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IN VITRO INHIBITION, BY LORATADINE AND DESCARBOXYETHOXYLORATADINE, OF HISTAMINE RELEASE FROM HUMAN BASOPHILS, AND OF HISTAMINE RELEASE AND INTRACELLULAR CALCIUM FLUXES IN RAT BASOPHILIC LEUKEMIA CELLS (RBL-2H3)

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Abstract—The effect of the H1-antihistamine drug loratadine and its active metabolite descarboxyethoxyloratadine upon histamine release was examined on anti-immunoglobulin E (IgE) triggered human basophils and 2,4-dinitrophenyl (DNP) triggered rat basophilic leukemia (RBL-2H3) cells. In both experimental systems, dose-dependent inhibition of histamine release was observed at descarboxyethoxyloratadine and loratadine doses above 2 and 7 μM, respectively. In the RBL-2H3 experimental system, inhibition by loratadine increased when the concentration of extracellular Ca^{2+} was reduced from 1.8 to 0.45 mM. We further investigated the effect of loratadine and descarboxyethoxyloratadine on the increase in cytosolic calcium concentration $(Ca^{2+})_i$, an early step in biochemical events leading to exocytosis. The effect of these two drugs upon $(Ca^{2+})_i$ changes was measured using the fluorescent probe fura-2 loaded into RBL-2H3 cells passively sensitized with DNP-specific IgE. Both drugs inhibited, in a dose-dependent manner (2.5–25 μM), the $(Ca^{2+})_i$ rise induced by DNP-BSA challenge in sensitized RBL cells, a process observed in both the presence and absence of extracellular Ca^{2+}. Loratadine also inhibited the Mn^{2+} influx into these cells, thus reflecting the Ca^{2+} influx. These results suggest that loratadine and descarboxyethoxyloratadine impair the increase in $(Ca^{2+})_i$ following cell activation by decreasing both the influx of extracellular Ca^{2+} and the release of Ca^{2+} from intracellular stores.

Key words: H_1-antihistamine; loratadine; histamine release; Ca^{2+} fluxes; human basophils; RBL cells

Inhibition of IgE§-dependent histamine release indicates that antihistamine drugs exert their effect not only upon histamine receptors but also upon histamine-containing cells [1]. These initial observations were confirmed by other *in vitro* data demonstrating the inhibitory action of these drugs on mediator release from mast cells and basophils on both human [2] and animal [3, 4] models. Recent work on new non-sedating antihistamine drugs also demonstrated an inhibitory effect on antigen- and ionophore-induced mediator release from mast cells [5, 6]. Clinical trials combined with *in vivo* experiments indicated the inhibitory effect of some H1-antihistamine drugs on allergen-induced increases in histamine levels in nasal washings [7–9]. Evidence for *in vivo* inhibition of mediator release related to therapeutic benefits necessitated further analysis of the effect of these drugs upon IgE-dependent mediator release.

The effect of H1-antihistamines and other anti-allergic drugs, on mediator release from mast cells and basophils, as well as the influence of these drugs on degranulation-associated biochemical events, namely superoxide formation [10], calmodulin and protein kinase C expression [11], intracellular cAMP level [1, 4, 12] and membrane stabilization [2, 4], have been analysed previously. However, the mechanism by which these effects occur is not fully understood. Earlier results showing increased inhibition of mediator release by antihistamine drugs when the extracellular Ca^{2+} concentration was lowered [4, 13] support the hypothesis that such drugs may interfere with the rise in cytosolic Ca^{2+} accompanying the secretion event [14].

The present study reports the inhibitory effect of loratadine, a new non-sedating H1-antihistamine, and one of its active metabolites, descarboxyethoxyloratadine, on anti-IgE-induced histamine release from human blood basophils. Possible inhibition, by loratadine and descarboxyethoxyloratadine, of calcium fluxes initiating histamine secretion was investigated in RBL-2H3 cells, since obtaining sufficient quantities of highly purified human basophils required large quantities of blood. Although RBL-2H3 cells are not exactly analogous to normal mast cells [15, 16] and basophils,

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§ Abbreviations: RBL-2H3, rat basophilic leukemia; DNP, 2,4-dinitrophenyl; fura-2-AM, fura-2-penta(acetoxymethyl) ester; IgE, immunoglobulin E.

they are widely used as an *in vitro* model for investigating the mechanisms of immediate hypersensitivity reactions. We observed here that loratadine and descarboxyethoxyloratadine inhibit the antigen-induced $(Ca^{2+})_i$ rise in DNP-sensitized RBL-2H3 cells.

