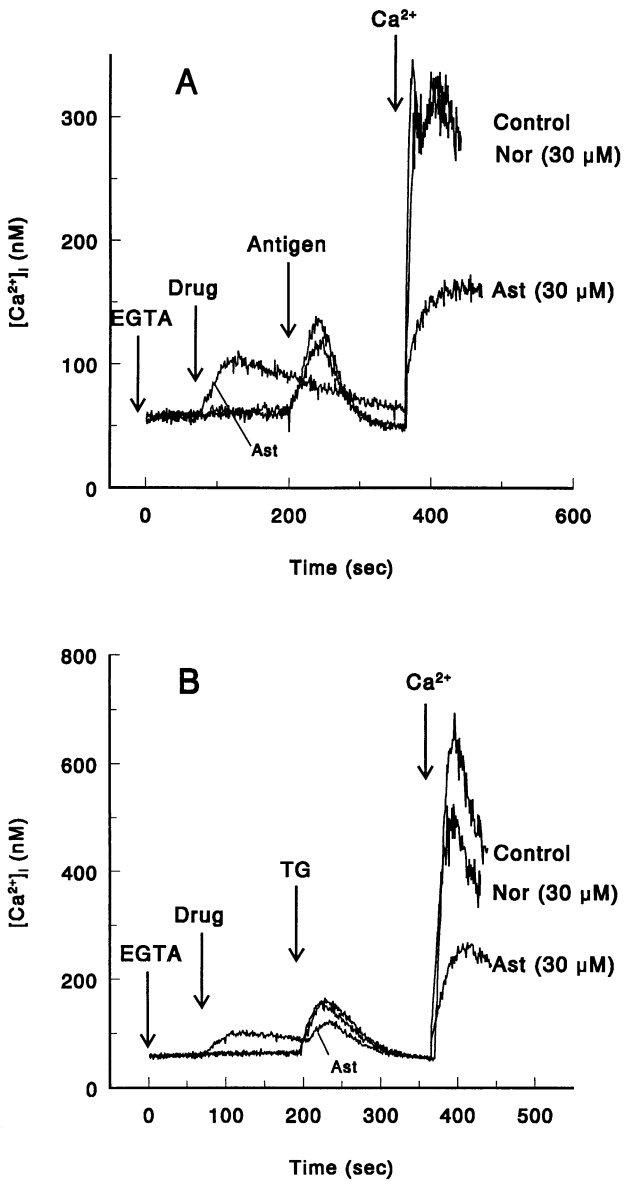

FIG. 4. Influence of astemizole (Ast) and norastemizole (Nor) on $[Ca^{2+}]_i$ in RBL-2H3 cells without free extracellular calcium, which was bound to EGTA (3 mM) prior to the addition of drug (30 μM). The sensitized and suspended RBL-2H3 cells were either stimulated with antigen (A) or thapsigargin (B) as indicated by the arrow. Finally, Ca^{2+} (5 mM) was added to the cells to restore the extracellular concentration (approximately 2 mM).

that astemizole inhibited SOC influx. The antigen-induced rise in $[Ca^{2+}]_i$ was absent in the presence of astemizole and EGTA (Fig. 4A). Apparently, upon addition the drug had already emptied the IP_3-sensitive Ca^{2+} stores.

With TG stimulation, a reduction in $[Ca^{2+}]_i$ was observed in the presence of astemizole (Fig. 4B). TG also empties non-IP_3-sensitive stores [22], and it is likely that astemizole did not deplete all these stores. The structurally related drug norastemizole exhibited a different behaviour in this experiment: it did not induce store depletion and had no significant effect on the inhibition of the FcεRI- or

TG-activated emptying of intracellular Ca^{2+} stores. However, after restoration of extracellular Ca^{2+}, norastemizole inhibited Ca^{2+} influx. From this, we conclude that norastemizole does not affect intracellular Ca^{2+} stores but only inhibits SOC channels.

The decrease in the $[Ca^{2+}]_i$ beyond the maximum induced by TG activation (Fig. 4B) proceeded with first-order kinetics, with a half-life of approximately 60 sec. Neither drug had any effect on this half-life time, from which we conclude that the drugs have no effect on the efflux of Ca^{2+} to the extracellular space or the uptake into intracellular stores. In the control experiment, the response after depletion of the stores using antigen trigger rapidly returned to basal values due to the absence of Ca^{2+} influx. In contrast, with 30 μM of astemizole the initial drug-induced rise in $[Ca^{2+}]_i$ decreased fairly slowly (Fig. 4A). A possible explanation for this slow decay is that Ca^{2+} is pumped back into the stores, from where it is re-released by the effect of the drug.

