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Effects of Oxatomide and Derivatives on High Affinity IgE Receptor-activated Signal Transduction Pathways in Rat Basophilic Leukemia Cells: Role of Protein Tyrosine Hyperphosphorylation and Inhibition of Extracellular Calcium Influx

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ABSTRACT. The antiallergic drug oxatomide and analogs inhibit mediator release from a rat basophilic leukemia (RBL-2H3) cell line, which is frequently used as a mast cell model. By investigating a series of derivatives of oxatomide with different inhibiting activities on exocytosis, we aimed to evaluate the role of their effects on the early steps of the signal transduction cascade in the inhibition of exocytosis. The active compounds induced hyperphosphorylation of tyrosine residues both in stimulated as well as in resting cells. Furthermore, some elevation of the inositol 1,4,5-trisphosphate (IP_3) formation upon antigen activation was observed for the active derivatives. Ca^{2+} fluxes were also studied. The inhibition of the antigen-induced $^{45}Ca^{2+}$ influx correlated with the effects of the drugs on exocytosis. Furthermore, the inhibitory activity on antigen- and thapsigargin-mediated exocytosis correlated well. Adherence of the cells to fibronectin, stimulating cellular integrin receptors, was synergistic to antigen activation of the RBL cells. However, oxatomide did lack any effect on integrin-mediated processes, as the IC_{50} value for exocytosis was identical for fibronectin-adhered cells and standard cultured cells. We conclude that oxatomide and its analogs inhibit exocytosis, mainly by inhibiting Ca^{2+} influx over store-operated Ca^{2+} (SOC) channels. The drugs have a direct effect on the store-operated Ca^{2+} channels or affect the direct regulation of these channels. BIOCHEM PHARMACOL **56**;6:693–701, 1998.

KEY WORDS. oxatomide; protein tyrosine phosphorylation; calcium influx; signal transduction; antiallergics; mast cell exocytosis

Inhibition of mast cell mediator release may be a mechanism by which the antiallergic drug oxatomide exerts its activity [1]. We reported that derivatives of oxatomide inhibit mediator release from a mast cell model [1]. We concluded that the inhibition of extracellular calcium influx by oxatomide is a cause for the inhibitory effect on exocytosis in RBL-2H3‖ cells [2].

Several causes for inhibition of extracellular calcium influx are possible, e.g. a direct effect on the activity of the Ca^{2+} channels [2, 3], or effects on earlier signal transduction processes that precede and regulate Ca^{2+} influx. The investigation described here was aimed at studying the early steps of the signal transduction cascade leading to exocytosis. To this end, we used a series of closely related derivatives of oxatomide. These derivatives differ in their activity to inhibit exocytosis [1]. Relating this activity to their cellular effects may provide insight into the mechanism(s) by which the antiallergic drug oxatomide and its derivatives inhibit mediator release.

Upon cross-linking of FcεRI receptors, RBL-2H3 cells are activated and a cascade of signal transduction processes occurs. Proteins are phosphorylated at different stages of mast cell activation [4]. In RBL cells, phosphorylation of the protein tyrosine kinases Lyn and Syk is one of the earliest events after FcεRI aggregation [5], and PLCγ1 (EC1.4.3) is phosphorylated as well [6, 7]. Subsequently,

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‖ *Abbreviations:* anti-DNP-IgE, IgE directed against the dinitrophenyl hapten; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; DNP_{30}-HSA, antigen dinitrophenylalbumin conjugate; FAK, focal adhesion kinase; FcεRI, high affinity IgE receptor; IP_3, inositol 1,4,5-trisphosphate; SOC channel, store-operated Ca^{2+} channel; PLCγ1, phospholipase C γ1; and RBL-2H3, rat basophilic leukemia cell line.

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IP_3 is generated upon PLCγ1-induced hydrolysis of membrane phospholipids. The $[Ca^{2+}]_i$ is increased, which ultimately results in the release of preformed and newly formed mediators. The antigen-induced increase in $[Ca^{2+}]_i$ in RBL cells is biphasic: an initial rise is caused by the release of Ca^{2+} from IP_3-sensitive stores [2, 8], which is followed by an influx of extracellular Ca^{2+} via SOC channels [9, 10]. Inhibition of Ca^{2+} influx via these SOC channels in the plasma membrane is related to inhibition of mediator release by oxatomide [2].

We studied effects of the drugs on FcεRI aggregation-induced protein tyrosine phosphorylation and IP_3 generation. Furthermore, we studied the influence of these compounds on thapsigargin- and antigen-mediated exocytosis and Ca^{2+} fluxes.

RBL-2H3 cells can adhere to an extracellular matrix, in which cell surface receptors such as integrins are involved. The activation of integrins by adherence of cells to fibronectin results in many cellular processes, such as reorganization of the cytoskeleton, protein tyrosine phosphorylation, and enhanced secretion [11, 12]. We investigated how this adherence might influence exocytosis and phosphorylation of RBL cells and how the antiallergic compounds might affect these processes.

The oxatomide derivatives appear to affect several processes, e.g. tyrosine phosphorylation of cellular proteins, IP_3 formation, and influx of extracellular Ca^{2+} via SOC channels. From the correlation between effects of the drugs on antigen-induced exocytosis and Ca^{2+} influx, it is concluded that inhibition of Ca^{2+} influx is the major cause for inhibition of exocytosis induced by the drugs. Furthermore, the drugs affect these channels directly or impede direct activation of these channels.