MATERIALS AND METHODS

Cells

RBL-2H3 cells. RBL-2H3 cells [15] were maintained in monolayer cultures. Cell viability was greater than 95% as determined by the Trypan blue exclusion method. Subconfluent adherent RBL-2H3 cells, cultured in minimal essential medium supplemented with 15% fetal calf serum as described earlier [17], were removed upon trypsin treatment and suspended in a standard isotonic saline solution (see below).

Human peripheral blood leukocytes. Human blood was collected on heparin and mixed with glucose–dextran for red blood cell sedimentation. After centrifugation leukocytes were washed twice in Tris–albumin buffer as described previously [18, 19].

Reagents

Loratadine and descarboxyethoxyloratadine were provided by Schering-Plough (France). Stock solutions were prepared in DMSO at 10 mg/mL. Because of the poor solubility of these drugs in water (0.024 mg/mL at 37°), the initial dilution factor was at least 500, leading to a final concentration of 0.02 mg/mL in the assay medium. DNP-specific mouse IgE and $DNP_{43}BSA$ (DNP-BSA) were a generous gift of Dr Annie Prouvost-Danon [20]. Anti-human IgE (Nordic anti-Fcε serum) was used for histamine release experiments on human basophils at 1×10^4- and 1×10^3-fold final dilutions. Fura-2-AM (Molecular Probes) was dissolved in DMSO at 1 mM and stored at 4°.

Buffers

For all experiments with RBL cells, a standard isotonic saline solution (NaCl 135 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM, glucose 5.6 mM, 0.5 mg/mL gelatin, pH 7.4) was used. In order to block the effect of Ca^{2+} present in the medium, EGTA at a final concentration of 3 mM was added. Blood leukocytes were washed twice in Tris-albumin buffer, and the histamine release assays were performed in Tris-albumin-Ca^{2+}-Mg^{2+} buffer [18].

Histamine release experiments

Human blood leukocytes. Histamine release assays were performed on twice washed human blood leukocytes, resuspended in Tris-albumin-Ca^{2+}-Mg^{2+} at 2×10^6 cells/mL. Anti-IgE (10 μL) and the drugs (30–500 μL) were added to the cells at a final volume of 1 mL and incubated for 30 min at 37°. The reactions were terminated by addition of 0.1 mL EDTA 125 mM. The supernatants and lysed cell aliquots acidified with 0.4 N perchloric acid were analysed for histamine content. Controls for possible direct histamine release by the drugs alone and by DMSO were also included.

RBL-2H3 cells. RBL-2H3 cells (2×10^6 cells/mL) were incubated for 45 min at 37° in buffered saline medium containing 0.05% gelatin (pH = 7.4) and mouse DNP-specific IgE (2 μg/mL). At the end of this period, cells were washed with the same buffer and resuspended at 6×10^5 cells/mL. Aliquots of 3×10^5 cells were incubated in the presence of 20 ng/mL DNP-BSA with or without loratadine or descarboxyethoxyloratadine (final volume 0.5 mL) for 30 min at 37°. The reaction was terminated by the addition of ice-cold buffered salt solution. The following steps were performed as described for the human basophils. Controls for possible direct histamine release by the drugs alone and by DMSO were included.

Histamine measurement and expression of the results

Histamine determination was performed by the fluorometric method described by Lebel [21]. In preliminary experiments, the drugs were analysed for their autofluorescence in the histamine assay and also for their possible interference with quantification of the standard histamine solutions. Then, histamine release from experiments with anti-IgE or DNP-BSA as the inducing agent was compared with histamine release from experiments with the inducing agent and drugs. The percentage of histamine release inhibition due to the drugs is given in the figures.

Sensitization and cytosolic calcium concentration $(Ca^{2+})_i$ measurement in RBL-2H3 cells

Determination of $(Ca^{2+})_i$ was performed according to Grynkiewicz *et al.* [22]. Aliquots (1×10^6 cells) of RBL-2H3 cells were incubated for 45 min at 37° in buffered saline medium containing 0.05% gelatin (pH = 7.4) with the fluorochrome fura-2-AM (4 μM) and DNP-specific IgE (1 μg/mL). At the end of this period, cells were washed and fluorescence was monitored in a spectrofluorimeter (Hitachi F 2000—stirred and thermostated). Because of the leakiness of fura-2-loaded cells, fluorochrome loading and sensitization were performed on new cell samples for each experiment.