Effect of Ba^{2+} on the $[Ca^{2+}]_i$ Signal

The effects of the drugs on SOC channels can be further characterized using Ba^{2+}. This cation enters the cell specifically via SOC channels of RBL and mast cells [11] and shifts the 340/380 fluorescence ratio of Fura-2. The entrapped Ba^{2+} cannot be pumped back into stores or out of the cell [18]. To avoid coentry of Ca^{2+}, extracellular Ca^{2+} is complexed with EGTA. In the presence of Ca^{2+}, Ba^{2+} is not expected to be complexed with EGTA, as it has a much lower complex constant [23]. Basal influx of Ba^{2+} in resting cells appeared to be negligible (results not shown), in agreement with Lee *et al.* [18]. The cells were stimulated with TG to open SOC channels (Fig. 5). After the response returned to basal level, addition of Ba^{2+} (0.5 mM) steadily increased the 340/380 fluorescence ratio, reflecting Ba^{2+} accumulation into the cytosol via the open SOC channels. The influx of Ba^{2+} was discerned from that of Ca^{2+} in that it was slower (see also Figs. 3 and 4). When astemizole was present at a concentration of 30 μM, Ba^{2+} influx was strongly reduced.

Inhibition of SOC Influx by Ni^{2+}

It was found in various cell types that the transition metal Ni^{2+} inhibited Ca^{2+} entry by nonspecifically blocking voltage-gated Ca^{2+} channels [24] and voltage-independent Ca^{2+} channels [25], and by inhibiting I_{CRAC} in rat peritoneal mast cells [12], which is similar to I_{CRAC} in RBL cells [11]. Table 1 shows the results of the effect of 0.1 and 1 mM of Ni^{2+} on $^{45}Ca^{2+}$ influx after FcεRI stimulation. A somewhat higher inhibiting activity of Ni^{2+} on influx was found compared to the IC_{50} (concentration of drug for 50% inhibitory activity) of 0.35 mM reported previously [25]. Addition of 30 μM of astemizole in the presence of Ni^{2+} had no influence on Ca^{2+} influx either in antigen-activated or in resting cells. We studied the possibility of using

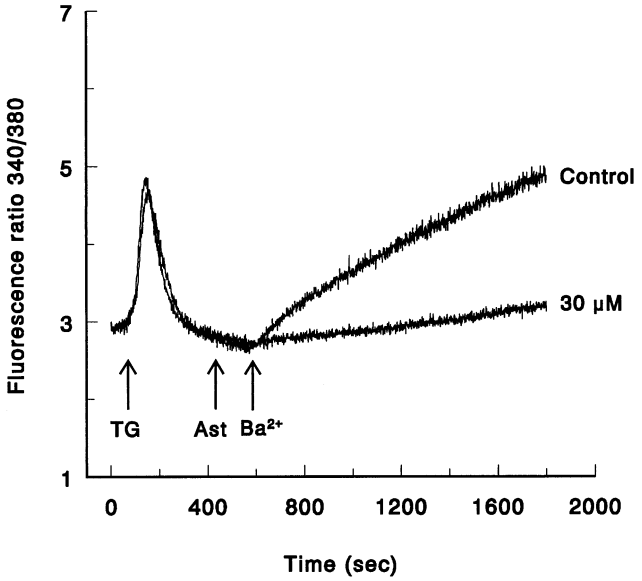

FIG. 5. Effects of astemizole on Ba^{2+} influx in RBL-2H3 cells in monolayer. After removal of extracellular Ca^{2+} with EGTA (3 mM), the cells were triggered with thapsigargin (0.2 μM). Astemizole was added at the indicated concentration after depletion of intracellular Ca^{2+} stores by thapsigargin and return of $[Ca^{2+}]_i$ to base line values. Immediately thereafter, Ba^{2+} (0.5 mM) was added (see arrow) and the course of Ba^{2+} influx was monitored in fluorescence ratio mode. The shift of the Fura-2 signal by binding of Ba^{2+} is recorded as an increase in the 340/380 fluorescence ratio.