MATERIALS AND METHODS

Materials

Oxatomide, 1-{3-[4-(diphenylmethyl)-1-piperazinyl]propyl}-1,3-dihydro-2H-benzimidazol-2-one, and derivatives were generously provided by Janssen Pharmaceutica. Anti-DNP-IgE, DNP_{30}-HSA and Fura-2-AM were purchased from Sigma. Bovine plasma fibronectin was from Life Technologies (Breda). Thapsigargin was obtained from Calbiochem. Anti-phosphotyrosine antibody coupled to horseradish peroxidase was purchased from Transduction Laboratories. ^{45}Calcium chloride and D-myo-[^{3}H] inositol 1,4,5-trisphosphate potassium salt were obtained from Amersham.

Standard Cell Culture

RBL-2H3 cells were maintained in culture as described previously [1]. For experiments, cells were incubated overnight at 37° and 5% CO_2. The release of β-hexosaminidase (EC 2.1.52) was measured with 1×10^4 cells per well in a 96-well plate. For determination of protein tyrosine phosphorylation and $^{45}Ca^{2+}$ influx, 4×10^5 cells/well were plated in a 24-well plate, and for IP_3 measurements 2×10^6 cells were plated per well in a 6-well plate. Cells were sensitized with 1 μg/mL of anti-DNP-IgE. For intracellular Ca^{2+} measurements, cells were grown in a 12-well plate (5×10^5/well) on glass coverslips and sensitized with 0.4 μg/mL of IgE. After 1 hr of sensitization, cells were washed with Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.31 mM NaH_2PO_4, 5.6 mM D-glucose, 10 mM HEPES, 12 mM $NaHCO_3$, 0.1% BSA, pH 7.4) and triggered with the antigen DNP_{30}-HSA or thapsigargin. Ten minutes before triggering, antiallergic compound was added.

Fibronectin-adhered Cells

A high binding 96-well ELISA plate (Costar) was coated with fibronectin by incubating wells with 100 μL of fibronectin in PBS (10 μg/mL) at 37° and 5% CO_2. As a control, wells were coated with 2% BSA in PBS. After 3.5 hr of incubation, plates were washed with PBS containing 2% BSA. Meanwhile, cells were harvested as described [1], and 1×10^5 cells/mL were sensitized with anti-DNP-IgE (10 ng/mL) for one hr at 37° under continuous shaking. Cells were then washed with Tyrode's buffer. A 50-μL aliquot of the cell suspension (8×10^5 cells/mL) was added to each coated well and incubated for 20 min at 37° to allow adherence of the cells. Cells were triggered with antigen DNP_{30}-HSA, and antiallergic agents were added 10 min before challenge.

Assay of β-Hexosaminidase

This assay was performed as described perviously [1]. Briefly, cells were triggered for 30 min with antigen DNP_{30}-HSA (20 ng/mL) or thapsigargin (0.2 μM), with or without antiallergic compounds. When thapsigargin was used as a trigger, cells were not sensitized with IgE. Samples of supernatant and Triton-X-100 (1%)-generated cell lysate were collected, and the β-hexosaminidase substrate 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide was added. After incubation at 37° for 1 hr, fluorescence was measured at excitation and emission wavelengths of 360 and 452 nm, respectively.

Protein Tyrosine Phosphorylation

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 of lysis buffer/well. Lysis buffer contained 50 mM Tris-HCl, 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 phenylmethanesulfonyl fluoride, 10 μg/mL of leupeptin, antipain and aprotinin. For fibronectin-adhered cells, 20 ng/mL of antigen was used. After centrifugation of the lysates, Laemmli buffer containing dithiothreitol (DTT) was added to the supernatants and boiled for 5 min. Ten microliters of each sample was analyzed by SDS-polyacrylamide gel electrophoresis (7.5%) and after-

wards electro-transferred to a polyvinylidene difluoride membrane. This membrane was treated with blocking buffer (Tris, NaCl, pH 7.6, 0.05% Tween-20, 5% BSA) for 1.5 hr. After washing, the blot was treated with 1:1000 anti-phosphotyrosine antibody coupled to horseradish peroxidase (PY-20-HRP) for 1.5 hr. After extensive washing, the blots were treated with the enhanced chemiluminescence ECL kit (Amersham) and the tyrosine-phosphorylated proteins visualized on a Kodak X-AR-2 film.

IP_3 Assay

After 5 min of triggering with antigen (40 ng/mL) in the presence or absence of compound, supernatant was removed and 150 μL of 3.5% $HClO_4$ added. This was allowed to stand for 30 min at 4°. IP_3 levels were determined according to the isotope dilution method of Bominaar and Van Haastert [13]. Briefly, samples were neutralized and centrifuged. The supernatant was added to a fixed amount of [^{3}H]IP_3, and IP_3-binding protein isolated from bovine liver was added. Then the sample was centrifuged and the supernatant aspirated. The pellet was resuspended in water, scintillation cocktail was added, and the sample was counted.

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

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 and 10 μCi/mL, respectively). The reaction was stopped on ice, supernatant was removed, and cells were subsequently washed with ice-cold Tyrode's buffer. Triton-X-100 (1%)-generated cell lysates were collected, liquid scintillator was added to all samples, and radioactivity was 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 (approximately 7% of total).