Fluorescence intensity was monitored at two excitation wavelengths, 340 nm and 380 nm (emission: 505 nm). $(Ca^{2+})_i$ was calculated from the ratio (R) of the fluorescence values obtained at the two wavelengths by the following equation:

$$(Ca^{2+})_i = K_d(R - R_{min}/R_{max} - R) \times F_o/F_s$$

where R_{max} and R_{min} were the maximum and minimum values of 340/380 nm ratios obtained at saturating (after addition of Triton X-100) or zero (after addition of EGTA) Ca^{2+} concentrations, respectively. K_d (224 nM) is the effective dissociation constant of fura-2 for Ca^{2+} at 37°, F_o is the 380 nm excitation efficiency in the absence of Ca^{2+} and F_s is the 380 nm excitation value at saturating Ca^{2+} concentration. No significant autofluorescence of the cells was noted.

The effects of loratadine and descarboxyethoxyloratadine on $(Ca^{2+})_i$ concentration were measured by variation in the 340/380 nm fluorescence ratio after the addition of DNP-BSA, and data obtained in the presence of the drugs were expressed

as per cent inhibition compared to control values observed in their absence. Similar experiments were performed in the absence of free Ca^{2+} by addition of 3 mM EGTA to the medium, enabling measurement of the contribution of intracellular Ca^{2+} stores to the variation noted in the cytosolic concentration of this divalent cation.

Use of $MnCl_2$ in determining Ca^{2+} influx in RBL-2H3 cells

In RBL-2H3 cells, Mn^{2+} competes with Ca^{2+} for entry into the cells [23]. This influx of Mn^{2+}, which has been shown to reflect the Ca^{2+} influx [24], was measured in RBL-2H3 cells. The technique used is based on the fact that Mn^{2+} binds to the fluorescent Ca^{2+} indicator fura-2 with a higher affinity than Ca^{2+}. Subsequent quenching of the fluorescent signal was caused by Mn^{2+} acting as a Ca^{2+} surrogate in the influx pathways, and was monitored with excitation at 360 nm, where fluorescence was independent of Ca^{2+} activity [22] and the emission wavelength at 505 nm.

RBL-2H3 cells, previously sensitized with DNP-specific IgE and loaded with fura-2, were incubated with DMSO alone or with loratadine. After 3–4 min incubation at 37° in the cell of the spectrofluorimeter, 100 μM $MnCl_2$ were added. One minute later, sensitized cells were triggered by the addition of DNP-BSA (20 ng/mL).

Statistics

All experimental values are given as mean values ± SEM.

RESULTS

Effects of loratadine and descarboxyethoxyloratadine on anti-IgE-induced histamine release in human basophils

For each blood sample, positive control experiments were performed at 1×10^4- and 1×10^3-fold dilutions of the anti-IgE serum, with these concentrations leading to suboptimal or optimal histamine release responses in most subjects. The mean control value (±SEM) for histamine release on human basophils in the absence of drug was 44.3 ± 3.9% (N = 8). In parallel experiments, either loratadine or descarboxyethoxyloratadine was added to the incubation medium at defined concentrations and anti-IgE-dependent histamine release was investigated. At descarboxyethoxyloratadine concentrations over 2 μM, and up to the highest concentration investigated, i.e. 16 μM, significant inhibition of histamine release from anti-IgE stimulated human blood leukocytes was noted (Fig. 1). Inhibition of anti-IgE-induced histamine release from human basophils by loratadine was noted only at the highest concentration studied (45 μM) (Fig. 1).

