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Influence of the anti-allergic drug oxatomide on the signal transduction mechanism in a mast cell model

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Abstract

In a mast cell model, oxatomide displays inhibition of mediator release which is not related to its histamine H_1 receptor antagonistic activity. From a previous study it appeared that especially early steps in the signal transduction leading to exocytosis were influenced by oxatomide. We now studied effects of oxatomide on those early steps in more detail. The antigen- and thapsigargin-mediated exocytosis in rat basophilic leukemia (RBL-2H3) cells were both inhibited by oxatomide. After aggregation of high affinity receptors for immunoglobulin E (FcεRI), protein tyrosine phosphorylation is induced. Oxatomide caused remarkable changes in the tyrosine phosphorylation pattern in resting cells. Also after antigen and thapsigargin activation, changes in the tyrosine phosphorylation of cellular proteins are observed. In addition, Ca^{2+} fluxes were studied by means of the net influx of $^{45}Ca^{2+}$ and by measuring intracellular free Ca^{2+} concentrations ($[Ca^{2+}]$) with the fluorescent probe fura-2. Oxatomide inhibited the $^{45}Ca^{2+}$ influx and the increase in $[Ca^{2+}]_i$ upon antigen and thapsigargin activation of the cells. Neither the release of Ca^{2+} from internal stores nor the efflux of Ca^{2+} over the plasma membrane seems to be affected. The effect of oxatomide on Ca^{2+} influx was further characterized by studying Ba^{2+} influx in the absence of extracellular free Ca^{2+}. We conclude that inhibition of mediator release is mainly caused by inhibition of influx of extracellular Ca^{2+}, via plasma membrane Ca^{2+} channels that are activated by depletion of intracellular Ca^{2+} stores. The molecular mechanism with which oxatomide might interfere with these channels is discussed.

Keywords: Oxatomide; RBL-2H3 cell; Signal transduction; Exocytosis; Protein tyrosine phosphorylation; Ca^{2+} flux; Ca^{2+} channel

1. Introduction

The primary event in immediate hypersensitivity reactions is the release of histamine and other inflammatory mediators from mast cells and basophils (Massey and Lichtenstein, 1992; Marshall and Bienenstock, 1994). This release is initiated when antigen binds to immunoglobulin E (IgE) occupying high affinity IgE receptors (FcεRI). After FcεRI receptors are aggregated, a cascade of intracellular biochemical processes occurs. The cellular protein tyrosine phosphorylation increases at different stages of the signaling cascade (Benhamou and Siraganian, 1992) and phospholipase C is activated, which generates the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol. These messengers respectively induce the release of Ca^{2+} from intracellular stores and the translocation and activation of protein kinase C (Beaven and Metzger, 1993). This release of Ca^{2+} from internal stores leads to a rise in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and an increased Ca^{2+} influx into the cell. These intracellular biochemical events ultimately result in the release of preformed and newly formed mediators.

Oxatomide (Fig. 1) is a histamine H_1 receptor antagonist, which suppresses hypersensitivity reactions in different species (Awouters et al., 1980) and in patients with chronic urticaria and allergic rhinitis (Richards et al., 1984). Furthermore, it inhibits the histamine, serotonin and leukotriene release from mast cells and basophils in vivo and in vitro (De Clerck et al., 1981; Truneh et al., 1982; Tasaka et al., 1987; Awouters et al., 1980). In a previous study we reported on the inhibitory activity of oxatomide and derivatives on the release of β-hexosaminidase from a

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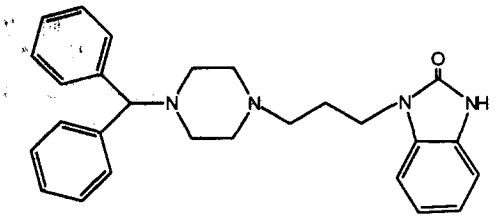

Fig. 1. Structure of oxatomide.

rat basophilic leukemia (RBL-2H3) cell line. We concluded that the inhibition of exocytosis in this mast cell model was not mediated by histamine H_1 receptor antagonism and that especially early signal transduction processes are affected by oxatomide (Paulussen et al., 1996).

The present paper is aimed at the elucidation of the effects of oxatomide on the early steps of the signal transduction cascade leading to exocytosis. In particular, we studied effects at the level of FcεRI- and FcεRI aggregation-induced protein tyrosine phosphorylation. Furthermore, we studied the influence of oxatomide on IP_3 generation and Ca^{2+} fluxes. Cells were activated on the FcεRI level with antigen or with triggers that bypass FcεRI aggregation. Such triggers are the calcium ionophore A23187, whether or not in combination with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and thapsigargin, a Ca^{2+}-ATPase inhibitor of the endoplasmic reticulum that prevents reuptake of Ca^{2+} in the endoplasmic reticulum subsequently leading to influx of extracellular Ca^{2+} and elevation of $[Ca^{2+}]_i$ (Thastrup et al., 1990; Alfonso et al., 1994).

We report the inhibitory activity of oxatomide on the release of both preformed and newly formed mediators from RBL cells. Even without cell activation oxatomide markedly increased the tyrosine phosphorylation of cellular proteins that might be involved in the exocytosis process. Oxatomide inhibited the influx of extracellular Ca^{2+}, which is a major cause of the inhibitory effect on mediator release.

2. Materials and methods

2.1. Materials

Oxatomide, 1-{3-[4-(diphenylmethyl)-1-piperazinyl]propyl}-1,3-dihydro-2*H*-benzimidazol-2-one, was generously provided by Janssen Pharmaceutica, Belgium. Monoclonal IgE directed against the dinitrophenyl (DNP) hapten, antigen DNP-albumin conjugate, calcium ionophore A23187 and fura-2-AM were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was from Gibco BRL (Breda, Netherlands). Thapsigargin and Fluorescein isothiocyanate (FITC) were obtained from Calbiochem (San Diego, CA, USA). Anti-phosphotyrosine coupled to horse radish peroxidase (PY-2-HRP) was purchased from Transduction Laboratories (Lexington, KY, USA). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid, $^{45}CaCl_2$, D-myo-[^{3}H]inositol 1,4,5-trisphosphate potassium salt and 5-hydroxy[G-^{3}H]tryptamine creatinine sulphate (serotonin) were obtained from Amersham ('s-Hertogenbosch, Netherlands).