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

Before activating, the cells were loaded with 1 μM Fura-2-AM in Tyrode's buffer supplemented with 0.1 mg/mL of sulfinpyrazone at 37° for 30 min. After a 10-min resting period at room temperature, the glass coverslip was transferred into a cuvette and Tyrode's buffer containing 0.1 mg/mL of sulfinpyrazone and 0.05% gelatine instead of BSA was added. Fluorescence emission (510 nm) was monitored, using a dual-wavelength filter fluorometer (Photon Technology) at 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 antiallergic compound was added 5 min before challenge. Similar experiments were performed in the absence of free extracellular calcium by addition of 3 mM EGTA to the buffer. Maximum and minimum fluorescence signals were obtained by addition of 3 μM ionomycin and

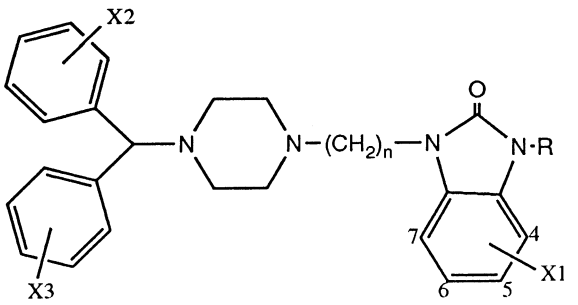

FIG. 1. Basic structure of benzimidazolone analogs of oxatomide.

20 mM EGTA, respectively. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz *et al.* [14] using a K_d of 224 nM. This calibration method does not yield absolute $[Ca^{2+}]_i$, however we compared the $[Ca^{2+}]$ in the presence of drug to that without. Upon antigen activation under standard conditions (see above), the observed increase in $[Ca^{2+}]_i$ was ~400 nM, which is in accordance with reported values [15]. Effects of some drugs on Ba^{2+} influx into RBL cells without free extracellular calcium were performed as previously described [2]. Briefly, fura-2 loaded cells, in the presence of 3 nM EGTA, were triggered with thapsigargin. After return of the fluorescence ratio at 340 and 380 nM (R340/380) to basic levels, 30 μM of drug or DMSO (control) was added, followed by 1.9 mM Ba^{2+}. The change in R340/380 was then monitored.

RESULTS

The structures of the benzimidazolone analogs of oxatomide (basic structure, Fig. 1) used in this study are listed in Table 1.

Exocytosis

Thapsigargin inhibits Ca^{2+}-ATpase located at the endoplasmic reticulum [16]. This brings the Ca^{2+} stores in the endoplasmic reticulum in an emptied state, and a herewith related influx of extracellular Ca^{2+} into the cell ultimately

TABLE 1. Structures of benzimidazolone analogues of oxatomide

Compound	R	N		X_1	X_2	X_3
Oxatomide	H	3		H	H	H
R36415	H	3		5Cl	H	H
R36599	H	3		6Cl	H	H
R34058	H	3		H	F	F
R35918	H	4		H	H	H
R37907	H	4		H	F	H
R37477	H	5		H	H	H
R35873	H	*	CH_3 $-CH_2-CH-CH_2$	H	F	F

*Replaces $(CH_2)_n$.

TABLE 2. Effect of 30 μM oxatomide and derivatives on the release of β-hexosaminidase from RBL cells upon thapsigargin (0.2 μM) or antigen (20 ng/mL) triggering

Compound	Thapsigargin (%)	Antigen* (%)
Oxatomide	42.6 ± 1.1	40.1 ± 8.9
R36415	51.5 ± 3.3	52.5 ± 7.5
R36599	83.5 ± 1.1	94.1 ± 9.9
R34058	29.8 ± 0.3	53.1 ± 10.1
R35918	48.3 ± 1.8	32.5 ± 8.5
R37907	34.6 ± 0.8	14.6 ± 1.5
R37477	18.9 ± 0.5	5.9 ± 2.2
R35873	75.2 ± 1.6	82.0 ± 11.9

The release is expressed relative to control (no drug present). Values are means ± SD from at least two determinations in triplicate.

*Data from [1].

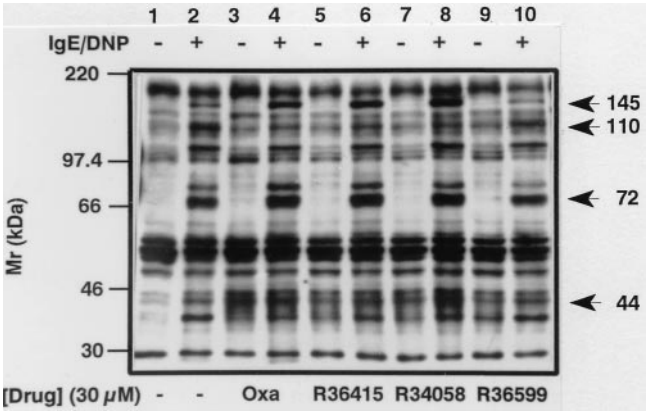

FIG. 2. Effect of oxatomide and some benzimidazolone analogs (30 μM) on tyrosine phosphorylation of cellular proteins in nonstimulated (−) as well as antigen (40 ng/mL)-stimulated (+) RBL cells. Electrophoresis was performed using a 7.5% gel. The results shown are representative of three independent experiments. The effects discussed in the text have been observed in all experiments.

leads to exocytosis [2, 17]. Thapsigargin was used as a trigger to discriminate between directly FcεRI-coupled events and post IP_3 events in the signal transduction. The inhibitory activity of a series of benzimidazolone derivatives of oxatomide on thapsigargin-mediated β-hexosaminidase release was determined in a drug concentration range from 7.5 to 60 μM (results not shown). Table 2 shows the activity of 30 μM compound. Most derivatives inhibited the exocytosis by at least 50% at 30 μM, although two compounds, R35873 and R36599, showed a small inhibitory effect at this concentration. The compound with a chlorine at position 6 of the benzimidazolone group (R36599) had a very small inhibitory activity, whereas the compound having a chlorine at the 5 position (R36415) was more active. The most active compound was R37477, with an alkyl chain length of five carbon atoms between the piperazinyl and the benzimidazolone moiety. Decreasing this chain length diminished activity. R35873, with a branched alkyl chain and two fluorines at the phenyl rings, was not active. This was probably not due to the presence of fluorines as R34058, which also has two fluorines, was even more active than oxatomide.