Effects of loratadine and descarboxyethoxyloratadine on histamine release from sensitized RBL-2H3 cells

Dose-dependent inhibition by loratadine and descarboxyethoxyloratadine of histamine release from DNP-sensitized RBL-2H3 cells challenged with 10 ng/mL DNP-BSA was investigated (Fig. 1). In

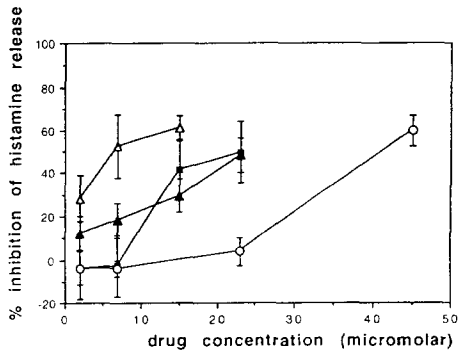

Fig. 1. Inhibition of histamine release in anti-IgE-triggered human basophils by loratadine (○) or descarboxyethoxyloratadine (△). Inhibition of DNP-BSA-induced histamine release in RBL-2H3 cells sensitized with DNP-specific IgE, by loratadine (■) or descarboxyethoxyloratadine (▲). In control experiments without drug, histamine release values were 49.0 ± 3.9% for RBL cells (N = 5) and 44.3 ± 3.9% (N = 8) for human basophils.

the control experiments without drug, histamine release was 49.0 ± 3.9% (N = 5). Increasing inhibition was observed in the concentration range from 2 to 25 μM and 7 to 25 μM, respectively, for descarboxy-ethoxyloratadine and loratadine.

At a single 20 μM concentration of loratadine, inhibition of histamine release due to the drug increased from 18.7 ± 3.7% to 63.5 ± 14.2% (N = 4) when the extracellular Ca^{2+} concentration was decreased from 1.8 to 0.45 mM (Table 1). In the absence of extracellular Ca^{2+} no histamine release was observed.

Determination of possible non-specific effects of the drugs

For descarboxyethoxyloratadine and loratadine, non-specific fluorescence was not observed in the range of concentrations (0–45 μM) used in histamine release experiments, nor was there any interference of these drugs when used alone, with histamine quantification (data not shown). Neither histamine release capacity nor cell toxicity, as evidenced by Trypan blue exclusion, was observed when cell suspensions (human basophils or RBL-2H3 cells) were incubated with the drugs at the concentration ranges studied.

Effects of loratadine and descarboxyethoxyloratadine on the $(Ca^{2+})_i$ increase induced by DNP-BSA in sensitized RBL-2H3 cells

Experiments were performed on RBL-2H3 cells sensitized with DNP-specific IgE. DNP-BSA (10 ng/mL) was added after an initial temperature equilibration period (3 min). Within 2 min of antigen addition, an increase in $(Ca^{2+})_i$ from 159 ± 5.5 (N = 29) (steady state) to 538 ± 29 (N = 18) nM was observed. Figure 2 displays the inhibitory effect of loratadine and descarboxyethoxyloratadine on $(Ca^{2+})_i$ increase induced by antigen challenge. At concentrations between 2.5 and 50 μM, these drugs inhibited the $(Ca^{2+})_i$ increase in a dose-dependent

Table 1. Inhibition by loratadine of histamine release observed in RBL-2H3 cells sensitized with DNP-specific IgE, after triggering with DNP-BSA, at various external Ca^{2+} concentrations

Extracellular Ca^{2+} concentration (mM)	Control histamine release (%)	Histamine release at 20 μM loratadine (%)	Inhibition (%)
1.8	46.4 ± 7.2	37.7 ± 5.0	18.7 ± 3.7
0.9	40.5 ± 9.8	27.2 ± 6.4	32.8 ± 8.0
0.45	36.2 ± 7.4	13.2 ± 6.8	63.5 ± 14.2

Values are means ± SEM (N = 4).

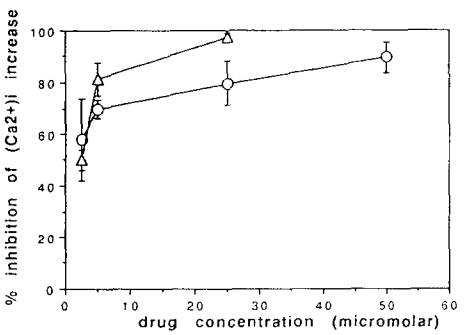

Fig. 2. Inhibition of the $(Ca^{2+})_i$ variation observed after DNP-BSA triggering in RBL-2H3 cells sensitized with DNP-specific IgE, by loratadine (○) or descarboxyethoxyloratadine (△). Exterior Ca^{2+} concentration was 1.8 mM. In control experiments without drug, the $(Ca^{2+})_i$ was raised from 159 ± 5.5 (N = 29) to 583 ± 29 nM (N = 18).