2.2. Cell culture

RBL-2H3 cells were grown in Eagles's Minimal Essential Medium with Earle's salts supplemented with 0.22% $NaHCO_3$, 15% fetal calf serum, 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 60 μg/ml tylosin, pH 7.2. Cultures were maintained under 5% CO_2 at 37°C tissue culture flasks. After 4–7 days cells were harvested by detaching them for 10 min with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid. After trypsinization they were used for experiments.

2.3. Assay of β-hexosaminidase and arachidonic acid release

These assays were performed as described before (Paulussen et al., 1996). When thapsigargin (0.2 μM) was used as a trigger, cells were not sensitized withe IgE.

2.4. Release of serotonin

Serotonin release was measured by incubating 2×10^5 cells/well with 1 μCi ^{3}H-serotonin/ml in a 24-well plate overnight at 37°C and under 5% CO_2. Cells were washed thoroughly with Tyrode's 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). Conditions for cell triggering were the same as described for β-hexosaminidase release (Paulussen et al., 1996) except that concentrations used here for IgE and antigen were different, 1 μg/ml and 20 ng/ml, respectively. Supernatant and Triton-X-100 (1%) generated cell lysate were collected, liquid scintillator was added and radioactivity was counted in a 1450 MicroBetaPLUS liquid scintillation counter from Wallac (Zeist, Netherlands).

2.5. Binding of FITC-IgE to FcεRI

Fluorescein isothiocyanate (FITC) was conjugated to IgE, according to Mason et al. (1987), but instead of a Sephadex G-50 column a ultrafiltration system was used. Labeling resulted in 7 ng FITC/μg IgE. Cells in suspension (10^6 cells/ml) were incubated with 50 μM oxatomide (or medium as a control) and 2 μg/ml IgE-FITC for 1 h at 37°C. Non-labeled IgE was used as a control. Cells were washed thoroughly with Tyrode's buffer without bovine serum albumin, supplemented with 1% fetal calf

serum and 0.1% azide and subsequently analyzed by flow cytometry (FACScan, Beckton Dickinson).

2.6. Tyrosine phosphorylation

Cells were plated at a concentration of 4×10^5 cells per well in a 24-well plate. They were kept overnight at 5% CO_2 and 37°C. Cells were sensitized with IgE (1 μg/ml) for 1 h and washed twice afterwards with Tyrode's buffer. After stimulation with antigen (40 ng/ml) or thapsigargin (0.2 μM) for 30 min the cell monolayers were washed twice and lysed in 40 μl lysis buffer/well (50 mM Tris, 150 mM NaCl, pH 7.4, 0.5% Triton-X-100, 0.4 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM phenylmethanesulfonylfluoride, 10 μg/ml leupeptin, antipain and aprotinin). After centrifugation of the lysates, Laemmli buffer containing dithiothreitol was added to the supernatants and boiled for 5 min. From each sample 10 μl were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10 or 7.5%) and afterwards electrotransferred to a polyvinylidene difluoride membrane. This membrane was treated with blocking buffer (Tris buffered saline, pH 7.6, 0.05% Tween-20, 5% bovine serum albumin) for 1.5 h. After washing, the blot was treated with 1:1000 anti-phosphotyrosine antibody coupled to horse radish peroxidase (PY-20-HRP) for 1.5 h. After extensive washing the tyrosine phosphorylated proteins were developed with the enhanced chemiluminescence ECL kit (Amersham) and visualized on a Kodak X-AR-2 film.

2.7. IP_3 assay

In a 6-well plate 2×10^6 cells were plated per well. They were kept overnight at 5% CO_2 and 37°C. Cells were sensitized with IgE (1 μg/ml) for 1 h, washed afterwards and triggered with antigen (40 ng/ml) for 5 min. Ten minutes before triggering 30 μM oxatomide was added. The reaction was stopped by removing the supernatant and adding 150 μl of 3.5% $HClO_4$, which was allowed to stand for 30 min at 4°C. IP_3 levels were determined according to the method of Bominaar and Van Haastert (1994).

2.8. Assay of $^{45}Ca^{2+}$ influx

Cells were treated as described above for phosphorylation experiments. Cells were challenged with antigen (40 ng/ml) for 5 min or with thapsigargin (0.2 μM) for 10 min, both in the presence of $^{45}Ca^{2+}$ (30 μCi/ml or 10 μCi/ml, respectively). Oxatomide was added 10 min before challenge in a concentration range of 0.1–60 μM. The reaction was stopped on ice, supernatant was removed and subsequently cells were washed with ice-cold Tyrode's buffer. Triton-X-100 (1%) generated cell lysates were collected, liquid scintillator was added to all samples and the radioactivity measured. Results were expressed as a percentage of the $^{45}Ca^{2+}$ influx after stimulation without oxatomide and corrected for leakage of $^{45}Ca^{2+}$ into the cell (±7% of total).