There appeared to be a good correlation between the inhibition of antigen- and thapsigargin- mediated release (data Table 2, $N = 8$, $r^2 = 0.80$, $F = 23.37$, $P = 0.003$). We previously reported that antigen-induced release was more sensitive to inhibition by these compounds than release with A23187, whether or not in combination with the phorbol ester TPA [1]. This indicates that post-Ca^{2+} influx phases of the signal transduction processes leading to mediator release are less sensitive to inhibition. This in combination with the correlation between the effect on IgE- and thapsigargin-mediated release indicates that the benzimidazolones act especially at the level of Ca^{2+} fluxes.

Protein Tyrosine Phosphorylation

The FcεRI-mediated processes were further investigated by studying the phosphorylation of tyrosine residues of intracellular proteins, which occurs at different stages of the signal transduction pathway. Anti-phosphotyrosine immunoblotting revealed several tyrosine-phosphorylated proteins. After cross-linking of FcεRI, an increase in tyrosine-phosphorylated proteins was found (Fig. 2, lanes 1 and 2). Upon FcεRI aggregation, a 72-kDa protein is phosphorylated [4], while the bands around 110 kDa and 40 kDa are due to later processes: the 110-kDa protein is tyrosine-phosphorylated after influx of Ca^{2+} into the cell [18], and the increase of the bands around 40 kDa can be (partly) ascribed to phosphorylation of mitogen-activated protein kinase [19]. Upon activation protein bands also appeared around 125 kDa and 145 kDa. The 145-kDa band corresponds to the molecular weight of PLCγ1 [7]. The 125-kDa band could contain $pp125^{FAK}$, a focal adhesion-associated tyrosine kinase whose phosphorylation is enhanced in adherent cells after aggregation of FcεRI by an increased Ca^{2+} influx or by activation of protein kinase C [20].

The addition of 30 μM compound to nonstimulated cells (Fig. 2, lanes 3, 5, 7 and 9) already showed an effect on protein tyrosine phosphorylation. All benzimidazolones used induced an enhanced tyrosine phosphorylation of proteins in the molecular-weight range 35–45 kDa, although R35873, an inactive compound, had only a slight effect (results not shown). The phosphorylation of a band around 110 kDa was increased by pretreatment with a compound, except for R35873.

In antigen-activated cells, the level of phosphorylation in the presence of a drug generally remained above the level of the control without drug (Fig. 2, lane 2 compared to lanes 4, 6, 8 and 10), as was also seen in resting cells. Oxatomide increased the phosphorylation of proteins around 35–45 kDa, 70–75 kDa and 145 kDa in antigen-activated cells (Fig. 2) [2]. R35873 and R36599, the inactive compounds with regard to exocytosis, did not increase the phosphorylation of protein bands around 40 kDa, in contrast to the others. The two bands that appeared

TABLE 3. IP_3 generation upon antigen (40 ng/mL)-mediated activation of RBL cells and the influence of 30 μM of several benzimidazolone derivatives of oxatomide

Compound	$[IP_3]$ (pmol/2 × 10^6 cells)
Control	4.33 ± 0.34
Oxatomide	5.14 ± 0.34*
R36599	2.99 ± 0.63**
R34058	5.74 ± 1.25*
R35918	6.51 ± 1.08*
R35873	2.99 ± 0.69**

Values are means ± SD from at least three independent determinations.
*$P < 0.05$, increase vs control.
**$P < 0.05$, decrease vs control.

between 70–75 kDa upon activation were enhanced in the presence of the active compounds R34058 and R36415. Both compounds also induced the increase of the band at 145 kDa. The active compound did not affect the phosphorylation of the 110-kDa protein, which is typically activated after Ca^{2+} influx [18]. The intensity of a band around pp125, which was increased upon activation (lane 2), was strongly decreased, especially in the presence of the active compounds. The inactive compounds R36599 (Fig. 2, lane 10) and R35873 (results not shown) exhibited some remarkable changes in the tyrosine phosphorylation pattern compared to the more active compounds: markedly less phosphorylation of the bands at pp145, pp110, pp72 and around 40 kDa was observed, while the phosphorylation at 125 kDa was increased compared to the active compounds.

It is remarkable that the hyperphosphorylation observed with the active drugs, corresponded to the inhibition of exocytosis in RBL cells, as activation of the cells generally increases phosphorylation (compare Fig. 2, lanes 1 and 2). Santini and Beaven also reported hyperphosphorylation of a 40-kDa protein for several inhibitors of tyrosine phosphatases, causing inhibition of exocytosis [19].

IP_3 Formation

IP_3 is generated upon antigen activation of PLCγ1 from phosphatidyl inositol 4,5-bisphosphate. This is an important step in the early phases of the signal transduction resulting in the release of Ca^{2+} from the IP_3-sensitive intracellular Ca^{2+} stores. The concentration of IP_3 was increased from 1.16 pmol/2 × 10^6 cells in resting cells to 4.33 pmol/2 × 10^6 cells upon 5 min of antigen activation (Table 3). Oxatomide, R35918, and R34058 enhanced the $[IP_3]$, whereas the less active compounds R35873 and R36599 inhibited IP_3 formation (Table 3). The active compounds oxatomide and R34058 also enhanced the phosphorylation of a tentative PLCγ1 band at 145 kDa (Fig. 2, lanes 4 and 8), whereas the inactive compounds R35873 (results not shown) and R36599 did not (Fig. 2, lane 10).