Ni^{2+} instead of EGTA as a tool to investigate intracellular Ca^{2+} fluxes, because EGTA slowly extracts intracellular Ca^{2+} [26]. As Ni^{2+} quenches Ca^{2+}-induced Fura-2 fluorescence (data not shown), we first performed a control experiment to demonstrate that Ni^{2+} did not enter the RBL cells (Fig. 6). In the control response, it can be observed that antigen activation opened SOC channels, after which the Ca^{2+} ionophore ionomycin raised $[Ca^{2+}]_i$ to its maximum (ionomycin is normally used for calibration of the Ca^{2+} signal). Addition of the detergent TritonX-100 perforated the cell membrane and exposed intracellular Fura-2 to the extracellular buffer containing high $[Ca^{2+}]$. The fluorescence signal did not change, probably because saturation of Ca^{2+} binding to Fura-2 had already taken place after ionomycin addition. Finally, addition of EGTA

TABLE 1. Effect of Ni^{2+} on the influx of $^{45}Ca^{2+}$ in antigen-activated RBL cells

Inhibitor (μM)	$^{45}Ca^{2+}$ influx (% of control, N = 4)
Ni^{2+}	
100	25.3 ± 11.8
1000	−5.9 ± 0.9
Ni^{2+} with 30 μM of astemizole	
100	27.3 ± 4.2
1000	−1.8 ± 2.8

When astemizole was present, Ni^{2+} was added 10 min prior to the drug, after which cells were stimulated with DNP_{30}-HSA (40 ng/mL).

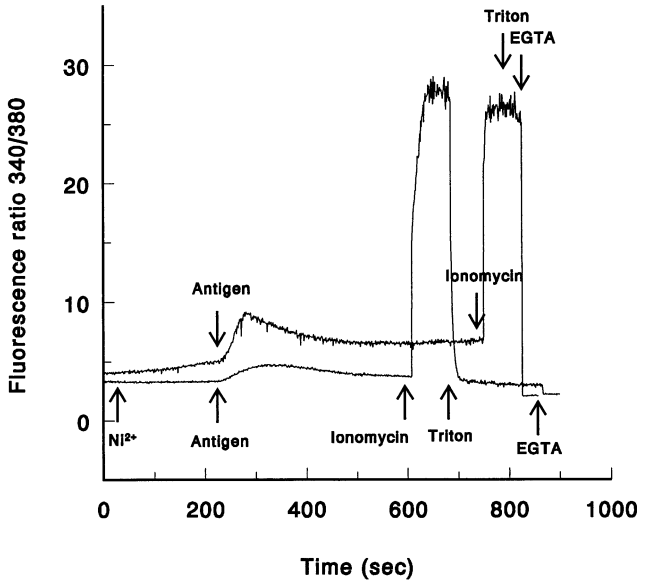

FIG. 6. Effect of Ni^{2+} on $[Ca^{2+}]_i$ in RBL-2H3 cells in suspension. The upper curve is a control trace of $[Ca^{2+}]_i$ of cells that were sensitized with IgE and triggered with DNP_{30}-HSA (40 ng/mL). Maximal elevation of $[Ca^{2+}]_i$ was obtained by applying ionomycin (3 μM). As indicated, cells were permeabilized with Triton X-100 (0.1%), after which Ca^{2+} was complexed with EGTA (20 mM) to obtain the minimal Fura-2 response. In the lower curve, Ni^{2+} (1 mM) was applied prior to antigen stimulation.