2.9. Measurements of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

In a 12-well plate, 5×10^5 cells per well were grown on glass cover slips. Conditions for sensitizing the cells were the same as described before. The concentration of IgE used in this assay was 0.4 μg/ml. Before activating, the cells were loaded with 1 μM fura-2-AM in Tyrode's buffer containing 0.1 mg/ml sulfinpyrazon at 37°C for 30 min. After a 10 min resting period at room temperature, the glass coverslip was transferred to a cuvette and Tyrode's buffer containing 0.1 mg/ml sulfinpyrazon and 0.05% gelatine instead of bovine serum albumin, was added. Fluorescence emission (510 nm) was monitored, using a dualwavelength filter fluorometer (Photon Technology Incorporation, New York, NY, USA) at the excitation wavelengths of 340 and 380 nm and a filter rotation frequency of 100 Hz. Cells were challenged using antigen (100 ng/ml) or thapsigargin (0.4 μM). The anti-allergic compound was added 5 min before challenge. Similar experiments were performed in the absence of free extracellular Ca^{2+} by the addition of 3 mM EGTA to the buffer. Maximum and minimum fluorescence signals were obtained by the addition of 3 μM ionomycin and 20 mM EGTA, respectively. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985) using a K_d of 224 nM. Ba^{2+} influx was studied in this assay by measuring the ratio of the 340/380 nm fluorescence signal. After thapsigargin activation (0.4 μM), under conditions where free extracellular Ca^{2+} was complexed, 30 μM oxatomide was added, one min before the addition of 0.5 mM Ba^{2+}, or after the addition of 1.9 mM Ba^{2+}.

3. Results

3.1. Effect of oxatomide on mediator release

RBL-2H3 cells were activated in four different ways and release of β-hexosaminidase was measured. IgE sensitization and triggering with antigen (IgE/DNP) starts the exocytosis process by aggregation of FcεRI. Mediator release is also induced by the calcium ionophore A23187 or a combination of A23187 and TPA, which both bypass FcεRI-coupled processes and activate post Ca^{2+} influx phases in the signal transduction. A23187 and TPA, a phorbol ester known to activate protein kinase C, act synergistically and in the presence of TPA less A23187 is needed to trigger the exocytosis process. Upon triggering with thapsigargin, intracellular Ca^{2+} stores are depleted and a related influx of extracellular Ca^{2+} into the cell (Ca^{2+} release activated Ca^{2+} current, I_{CRAC}) occurs (Hoth

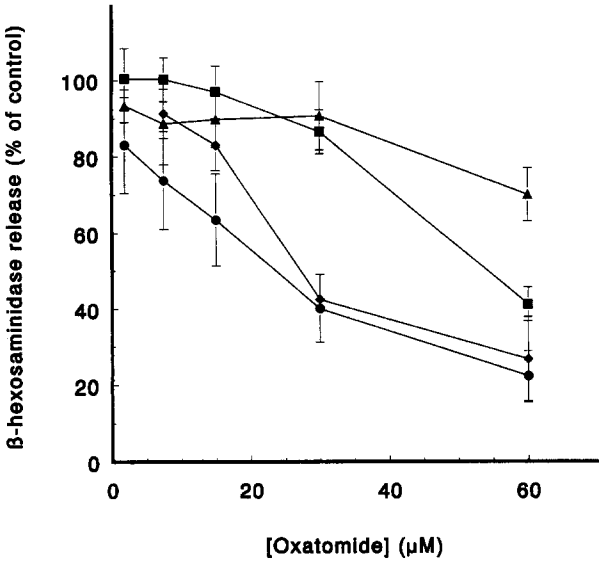

Fig. 2. Effect of oxatomide on β-hexosaminidase release from RBL-2H3 cells activated with different triggers. Cells are preincubated with oxatomide for 10 min. Release is induced by 0.2 μg/ml IgE and 5 ng/ml antigen (●), 0.4 μM thapsigargin (◆), 1 μM A23187 (■), 0.25 μM A23187 and 50 nM TPA (▲) and measured after 30 min. Values are means ± S.D. from at least two independent experiments in triplicate.

and Penner, 1993). These different stimulants were chosen such that all induced the release of β-hexosaminidase to the same extent, namely 70% for IgE/DNP, 80% for A23187, 80% for A23187/TPA and 75% for thapsigargin. The concentration-effect curves are shown in Fig. 2. The antigen- and thapsigargin-mediated release appeared to be more sensitive for inhibition by oxatomide than release with A23187, whether or not in combination with TPA. This indicates that post Ca^{2+} influx phases of the signal transduction processes leading to mediator release, are less sensitive for inhibition by oxatomide. In combination with this, the inhibition of the thapsigargin-induced release indicates that oxatomide acts especially at the level of Ca^{2+} fluxes.

The influence of oxatomide on the antigen-mediated release of serotonin and arachidonic acid and its metabolites was also investigated. The inhibitory activity of 30 μM oxatomide is shown in Table 1. The inhibition of serotonin and β-hexosaminidase release, both preformed mediators, is comparable with the effect on arachidonic acid release, which is a newly formed mediator upon activation of phospholipase A_2 (Beaven and Cunha-Melo, 1988; Holowka and Baird, 1990).

3.2. Effect of oxatomide on binding of FITC-IgE to RBL cells

An initial step in the antigen-mediated exocytosis is binding of IgE to its receptor. The effect of oxatomide on binding of FITC-labeled anti-DNP-IgE to FcεRI was studied using FACScan. FITC-labeled IgE did not affect the

Table 1
Inhibitory effect of 30 μM oxatomide on the release of β-hexosaminidase (β-hexo), serotonin and arachidonic acid (Araa) from RBL cells upon various ways of cell triggering. The release was expressed relative to control (no drug present)

Assay/triggering	Release (% of control)
β-hexo/DNP	40.1 ± 8.9 [a]
β-hexo/A23187	86.5 ± 5.8 [a]
β-hexo/A23187 + TPA	90.7 ± 8.9
β-hexo/thapsigargin	42.6 ± 1.1 [a]
Serotonin/DNP	48.6 ± 10.1 [a]
Araa/DNP	57.1 ± 17.3 [a]

Values are means ± S.D. from at least two independent experiments in triplicate. [a] $P < 0.05$ as tested versus control with Student's t-test.

release of β-hexosaminidase in RBL cells and the inhibition of this by oxatomide (data not shown). Cells were incubated with oxatomide and labeled or non-labeled IgE. The fluorescence signal produced by cells with labeled IgE bound to its receptor was not influenced by the addition of 50 μM oxatomide (Fig. 3). This demonstrates that the amount of IgE bound to the cells is not influenced by oxatomide.