TABLE 4. Effect of 30 μM oxatomide and derivatives on $^{45}Ca^{2+}$ influx

Compound	Ca^{2+} influx (%) IgE/DNP	Thapsigargin
Oxatomide	16.1 ± 6.0	13.2 ± 3.6
R36415	39.3 ± 10.6	n.d.*
R36599	n.d.	54.9 ± 5.5
R34058	29.6 ± 6.7	11.2 ± 3.5
R35918	13.7 ± 2.7	6.0 ± 0.7
R37907	10.9 ± 0.3	1.8 ± 2.0
R37477	11.2 ± 2.6	n.d.
R35873	43.1 ± 5.7	52.7 ± 3.6

$^{45}Ca^{2+}$ influx was induced by antigen (40 ng/mL) or thapsigargin (0.2 μM). Influx is expressed as % of control (in the absence of drug), Values are means ± SD from at least two independent determinations in duplicate.
*n.d. = not determined.

$^{45}Ca^{2+}$ Influx and Intracellular Calcium Concentration

Ca^{2+} fluxes play a central role in the processes leading to exocytosis. As described previously by us, oxatomide affects Ca^{2+} fluxes, especially the Ca^{2+} influx via SOC channels [2]. In the present study using closely related oxatomide derivatives, we studied their influence on these fluxes by measuring the net influx of $^{45}Ca^{2+}$ and the $[Ca^{2+}]_i$ using the fluorescent probe Fura-2.

Upon thapsigargin or antigen activation, the $^{45}Ca^{2+}$ influx was effectively inhibited by 30 μM of the benzimidazolones (Table 4). All compounds inhibited the antigen-induced influx by at least 50%. In a detailed study of the effect on various Ca^{2+} fluxes, we previously demonstrated that oxatomide inhibition of the net $^{45}Ca^{2+}$ influx is caused by inhibition of SOC channels [2]. The inhibition of antigen-activated exocytosis and Ca^{2+} influx appeared to correlate well ($N = 7$, $r^2 = 0.82$, $F = 22.36$, $P = 0.005$) (Fig. 3), which points to the major role of inhibition of Ca^{2+} influx in the inhibition of exocytosis.

Previously, we described the concentration-dependent correlation between the inhibition of antigen-mediated

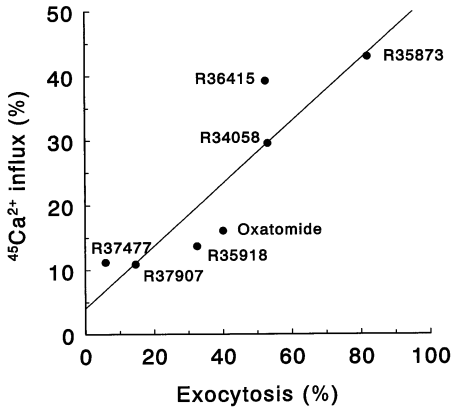

FIG. 3. Relation between inhibition of antigen-induced exocytosis and $^{45}Ca^{2+}$ influx by oxatomide and some analogs. The inhibition is expressed as the percentage compared to the absence of drug.

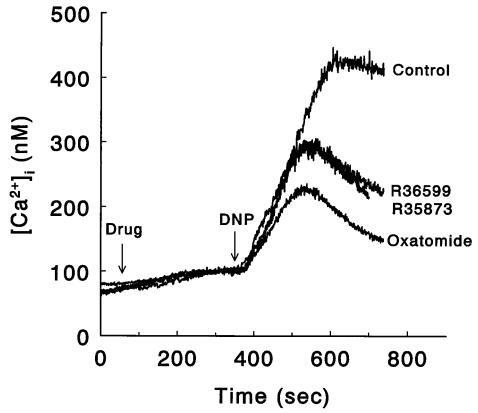

FIG. 4. Effect of oxatomide and some of its derivatives on the antigen-induced $[Ca^{2+}]_i$ increase. Fura-2 loaded cells were incubated (at t = 50 sec) with DMSO as a control or drug (30 μM) for 5 min and subsequently stimulated with antigen (100 ng/mL of DNP). The tracings are representative of at least two independent experiments.

exocytosis and Ca^{2+} influx by oxatomide [2]. The calcium influx had to be inhibited by more than 40% before exocytosis was influenced. Thapsigargin-mediated $^{45}Ca^{2+}$ influx was inhibited by almost 50% by 30 μM R36599 and R35873, (Table 4), which is not sufficient to inhibit exocytosis. From the regression line as depicted in Fig. 3, it appears that a threshold value for inhibition of Ca^{2+} influx also exists for the other benzimidazolones.

We measured the influence of oxatomide and two inactive derivatives, R35873 and R36599, on the antigen-induced increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (Fig. 4). Upon activation without drug, the initial rise to approximately 420 nM was followed by a sustained level of $[Ca^{2+}]_i$, which slowly decreased. In the presence of 30 μM drugs, the peak level of the $[Ca^{2+}]_i$ was strongly decreased. In addition, the decline of the plateau phase was markedly increased (Fig. 4).

We recently demonstrated that oxatomide also inhibits the thapsigargin-induced rise in $[Ca^{2+}]_i$ [2]. Other oxatomide derivatives also inhibited the thapsigargin-induced rise in $[Ca^{2+}]_i$, (results not shown).