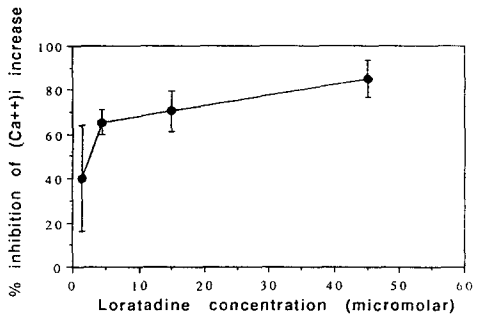

Fig. 3. Inhibition of the $(Ca^{2+})_i$ variation observed after DNP-BSA triggering in RBL-2H3 cells sensitized with DNP-specific IgE by loratadine (●) in the absence of external Ca^{2+}. External Ca^{2+} was complexed by EGTA 3 mM. In control experiments without drug, the $(Ca^{2+})_i$ was raised from 92.9 ± 6.7 to 185 ± 15 nM (N = 11).

manner. No variation in the 340/380 nm fluorescence ratio after addition of loratadine, descarboxyethoxyloratadine on DMSO alone, or after addition of DNP-BSA to non-sensitized cells, was noted (data not shown).

Addition to the incubation medium of EGTA (3 mM), which decreases the signal slightly by chelating Ca^{2+} linked to traces of fura-2 in the external medium, did not abolish the increase in $(Ca^{2+})_i$ induced by the antigen. The $(Ca^{2+})_i$ was raised from 92.9 ± 6.7 to 185 ± 15 nM (N = 11) after addition of the antigen. This response was also inhibited in a dose-dependent manner in the presence of loratadine, with no effect of DMSO alone (Fig. 3). These results indicate that mobilization of internal stores of Ca^{2+} is inhibited by loratadine.

Effect of loratadine on Mn^{2+} influx induced by DNP-BSA in sensitized RBL-2H3 cells

The Mn^{2+} influx, which reflects Ca^{2+} influx into the cells, was measured by recording the emission signals (505 nm) of fura-2 at a 360 nm excitation wavelength. Fura-2-loaded RBL-2H3 cells sensitized with DNP-specific IgE were preincubated with DMSO alone or loratadine for 3–5 min. Upon addition of $MnCl_2$ (100 μM), an immediate drop in fluorescence intensity due to the binding of Mn^{2+} to trace amounts of extracellular fura-2 was noted (Fig. 4). Then, a slow decrease in fluorescence (0.47 arbitrary units/sec in DMSO-incubated cells), presumably due to basal entry of Mn^{2+} into the RBL-2H3 cells at a slow rate, was observed. In RBL cells preincubated with DMSO, addition of DNP-BSA (20 ng/mL) caused a rapid and non-specific drop in fluorescence followed by a marked increase in the rate of fura-2 fluorescence quenching. This result is consistent with antigen-induced influx of Mn^{2+} into the sensitized RBL-2H3 cells. When cells were preincubated with loratadine (15 and 45 μM), no such increase in the rate of fluorescence quenching was observed following the addition of antigen. The results show that loratadine inhibits the antigen-induced influx of Mn^{2+}, and thus of Ca^{2+}, into sensitized RBL-2H3 cells.

DISCUSSION

In preliminary experiments, dose-dependent inhibition by loratadine and descarboxyethoxyloratadine of histamine release from anti-IgE-triggered human basophils was observed. Studies on IgE-sensitized RBL cells showed a similar inhibitory effect of these