3.3. Protein tyrosine phosphorylation

After cross-linking of FcεRI, tyrosine phosphorylation of cellular proteins increases at different stages of mast cell activation (Benhamou and Siraganian, 1992). Anti-phosphotyrosine immunoblotting revealed changes in tyrosine-phosphorylated proteins upon stimulation of the cells (Fig. 4A, lane 1 and 2). The increase in phosphorylation around 72 kDa is related to FcεRI aggregation, while the band around 110 kDa is observed after influx of Ca^{2+} into the cell (Benhamou and Siraganian, 1992; Yu et al., 1991).

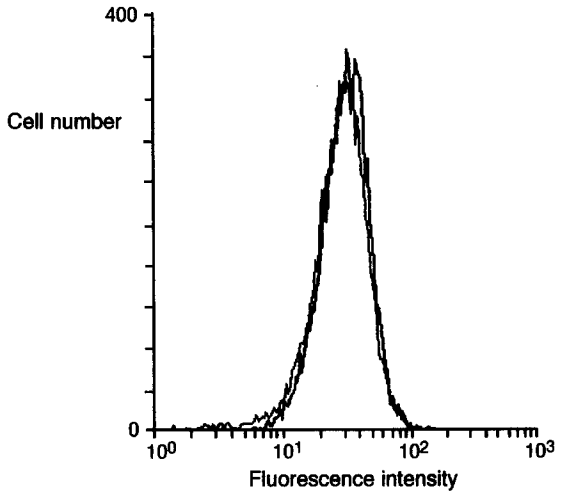

Fig. 3. Flow cytometry analysis of FITC-IgE-labeled RBL-2H3 cells in the presence or absence of oxatomide. Cells were incubated with 50 μM oxatomide and 2 μg/ml IgE-FITC. Representative histograms from three experiments are shown.

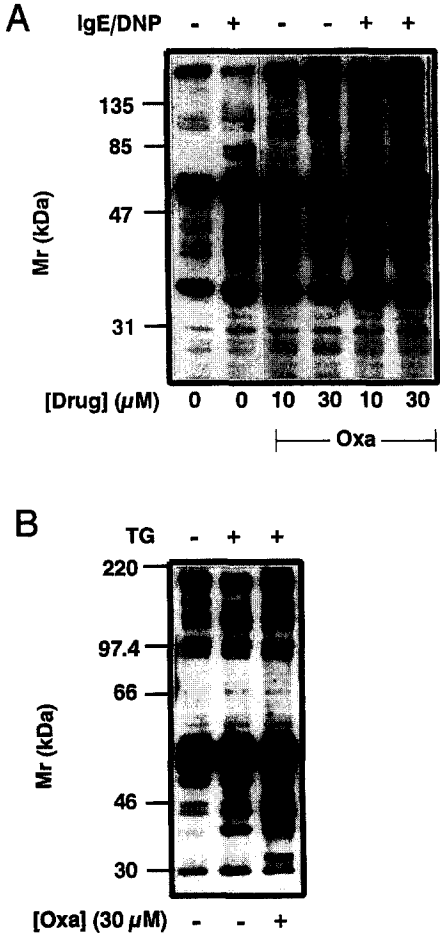

Fig. 4. Effect of oxatomide on tyrosine phosphorylation of cellular proteins in RBL-2H3 cells upon antigen or thapsigargin stimulation. The presence and concentration of oxatomide is as indicated. (A) Antigen stimulation (+IgE/DNP) or resting cells (−IgE/DNP). Electrophoresis was performed using a 10% gel. (B) Thapsigargin stimulation (lane 2 and 3) and resting cells (lane 1). Electrophoresis was performed using a 7.5% gel.

The increase in the bands of around 40 kDa can be (partly) ascribed to tyrosine phosphorylation of mitogen-activated protein (MAP) kinase, which occurs in late phases of the exocytosis process (Santini and Beaven, 1993).

Pretreatment with 10 or 30 μM oxatomide already showed a marked effect on non-stimulated cells (Fig. 4A, lane 3 and 4, respectively). Oxatomide increased the phosphorylation of proteins in different molecular weight regions dose dependently, especially the regions of 35–45 kDa and 105–115 kDa. Also in antigen-activated cells, the level of phosphorylation in the presence of oxatomide remains above the level observed in the absence of oxatomide. Phosphorylation of the proteins in the molecular weight regions of 35–45 kDa, 70–75 kDa, 105–115 kDa and approximately pp145 (Fig. 4A, lane 5 and 6) were increased by oxatomide. The phosphorylation of the 70–75 kDa proteins includes the pp72, which is only enhanced by oxatomide after FcεRI crosslinking. Upon antigen activation in the presence of oxatomide a band at 145 kDa becomes visible (Fig. 4A, lane 6). The position of this band corresponds to that of PLCγ1 (Park et al., 1991).

Thapsigargin was also used as a stimulant, to discriminate between the directly FcεRI coupled events and the post IP_3 events in the signal transduction. Compared to antigen activation a different phosphorylation pattern is observed upon activation of the cells with thapsigargin (Fig. 4B, lane 2). It does not show an increase in a pp72 band, which confirms that receptor coupled events are bypassed (Fig. 4B, lane 1 and 2). Furthermore, increased phosphorylation of proteins around 40 kDa and of 110 kDa is observed, which is in accordance with post Ca^{2+} events (Yu et al., 1991; Santini and Beaven, 1993). In thapsigargin stimulated cells, oxatomide also increased the phosphorylation of the 145 kDa band (Fig. 4B, lane 3). In the region of 40–45 kDa generally an increase in phosphorylation is seen in the presence of oxatomide. However, the pp42 and pp110 bands show a slight decrease in phosphorylation after thapsigargin activation in the presence of oxatomide.