To study possible effects of the drugs on Ca^{2+} release from intracellular stores or on the efflux of calcium over the plasma membrane, experiments were performed in medium without free extracellular Ca^{2+}. This was achieved by complexing extracellular Ca^{2+} with EGTA. Because of the small rise in $[Ca^{2+}]_i$ upon antigen trigger without free extracellular Ca^{2+}, we could not observe an increased release of Ca^{2+} from intracellular stores due to increased IP_3 formation. Upon triggering with thapsigargin, an immediate rise in $[Ca^{2+}]_i$ was measured (Fig. 5). For oxatomide it was reported earlier that the peak level was affected slightly but not significantly [2]. R35918, another active inhibitor of the Ca^{2+} influx, also seemed to have a slight but not significant effect on the mobilization of Ca^{2+} from the internal stores (85.8 ± 8% compared to 100 ± 10.9%, $P > 0.05$). After reaching a peak, the $[Ca^{2+}]_i$ decreased

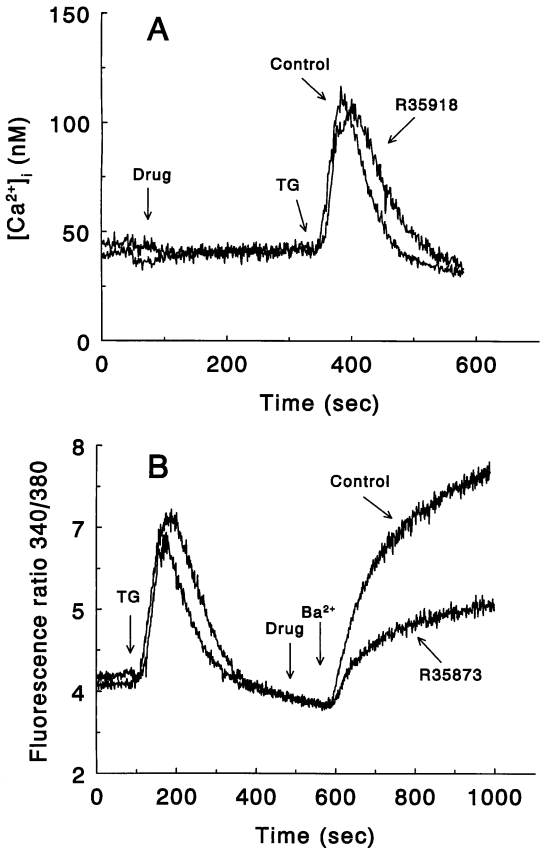

FIG. 5. Effect of R35918 on thapsigargin (0.4 μM)-induced change in $[Ca^{2+}]_i$, in fura-2 loaded RBL cells, with free extracellular calcium bound to 3 mM EGTA. Panel A: After 5 min of preincubation with 30 μM R35918 or DMSO (as a control), cells were triggered with thapsigargin. Panel B: After return of the fluorescence ratio to basic value, 30 μM R35873 was added, followed 1 min later by 1.9 mM Ba^{2+}. The tracings are representative of at least two independent experiments.

rapidly by efflux of Ca^{2+} over the plasma membrane. The rate of decrease of $[Ca^{2+}]_i$ to basal levels was identical for the control and for R35918 ($t_{1/2}$ approximately 60 sec), indicating that the extrusion of Ca^{2+} out of the cell was not influenced by the drug. Like oxatomide [2], its derivatives active in inhibiting $^{45}Ca^{2+}$ influx blocked the Ba^{2+} influx measured as a change in fluorescence ratio of fura-2, which is indicative of inhibition of Ca^{2+}channels [2].

Fibronectin-adhered Cells

To simulate the *in vivo* physiological situation more closely, we studied RBL cells adhered to fibronectin. Fibronectin adherence resulted in morphological changes not seen with BSA adherence, and more cells were adhered to fibronectin-coated surfaces compared to BSA-coated surfaces. For fibronectin-adhered cells, the release of β-hexosaminidase was more sensitive for IgE compared to BSA-adhered cells and cells adhered to plastic (standard culture). As can be seen in Fig. 6A, the IgE concentration evoking half-maximum release of β-hexosaminidase was 3 ng/mL for

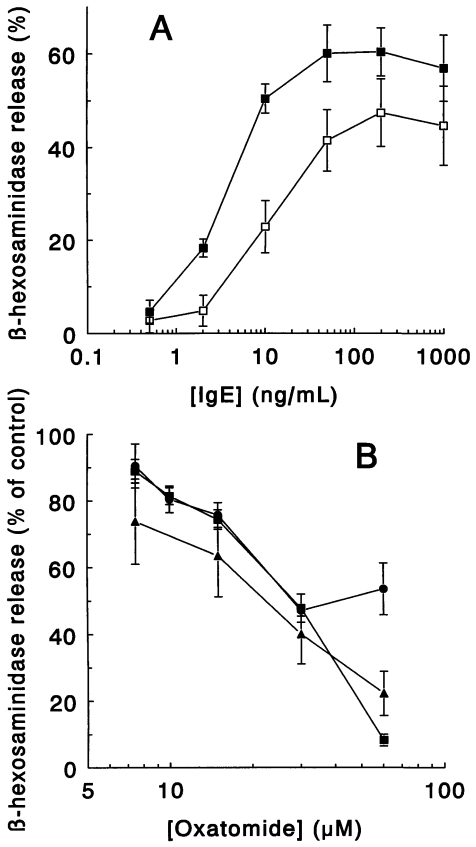

FIG. 6. Synergistic activation of RBL cells by fibronectin adherence and FcεRI activation. (A) Cells were adhered to fibronectin (10 μg/mL) (■) or BSA (2%) (□) coated wells, sensitized with anti-DNP-IgE and activated with 20 ng/mL of antigen. β-Hexosaminidase release is presented as % of total amount. (B) Concentration effect curves of oxatomide on antigen-mediated β-hexosaminidase release in RBL cells in standard culture (▲), adhered to fibronectin (■) or to BSA (●). Cells were sensitized with 10 ng/mL of IgE for fibronectin- and BSA-adhered cells and 0.2 μg/mL of IgE was used for standard cultured cells. β-Hexosaminidase release is expressed as % of control (no drug present).