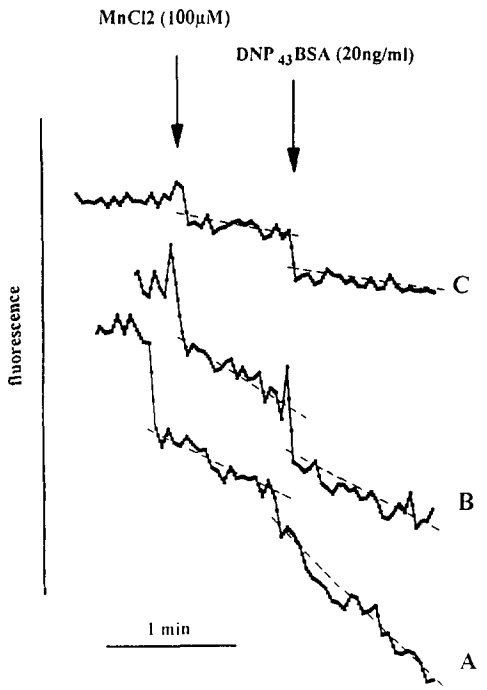

Fig. 4. A typical result representative of the inhibition of Mn^{2+} influx after DNP-BSA triggering in RBL-2H3 cells sensitized with DNP-specific IgE, by loratadine at 15 and 45 μM. The cells were incubated with DMSO (A), loratadine 15 μM (B) or 45 μM (C). After 3–5 min incubation at 37° in the cell of the spectrofluorimeter, 100 μM $MnCl_2$ were added and the basal $MnCl_2$ influx was followed for 1 min. Scales are identical for (A), (B) and (C) but, for clarity, traces have been shifted along the *y*-axis.

drugs on antigen-induced histamine release from these cells. While the concentration range for effective inhibition of RBL-2H3 cells by the two drugs was very similar, in the human basophils the inhibition pattern was 10-fold higher for descarboxyethoxyloratadine than for loratadine. These data suggest differences in the membrane characteristics of the two cell types, which could lead to different behavior of these cells in the presence of the drugs. For human basophils, experiments were performed on a mixed population of blood leukocytes, and thus the interference of cells other than basophils could also explain the observed difference between data on RBL cells and basophils.

In RBL-2H3 cells, a decrease in the external concentration of Ca^{2+} $(Ca^{2+})_e$ was shown to increase the extent of histamine release inhibition at the 20 μM concentration of loratadine. Similar results were reported by others on the effect of $(Ca^{2+})_e$ on histamine release inhibition induced by anti-H1 drugs in rat peritoneal mast cells [4, 13]. Recent studies on permeabilized RBL-2H3 cells have shown that antigen-induced exocytosis is dependent on an increase in $(Ca^{2+})_i$ [25]. In human basophils, the mobilization of Ca^{2+} from internal stores and the influx of external Ca^{2+} are necessary to induce IgE-mediated degranulation [26]. All these data, as well as the previous observation of a loratadine-induced decrease in the Ca^{2+} influx into mastocytoma cells [5], indicated that inhibition observed by loratadine and descarboxyethoxyloratadine at the histamine release level could result from impairment of the $(Ca^{2+})_i$ rise following IgE-mediated stimulation. These results stress the need for further investigation of the effect of the two drugs on Ca^{2+} fluxes. For this investigation, pure cell populations of IgE passively sensitized RBL-2H3 cells were used.

Our results demonstrated dose-dependent inhibition of the $(Ca^{2+})_i$ increase following antigen stimulation by loratadine and descarboxyethoxyloratadine. It has been shown that the cytosolic Ca^{2+} response in RBL-2H3 cells following IgE-mediated stimulation is related to two components [27]. The early component does not directly depend on the presence of extracellular Ca^{2+}, but is associated with release of Ca^{2+} from internal stores by the second messenger, inositol 1,4,5-triphosphate. This Ca^{2+} mobilization can be assessed in experiments performed in the absence of external Ca^{2+}. The second component is an influx of Ca^{2+} from the extracellular medium. This influx can be measured indirectly with the use of Mn^{2+} which enters the cell through the same pathway used by external Ca^{2+} and binds more avidly to the fluorescence probe (24).

Present results demonstrate that loratadine and descarboxyethoxyloratadine act on both components of the antigen-induced increase of $(Ca^{2+})_i$ in sensitized RBL-2H3 cells, namely, mobilization of Ca^{2+} from internal pools and Ca^{2+} influx. Indeed, in the absence of external Ca^{2+}, these drugs inhibit the rise in $(Ca^{2+})_i$ following antigen challenge, a result consistent with inhibition of Ca^{2+} release from internal stores. The antigen-induced influx of Mn^{2+} is inhibited after preincubation of the cells with loratadine, indicating a decrease in the Ca^{2+} influx.

Other anti-H1 drugs tested have been reported to exert an effect on Ca^{2+} fluxes in guinea pig macrophages [28] and in rat peritoneal mast cells [13, 29]. Further studies are required to elucidate the mechanism by which loratadine and descarboxyethoxyloratadine modify cell membrane components inhibiting Ca^{2+} fluxes, an important step in the numerous biochemical events implicated in exocytosis.

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