3.4. Inositol 1,4,5-trisphosphate formation

After FcεRI crosslinking, phospholipase Cγ1 is activated which enhances the turnover of phosphatidyl inositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3). As oxatomide has an effect on the phosphorylation of proteins that is possibly related to FcεRI aggregation, we investigated whether the IP_3 level is affected. Upon activation with antigen, the level of IP_3 in the cells increased towards a maximum at 5 min after stimulation (data not shown). The influence of oxatomide on the amount of IP_3 was measured 5 min after stimulation. The IP_3 level in resting cells was 1.16 pmol/2×10^6 cells and after antigen activation this value rose to 4.33 pmol/2×10^6 cells (Table 2). These values agree fairly well with those of Teshima et al. (1994). From Table 2 it can be concluded that oxatomide did have a slight but significant ($P < 0.05$) stimulating effect on the [IP_3] in RBL cells. This increased [IP_3] might have implications in the release of Ca^{2+} from IP_3-sensitive stores.

Table 2
Effect of 30 μM oxatomide on 1,4,5-IP_3 formation upon antigen activation (40 ng/ml) in RBL-2H3 cells

Compound	[IP_3] (pmol/$2 \cdot 10^6$ cells)	
	−IgE/DNP	+IgE/DNP
Control	1.16 ± 0.74	4.33 ± 0.34
Oxatomide	0.28 ± 0.12	5.14 ± 0.34 [a]

Values are means ± S.D. from at least three independent determinations.
[a] $P < 0.05$ as tested versus control, determined with Student's t-test.

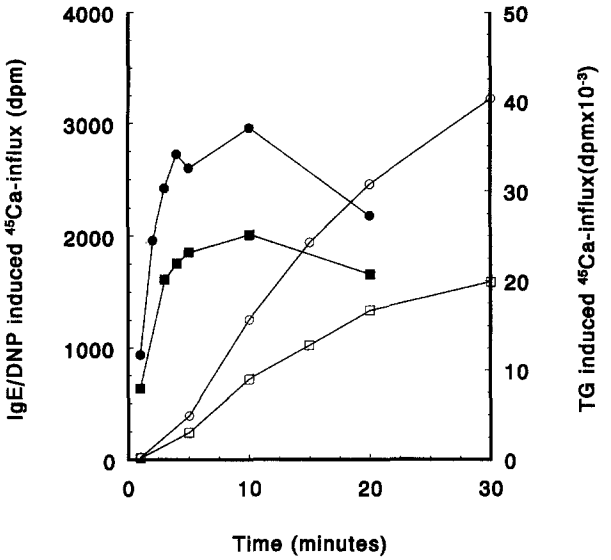

Fig. 5. Time dependency of $^{45}Ca^{2+}$ influx in RBL-2H3 cells, activated with antigen (closed symbols) or thapsigargin (open symbols). Cells were activated with 40 ng/ml antigen or 0.2 μM thapsigargin. Influx was measured in the absence (circles) and in the presence of 15 μM oxatomide (squares).

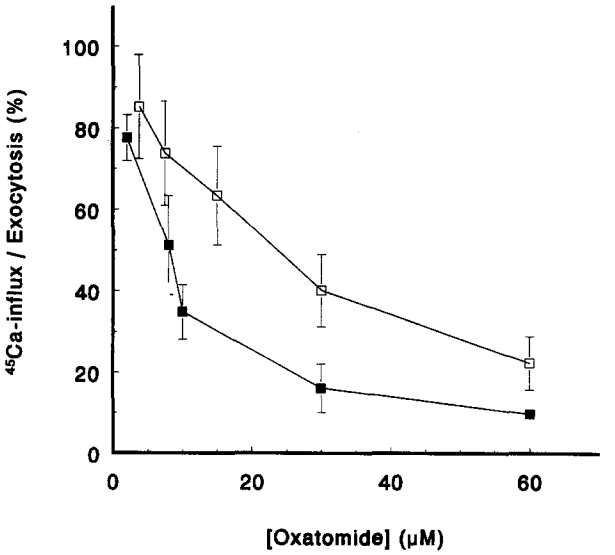

Fig. 6. Effect of oxatomide on antigen-induced $^{45}Ca^{2+}$ influx (■) and β-hexosaminidase release (□) in RBL-2H3 cells. After 10 min of preincubation with oxatomide, cells were stimulated with antigen. $^{45}Ca^{2+}$ influx is measured after 5 min and β-hexosaminidase release is measured after 30 min of stimulation. Values are means ± S.D. from at least two independent experiments in duplicate.

3.5. Ca^{2+} fluxes and the intracellular free Ca^{2+} concentration

As mentioned above, the effect of oxatomide on thapsigargin-induced exocytosis compared to A23187-induced exocytosis suggests that oxatomide affects Ca^{2+} fluxes. In principle several subprocesses contribute to $[Ca^{2+}]_i$: (1) release of Ca^{2+} from intracellular stores, (2) efflux of intracellular Ca^{2+} over the plasma membrane, (3) reuptake of Ca^{2+} in the internal stores and (4) influx of extracellular Ca^{2+} into the cell. We studied Ca^{2+} fluxes with two experimental approaches: net influx of $^{45}Ca^{2+}$, comprising the above mentioned processes 2 to 4, and measurement of the $[Ca^{2+}]_i$ using the fluorescent probe fura-2.

The $^{45}Ca^{2+}$ influx was measured upon triggering with either antigen or thapsigargin. In a concentration range of 0.1–2.0 μM, thapsigargin induced 60% release of β-hexosaminidase (results not shown). For further experiments the thapsigargin concentration was 0.2 μM.