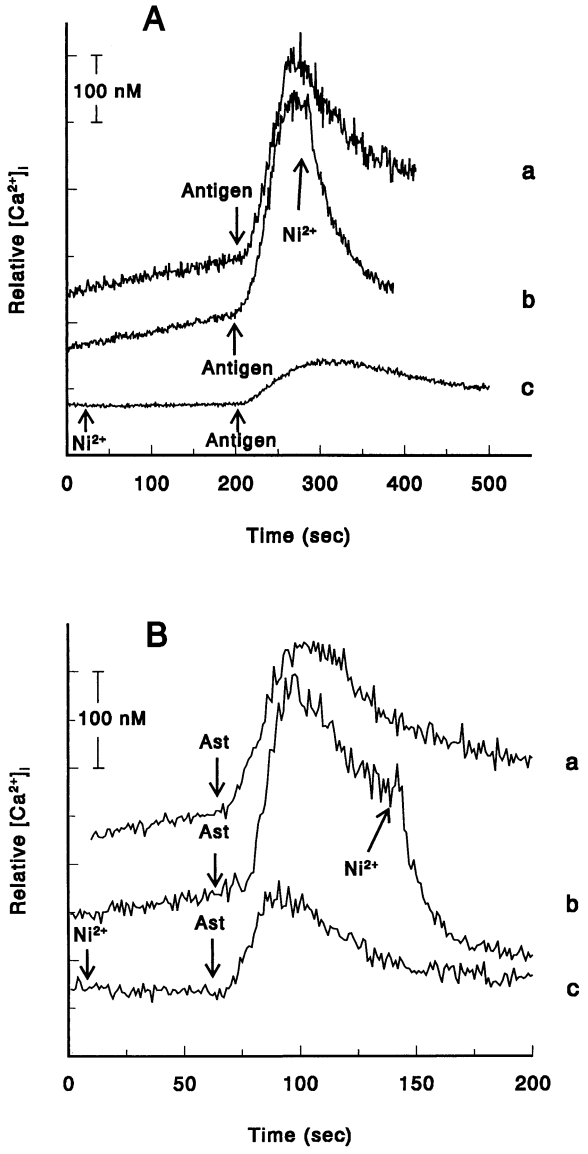

FIG. 7. The influence of Ni^{2+} on intracellular calcium concentration in DNP_{30}-HSA (40 ng/mL, A) and astemizole (30 μM, B) stimulated RBL-2H3 cells. The $[Ca^{2+}]_i$ responses are shifted relative to each other for clarity of presentation. Controls (without Ni^{2+} are shown in curves a, whereas Ni^{2+} (1 mM) was added in the sustained phase (curves b) or prior to the addition of drug or antigen (curves c).

brought the response to basal values. There was a marked difference in the response when Ni^{2+} was added prior to stimulation with antigen. Firstly, the response of the fluorescence ratio on antigen stimulation was much weaker. As 1 mM of Ni^{2+} inhibited $^{45}Ca^{2+}$ influx completely (Table 1), the increase in $[Ca^{2+}]_i$ upon antigen stimulation in the presence of Ni^{2+} may only be attributed to emptying of Ca^{2+} stores. Secondly, treatment with Triton X-100 immediately decreased the signal to almost basal values. Finally, the remaining slight Ca^{2+} signal disappeared upon binding of Ca^{2+} to EGTA. If Ni^{2+} had entered the cell, the increase in the fluorescence ratio upon addition of ionomycin would be much lower than that of the control due to quenching of Fura-2 fluorescence. This quenching does occur in the presence of Ni^{2+} after addition of detergent. Therefore, it can be concluded that Ni^{2+} does not interfere in the assay of $[Ca^{2+}]_i$.

In Fig. 7, the effect of Ni^{2+} on $[Ca^{2+}]_i$ was investigated either after antigen stimulation or with 30 μM of astemizole. In signal b, it was observed that Ni^{2+} immediately decreased the elevated $[Ca^{2+}]_i$ in either situation when introduced in the sustained phase. In both situations, opened SOC channels were blocked by Ni^{2+}. If Ni^{2+} was introduced prior to antigen (Fig. 7A, signal c), the maximum response in $[Ca^{2+}]_i$ decreased by 70%. The sustained phase very slowly returned to basal levels. The reduction in the $[Ca^{2+}]_i$ increase was due to a Ni^{2+} blockade of Ca^{2+} influx, whereas the remaining response only represented emptying of Ca^{2+} stores. When Ni^{2+} was added before astemizole (Fig. 7B, signal c), it was observed that the astemizole-induced rise in $[Ca^{2+}]_i$ was also reduced and that the rate of return to basal values was again comparable to the control without Ni^{2+} ($t_{1/2}$ of *ca.* 85 sec). Although different methods are used to prevent Ca^{2+} influx, the similarity of the results illustrated in Figs. 5 and 7 strengthen the conclusion that astemizole empties Ca^{2+} stores and inhibits SOC influx.

DISCUSSION

Whereas our previous work demonstrated that astemizole affected Ca^{2+} fluxes [16], we have now presented a more

detailed study on the effects of the antiallergic drug astemizole and its metabolite norastemizole on Ca^{2+} fluxes in RBL-2H3 cells.

Astemizole has a dual effect on Ca^{2+} fluxes, a phenomenon also reported for the lipophilic compounds econazole, miconazole and SKF 96365 in lymphocytes [27], and for the much less lipophilic compound tenidap in RBL-2H3 cells [28]. On the one hand, astemizole induces the release of Ca^{2+} from internal stores in resting cells leading to SOC influx (Figs. 4 and 7), while on the other it inhibits SOC influx as illustrated in Figs. 2, 3, 4, 5 and 7. Both effects occur independently of each other: the drug-induced release also takes place when there is no SOC influx activation (Figs. 4 and 7B, curve c), and conversely SOC influx is inhibited when the stores have been depleted by TG prior to the addition of astemizole (e.g. Fig. 5). We thereby conclude that astemizole affects two targets independently: the intracellular Ca^{2+} stores and SOC channels. The dual effects of astemizole on Ca^{2+} fluxes do not normally occur separately. The astemizole-induced rise in $[Ca^{2+}]_i$ shown in Fig. 1 consists of release from intracellular stores inducing activation of SOC influx, which is inhibited by the drug. The reduction in the antigen-induced elevation of $[Ca^{2+}]_i$ is a combined effect of less release from IP_3-sensitive stores, due to drug-induced depletion, and of inhibition of SOC influx. The structurally related drug norastemizole behaved quite differently by only inhibiting SOC influx, albeit somewhat less efficiently than astemizole.