fibronectin-adhered cells and 20 ng/mL for BSA-adhered cells. Cells from a standard culture had a maximum release of 60% with 200 ng of IgE/mL (data not shown). The synergism of FcεRI stimulation and fibronectin adherence has been reported previously and is associated with activation of cellular integrins, which recognize binding sites on fibronectin, leading to intracellular processes affecting tyrosine phosphorylation and changes in the cytoskeleton and morphology of the cell [11]. This synergism prompted us to investigate the inhibiting effect of oxatomide and some of its derivatives on exocytosis in fibronectin-adhered cells. As the difference in exocytosis between fibronectin and BSA-adhered cells was largest at 10 ng/mL of IgE, this concentration was used in further experiments.

We investigated the effect of oxatomide and two less active derivatives, R35873 and R36599, on β-hexosaminidase release. The results for oxatomide are shown in Fig. 6B,

TABLE 5. Effect of oxatomide and some derivatives (30 μM) on exocytosis induced by antigen in RBL cells under different conditions of adherence

Compound	Standard	Fibronectin	BSA
Oxatomide	40.1 ± 8.9	47.9 ± 4.2	47.2 ± 1.6
R36599	94.1 ± 9.9	102.5 ± 1.5**	117.3 ± 10.0*
R35873	82.0 ± 11.9	106.0 ± 3.2*	116.2 ± 19.5*

Cells were incubated overnight in a well plate (standard culture), on a fibronectin-coated well plate, or on a BSA-coated well plate. The release is expressed relative to control (no drug present). Values are means ± SD from at least two independent experiments in duplicate or triplicate.

*$P < 0.05$ for fibronectin or BSA vs standard culture.

**$P < 0.05$ for fibronectin vs BSA.

where we find comparable concentration effect curves for fibronectin- and BSA-adhered cells as well for standard cultured cells. From Table 5, it appeared that the inhibiting activity of the drugs was not affected by fibronectin adherence of the cells. R35873 and R36599 did not inhibit the exocytosis at all in fibronectin- and BSA-adhered cells. With 60 μM oxatomide, however, increased release from BSA-adhered cells was observed, not seen with the other culture conditions. Moreover, without antigen activation at 60 μM, all three drugs showed increased release only in BSA-adhered cells (results not shown). Such drug-induced release from nonactivated cells is generally observed in standard cultures at higher drug concentrations (>100 μM) and is related to influx of extracellular Ca^{2+} [21]. We suggest that in BSA-adhered cells the cellular membrane is more susceptible to perturbation induced by the drugs [1], resulting in a change in Ca^{2+} permeability.

Whether intracellular events were influenced by fibronectin adherence was studied by antiphosphotyrosine immunoblotting of antigen-activated cells (Fig. 7). The light band observed in every lane was due to nonphospho-

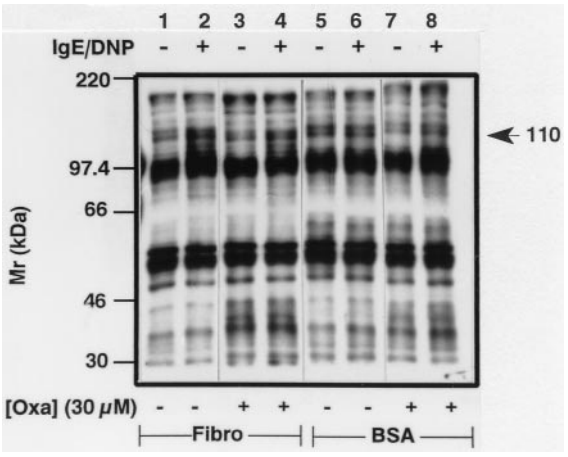

FIG. 7. Effect of fibronectin and BSA adherence on tyrosine phosphorylation of cellular proteins with or without 30 μM oxatomide in nonstimulated (−) as well as antigen (20 ng/mL)-stimulated (+) RBL cells. Electrophoresis was performed using a 7.5% gel. The results shown are representative of four independent experiments. The effects discussed in the text have been observed in all experiments.

rylated BSA at 66 kDa. With fibronectin- and especially BSA-adhered cells, the BSA could not be completely washed away without the loss of cells. Therefore, other tyrosine-phosphorylated proteins in this molecular weight region, e.g. pp72, were not visible. Fibronectin- and BSA-adhered cells, which were not stimulated, revealed a comparable phosphorylation pattern, except for a band around 60 kDA that only occurred in BSA-adhered cells (Fig. 7, lanes 1 and 5). Upon activation, fibronectin-adhered cells showed an increase in protein tyrosine phosphorylation in the molecular weight range of pp105-pp115 and pp120-pp130 (lane 2), whereas BSA-adhered cells did not (lane 6). This is in accordance with previous reports demonstrating that aggregation of FcεRI and adherence to fibronectin synergistically regulates tyrosine phosphorylation of the 105–115-kDa proteins [11], whereas aggregation of FcεRI in adherent cells enhances the phosphorylation of $pp125^{FAK}$ [19].

Pretreatment of resting cells with oxatomide induced phosphorylation of proteins in the molecular weight range of 40–45 kDa. This was observed in both fibronectin- and BSA-adhered cells (Fig. 7, lanes 3 and 7) and, as mentioned above, also in standard cultured cells (Fig. 2). In fibronectin-adhered antigen-activated cells, oxatomide seemed to somewhat decrease the phosphorylation of proteins in the molecular weight region 105–115 kDa and 125 kDa.