First the time dependency of the $^{45}Ca^{2+}$ influx was determined (Fig. 5). When cells were triggered with antigen the influx reached a plateau after 5 min, during which Ca^{2+} influx prevails (Ran and Rivnay, 1988), and at the time when the plateau equilibrium exists between influx and efflux. After longer time (> 10 min) the efflux prevails, resulting in a negative net influx of $^{45}Ca^{2+}$. The total influx upon activation of the cells with thapsigargin was much larger and did not reach a plateau within 30 min. This longer duration of net increase in $^{45}Ca^{2+}$ is in accordance with a more permanent opening of plasma membrane Ca^{2+} channels due to a sustained emptied state of intracellular Ca^{2+} stores by inhibition of Ca^{2+}-ATPase by thapsigargin. The Ca^{2+} current induced by thapsigargin can be characterized as being of the I_{CRAC} type (Fasolato et al., 1994). In the presence of 15 μM oxatomide $^{45}Ca^{2+}$ influx is decreased, but the form of the curves is similar to that in the absence of oxatomide. With antigen trigger especially the influx phase seems to be inhibited. Based on the time dependency shown in Fig. 5, in further experiments the influence of oxatomide on antigen and on thapsigargin-induced Ca^{2+} influx was measured after 5 and 10 min, respectively.

Fig. 6 shows the effect of oxatomide on the antigen-mediated $^{45}Ca^{2+}$ influx as well as on the release of β-hexosaminidase. The form of the curves are similar, although the influx of Ca^{2+} is more sensitive to the inhibitory activity of oxatomide. It appears that the Ca^{2+} influx can be inhibited for approximately 40%, without significant effect on the exocytosis. Below this threshold value the exocytosis is efficiently inhibited. When thapsigargin was used as a trigger, 30 μM oxatomide inhibited

Table 3
Influence of 30 μM oxatomide on $^{45}Ca^{2+}$ influx and $[Ca^{2+}]_i$ in RBL cells upon activation with antigen or thapsigargin

Trigger	$^{45}Ca^{2+}$ influx (% of control)	$[Ca^{2+}]_i$ (% of control)
IgE/DNP	16.1 ± 6.0 (n = 4)	38.6 ± 8.75 (n = 7)
Thapsigargin	19.6 ± 7.5 (n = 8)	43.7 ± 12.8 (n = 4)

Values are means ± S.D. from n experiments.

the Ca^{2+} influx for 80% which is similar to the effect on the antigen-induced $^{45}Ca^{2+}$ influx (Table 3).

Measurement of $[Ca^{2+}]_i$ gives additional information on how oxatomide affects Ca^{2+} fluxes. Fig. 7 shows the effect of 30 μM oxatomide on $[Ca^{2+}]_i$ with antigen as well as thapsigargin triggering. With antigen triggering an initial rise in $[Ca^{2+}]_i$ to ±420 nM is observed, followed by a plateau phase of elevated $[Ca^{2+}]_i$ which is characterized by a slow decrease in intracellular Ca^{2+} (Fig. 7A). The initial rise is caused only for a minor part by the release of Ca^{2+} from IP_3-sensitive stores (see below, Fig. 8A), the major contribution is from the influx of extracellular Ca^{2+}. In the presence of 30 μM oxatomide the onset of the initial rise is slowed down and the peak reaches only a level of approximately 240 nM (Table 3). Furthermore, the decline in the plateau phase is markedly increased (Fig. 7A). Triggering with thapsigargin shows a rapid increase in $[Ca^{2+}]_i$ caused by leakage of Ca^{2+} from the endoplasmic reticulum, due to inhibition of Ca^{2+}-ATPase, and influx of extracellular Ca^{2+} (Fig. 7B). Again a plateau phase is reached with a $[Ca^{2+}]_i$ of ±550 nM, which is higher than that after antigen trigger. Also with thapsigargin activation, oxatomide inhibits the initial rise of $[Ca^{2+}]_i$ and augments the decline of the plateau phase.

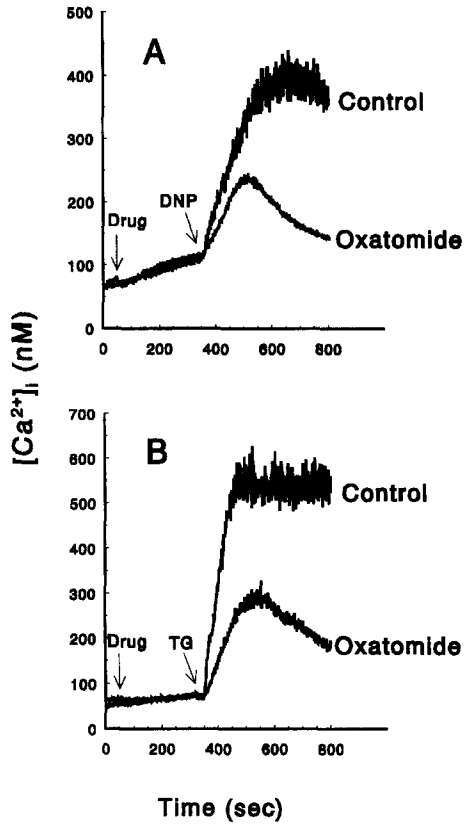

Fig. 7. Effect of oxatomide on the antigen (A)- and thapsigargin (B)-induced $[Ca^{2+}]_i$ in RBL-2H3 cells. Fura-2 loaded cells were incubated (at $t = 50$ s) with oxatomide (30 μM) or DMSO as a control for 5 min and subsequently stimulated ($t = 350$ s) with either antigen (100 ng/ml) or thapsigargin (0.4 μM). The tracings are representatives of at least four similar experiments.