In a previous study, we demonstrated that inhibition of Ca^{2+} influx was the major cause of the inhibition of mediator release [16]. In principle, the inhibition of SOC influx might be caused by several mechanisms, e.g. direct inhibition of SOC channels, inhibition of inward or outward rectifying K^+-channels, or by effects on Ca^{2+} efflux from the cell. The results in Fig. 4B demonstrate that astemizole has no significant effect on Ca^{2+} efflux. Furthermore, K^+-channels related to SOC influx are inhibited by 1 mM of Ba^{2+} [29]. This makes inhibition of the SOC influx by astemizole under the conditions in Fig. 5 through an effect on K^+-channels unlikely. Therefore, our results suggest a direct effect of astemizole on the SOC channels. Some other highly lipophilic drugs have been reported to inhibit various ion channels, e.g. econazole [30]. Therefore, the effect of astemizole is likely not specific for SOC channels alone. The relation between lipophilicity and aspecific action on ion channels is not unambiguous, as a less lipophilic compound such as tenidap also shows effects on several ion channels [30] and a lipophilic compound such as ketotifen has only low inhibitory activity [30].

Hoth and Penner [31] first introduced I_{CRAC}, distinguished from Ca^{2+} influx via other Ca^{2+} entry channels. Recently, great interest in SOC channels has arisen [for reviews see 32–34]. As yet, the SOC channel has not been characterized. SOC channels are different from voltage-operated (VOC) Ca^{2+} channels found in electrically excitable cells, but they share the extremely high selectivity for Ca^{2+} and Ba^{2+} [11]. Several suggestions have been made as to how the filling state of the Ca^{2+} store regulates the I_{CRAC}, including changes in protein phosphorylation. In a previous study, we observed that astemizole affects the tyrosine phosphorylation of cellular proteins in activated and resting RBL cells [16]. Another possibility is that the drugs affect SOC channels by way of membrane perturbation. The high lipophilicity of astemizole contributes to the distortion of membranes. This does not mean that the effect of astemizole is completely aspecific and not related to the detailed molecular structure. In addition, norastemizole inhibits SOC influx. In studying a series of astemizole derivatives, we found that inhibition of SOC influx depends on chemical structure [35]. Furthermore, a highly lipophilic and membrane-perturbing compound such as meclozine does not inhibit SOC influx [15].

The concentration range may seem high in comparison to *in vivo* situations. In our opinion, an *in vitro* cellular model and *in vivo* mast cells have different characteristics. For example, *in vivo* the drugs may also be able to interact with other cells involved in the allergic response that contain SOC channels (e.g. T cells) [36, 37].

The additional effect of astemizole on the stores, not observed with norastemizole, is not relevant in the inhibition of exocytosis, as FcεRI activation itself induces store depletion and exocytosis. Moreover, in contrast to the other antiallergic drugs we recently investigated, astemizole is able to induce exocytosis of resting cells at 30 μM (±15% degranulation of total) [16]. This astemizole-induced exocytosis can be explained by the influence of the drug on Ca^{2+} release from intracellular stores and the subsequent influx of Ca^{2+}, which ultimately leads to exocytosis. Several intracellular Ca^{2+} pools can be discerned, the location and structure of which are not known in great detail. It is generally assumed, however, that Ca^{2+} is stored in an endomembrane pool, indicating that membrane structures are relevant for these pools [38]. Astemizole is a weak base with high lipophilicity (the partition coefficient [log P] for the octanol/water system is 5.5). In model membranes, we demonstrated that astemizole, in contrast to norastemizole, affects the membrane structure [19]. Therefore, we suggest that interference with endomembrane structures is responsible for the astemizole-induced release of Ca^{2+}, which is not observed with the structurally related, but less lipophilic norastemizole.

In conclusion, astemizole simultaneously inhibits SOC channels and induces store depletion, whereas norastemizole, due to its different structure, only inhibits SOC channels. This inhibition of SOC channels is responsible for inhibition of exocytosis and is probably relevant for the antiallergic effect of astemizole and norastemizole.

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