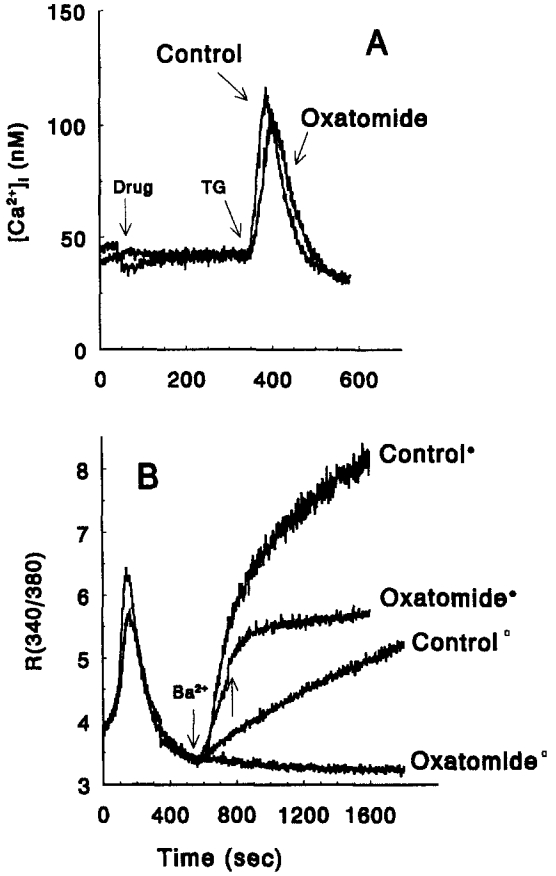

Fig. 8. Effect of oxatomide on thapsigargin (0.4 μM) induced fura-2 fluorescence signals in RBL-2H3 cells, with free extracellular Ca^{2+} bound to 3 mM EGTA. (A) After 5 min of preincubation with 30 μM oxatomide or DMSO (as a control) cells were triggered with thapsigargin. (B) After return of the thapsigargin-induced change in the fluorescence ratio (R(340/380)) to basic levels, 0.5 mM Ba^{2+} (□) or 1.9 mM Ba^{2+} (●) was added. 30 μM Oxatomide or DMSO (control) was added as indicated with an arrow, 1 min before (□) or 3 min after (●) addition of Ba^{2+}. All tracings are representatives from three independent experiments.

To study the effect of oxatomide on the various above-mentioned Ca^{2+} fluxes experiments were performed in which extracellular Ca^{2+} was bound to EGTA. When activation with antigen took place and no free extracellular Ca^{2+} was available, a slight but not significant temporarily increase in $[Ca^{2+}]_i$ occurred (data not shown). An effect of oxatomide could not be measured under these conditions. However, addition of thapsigargin immediately induces a rise in $[Ca^{2+}]_i$ from 40 to 110 nM (Fig. 8A), which is considerably less than that in the presence of free extracellular Ca^{2+} (see Fig. 7). The thapsigargin-induced rise in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, is larger than the antigen-induced rise, because thapsigargin also empties non-IP_3 sensitive Ca^{2+} stores (Thastrup et al., 1990). The mobilization of Ca^{2+} from the internal stores seems to be

slightly, but not significantly, affected by 30 μM oxatomide (84.5 ± 2.0% compared to 100 ± 10.9%, $P > 0.05$). After reaching the peak value, the $[Ca^{2+}]_i$ decreases rapidly, by efflux of Ca^{2+} to the outside of the cell. This decrease appeared to obey first order kinetics and is characterized by a half time of approximately 60 s in the presence or absence of oxatomide. This indicates that efflux of Ca^{2+} out of the cell is not influenced by oxatomide. Efflux was also studied using cells loaded with $^{45}Ca^{2+}$, triggering them with thapsigargin and measuring the amount of $^{45}Ca^{2+}$ which was extruded. The presence of oxatomide had no significant effect on the extrusion of $^{45}Ca^{2+}$ (data not shown). From this experiment and the efflux phase seen in Fig. 8A, we conclude that the efflux of intracellular Ca^{2+} is not affected by oxatomide.

The influence of oxatomide on the influx of Ca^{2+} was further investigated by studying the effect of Ba^{2+} influx. Influx of Ba^{2+} occurs through Ca^{2+} channels, after which Ba^{2+} is trapped in the cytoplasm as it is not pumped out of the cell or into the Ca^{2+} stores (Lee et al., 1993). Ba^{2+} increases the ratio of the 340/380 nm fura-2 fluorescence signal (Lee et al., 1993). In these experiments the RBL cells were triggered with 0.4 μM thapsigargin, while free extracellular Ca^{2+} was complexed with EGTA. A rise in the fluorescence ratio due to the release of Ca^{2+} from intracellular stores is seen (Fig. 8B). After the ratio had returned to basic level, 30 μM oxatomide was added followed 1 min later by 0.5 mM Ba^{2+}. As can be seen from Fig. 8B, oxatomide inhibits the influx of Ba^{2+} completely. Also from Fig. 8B it appears that the addition of 30 μM oxatomide during the influx of Ba^{2+} (1.9 mM), immediately stops the influx of Ba^{2+}. These results indicate that the activity of Ca^{2+} channels, regulated by depletion of intracellular Ca^{2+} stores, is blocked by oxatomide.

4. Discussion

In our previous study we reported on the inhibitory activity of oxatomide and derivatives on the release of β-hexosaminidase induced by different triggers from a rat basophilic leukemia (RBL-2H3) cell line (Paulussen et al., 1996). It was concluded that oxatomide affects especially early phases of signal transduction leading to exocytosis. The assay of total release of arachidonic acid and metabolites as applied in this study can be regarded primarily as an assay for phospholipase A_2 activity. In contrast to degranulation, activation of phospholipase A_2 is more closely related to the early events after FcεRI receptor aggregation and Ca^{2+} mobilization (Beaven and Cunha-Melo, 1988; Holowka and Baird, 1990). Therefore, the similar ability of oxatomide to inhibit antigen-induced degranulation and phospholipase A_2 activity can be understood by assuming effects of oxatomide on common early processes after FcεRI aggregation for both degranulation of β-hexosaminidase and serotonin as well as phospholipase A_2 activation. Moreover, the inhibitory activity of oxatomide on antigen- as well as thapsigargin-induced β-hexosaminidase release indicates that it primarily affects common processes in the signal transduction pathway, especially at the level of Ca^{2+} fluxes. Post Ca^{2+} processes are less sensitive to inhibition by oxatomide.

In view of the importance of the early steps in the signal-transduction cascade the FcεRI-related processes were studied in more detail to reveal how oxatomide inhibits mast cell mediator release. The first step in the activation of cells is the binding of IgE to its receptor. We demonstrated that there is no effect of oxatomide on the IgE-FcεRI binding.

After aggregation of FcεRI a cascade of biochemical processes occurs, including tyrosine phosphorylation of proteins, generation of inositol trisphospate (IP_3) and Ca^{2+} fluxes.

The observed changes in tyrosine phosphorylation induced by oxatomide in resting cells are remarkable. This change in phosphorylation might be caused by membrane distortion induced by oxatomide, as aggregation of membrane-bound protein tyrosine kinases is sufficient to activate phosphorylation processes (Rivera and Brugge, 1995). In resting cells, oxatomide induces changes in phosphorylation of proteins, which might also play a role in the processes leading to exocytosis: increased tyrosine phosphorylation of proteins in the 105–115 kDa range and proteins around 40 kDa. The proteins in the 105–115 kDa range are phosphorylated after Ca^{2+} influx (Yu et al., 1991) and proteins around 40 kDa, presumably MAP-kinases, are involved in late phases of the exocytosis process (Santini and Beaven, 1993).

Upon antigen activation this enhanced phosphorylation remains, and also the pp72 and pp145 phosphorylation band is increased in the presence of oxatomide. Proteins at 72 kDa are phosphorylated upon FcεRI aggregation (Benhamou and Siraganian, 1992) and the increased band at 145 kDa might be PLCγ1 (Park et al., 1991). However, increased phosphorylation generally points to activation of the cells (see Fig. 6, lane 1 and 2; Fig. 7, lane 1 and 2), which is not in line with the inhibiting activity of oxatomide on exocytosis. Nevertheless, it is also described in the literature that increased phosphorylation is involved in negative feedback mechabisms, which results in decreased exocytosis (Yamada et al., 1992). Furthermore, it is difficult to draw conclusions about individual proteins from blots of total cell lysates (Minoguchi et al., 1994).

Our study of Ca^{2+} fluxes shows that oxatomide does not affect Ca^{2+} efflux out of the cell, but that the decrease in $[Ca^{2+}]_i$ is caused mainly by inhibition of Ca^{2+} influx over I_{CRAC} channels in the plasma membrane. Much controversy remains on how depletion of Ca^{2+} stores activates Ca^{2+} influx (for reviews see Fasolato et al., 1994; Felder et al., 1994). The effect of the protein tyrosine phosphatase inhibitor okadaic acid on thapsigargin-induced Ca^{2+} influx

in HeLa cells (Berlin and Preston, 1993) and the effect of protein kinase inhibitors especially on influx of extracellular Ca^{2+} in RBL cells (Teshima et al., 1994) suggest that phosphorylation/dephosphorylation events play a role in the coupling of depletion of Ca^{2+} stores to Ca^{2+} influx, possibly by a phosphorylated second messenger, the Ca^{2+} influx factor (Randriamampita and Tsien, 1993). The inhibiting effect of oxatomide on I_{CRAC} might be due to phosphorylation, as we observe strong effects of oxatomide on tyrosine phosphorylation. However, other possibilities cannot be excluded, e.g. perturbation of the membrane structure that may affect the functionality of the membrane Ca^{2+} channels (Fischer et al., 1995). The immediate effect of oxatomide on Ba^{2+} influx, suggests that oxatomide has a direct effect on the activity of the Ca^{2+} channels.

In the literature, reports on effects of oxatomide on Ca^{2+} fluxes have appeared (Tasaka et al., 1987; Yoshii et al., 1991). Tasaka et al. (1987) reported that in rat peritoneal mast cells compound 48/80 induced a rise in $[Ca^{2+}]_i$ in Ca^{2+}-free medium which was inhibited by oxatomide. Oxatomide also inhibited $^{45}Ca^{2+}$ release induced by IP_3, from endoplasmic reticulum-rich fractions which were isolated from mast cells (Yoshii et al., 1991). Our results in complete cells indicate that release of Ca^{2+} from intracellular stores is hardly affected by oxatomide (see Fig. 8A). Besides, the amount of IP_3 is slightly increased by oxatomide (see Table 2). De Clerck et al. (1981) reported less effective inhibition by oxatomide of the compound 48/80-induced histamine release in rat peritoneal mast cells at increasing free extracellular Ca^{2+} concentration. This is in agreement with our results that inhibition of extracellular Ca^{2+} influx is a major cause for the inhibition of exocytosis by oxatomide. The inhibition of Ca^{2+} influx in bovine adrenal chromaffin cells over voltage-operated Ca^{2+} channels by oxatomide (Tachikawa et al., 1993) indicates that oxatomide might not only affect I_{CRAC} channels, but also other types of Ca^{2+} channels. Voltage-operated channels are not involved in Ca^{2+} influx in mast cells and RBL cells (Matthews et al., 1989; Zhang and McCloskey, 1995).

In conclusion, inhibition of Ca^{2+} influx over I_{CRAC} channels is the major cause of inhibition of exocytosis by the anti-allergic drug oxatomide. This inhibition occurs at oxatomide concentration of around ± 10 μM. The same concentration range has been reported for in vitro inhibition of mediator release by oxatomide in other mast cell types (De Clerck et al., 1981; Tasaka et al., 1987; Truneh et al., 1982). This concentration is rather high compared to effects of oxatomide on histamine release under in vivo conditions (Awouters et al., 1980). Apparently under physiological conditions mast cells are more sensitive. It is known that mast cell behavior is sensitive to the micro-environment (Galli, 1990). Furthermore, other cells in the cellular network leading to hypersensitivity reactions may be affected by oxatomide. In a subsequent study the role of membrane distortion in the inhibition of Ca^{2+} influx by oxatomide and derivatives will be explored.

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