DISCUSSION

The results of this study show that inhibition of exocytosis by oxatomide is mainly caused by inhibition of extracellular Ca^{2+} influx. This appears from the correlation between the inhibition of antigen- and thapsigargin-mediated release (Table 2) and from the fact that the drugs are much less effective in influencing post Ca^{2+} influx phases of the signal transduction process. In addition, we have demonstrated a relation between inhibition of exocytosis and inhibition of Ca^{2+} influx for a series of oxatomide derivatives (Fig. 3). The $[Ca^{2+}]_i$ is also decreased in the presence of the drugs (Fig. 4). This is not caused by effects on release from intracellular Ca^{2+} stores or efflux of Ca^{2+} out of the cell (Fig. 5B). However, various cellular processes are affected by the drugs, e.g. IP_3 formation and tyrosine phosphorylation of cellular proteins. The observed increased IP_3 formation by the active compounds is adversative to inhibition of exocytosis, as it enhances the generation of calcium fluxes from Ca^{2+}stores and exocytosis [22]. As mentioned above, we have not observed such effects on Ca^{2+}fluxes (Fig. 5B). The decrease in $[Ca^{2+}]_i$ and the observed effects on tyrosine phosphorylation may be related, as Ca^{2+} is known to regulate tyrosine kinases as well as phosphatases [23], and a protein kinase-mediated phosphorylation, PKC, has been found to regulate SOC channels [24]. On the other hand, inhibition of Ca^{2+} influx can affect protein phosphorylation [18]. The thapsigargin-induced exocytosis strongly suggests that only effects of the drugs on processes after emptying of the intracellular Ca^{2+} stores are relevant for the inhibition of Ca^{2+} influx. This includes a direct effect on the SOC channels or its direct regulation mechanism. The mechanism with which SOC channels are regulated has not yet been elucidated and is the subject of considerable debate (see [25] for a review). Blocking of Ba^{2+}influx by oxatomide and its active derivatives (Fig. 5B) strongly suggests that the drugs affect the SOC channels.

The similarity in the inhibitory effect of oxatomide on exocytosis in fibronectin-adhered cells and cells in a standard culture (Fig. 6B) indicates that oxatomide does not affect the processes directly related to integrin activation by binding to fibronectin. This similarity suggests the same mechanism is operative for inhibition of exocytosis for fibronectin-adhered and standard cultured cells. This is in line with our conclusion that effects on the level of the SOC channel are the major cause of inhibition of exocytosis, as Ca^{2+} influx occurs after convergence of the synergistic integrin-mediated pathway and the FcεRI-mediated pathway [11].

In principle, there are several possibilities as to how the drugs affect the activity of SOC channels. It has been suggested that phosphorylation of these channels or of PKC might regulate the activity of SOC channels [23, 24, 26]. The effect of the drugs on the phosphorylation of cellular proteins is in line with this possibility, especially as the more active compounds enhance the tyrosine phosphorylation of several proteins. Addition of oxatomide immediately blocks the SOC channels [2]. This also suggests a direct effect of the drugs on the SOC channels. It is conceivable that drug-membrane interactions are involved in the effects of the drugs reported in this study [3]. Depolarization of the RBL cell membrane results in inhibition of Ca^{2+} influx and exocytosis [27], thereby indicating that the activity of the SOC channel is sensitive to changes in the membrane environment.

Therefore, the effect of the drugs on the SOC channel could also be indirect by influencing the membrane environment. The drugs indeed do change several membrane properties (Paulussen JJC, Fischer MJE, Zuidam NJ, van Miltenburg JC, de Mol NJ and Janssen LHM, manuscript in preparation). The effects of the drugs do not seem to be completely aspecific, as small changes in the structure which do not modify the overall lipophilicity of the molecule (e.g. a chlorine in position 5 or 6, compare R36415 and R36599) affect the activity of the drugs.

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References

1. Paulussen JJC, Fischer MJE, Roelofsen EPW, Horbach DA, de Mol NJ and Janssen LHM, Oxatomide and derivatives as inhibitors of mediator release from a mast cell model; structure activity relationships. *Drug Res* **46:** 496–501, 1996.
2. Paulussen JJC, Fischer MJE, Kok-van Esterik JAE, Tiemessen

RC, de Mol NJ and Janssen LHM, Influence of the anti-allergic drug oxatomide on the signal transduction mechanism in a mast cell model. *Eur J Pharmacol* **312:** 121–130, 1996.
3. Paulussen JJC, Influence of oxatomide and derivatives on membrane structures: relation with inhibition of calcium influx in RBL cells. In: *The antiallergic drug oxatomide: Mechanism of action in a mast cell model,* pp. 71–89, Thesis, Utrecht University, 1996.
4. Benhamou M and Siraganian RP, Protein-tyrosine phosphorylation: an essential component of FcεRI signaling. *Immunol Today* **13:** 195–197, 1992.
5. Stephan V, Benhamou M, Gutkind JS, Robbins KC and Siraganian RP, FcεRI-induced protein tyrosine phosphorylation of pp72 in rat basophilic leukemia cells (RBL-2H3). *J Biol Chem* **267:** 5434–5441, 1992.
6. Li W, Deanin GG, Margolis B, Schlessinger J and Oliver JM, FcεRI-mediated tyrosine phosphorylation of multiple proteins, including phospholipase Cγ1 and the receptor $\beta\gamma_2$ complex, in RBL-2H3 rat basophilic leukemia cells. *Mol Cell Biol* **12:** 3176–3182, 1992.
7. Park DJ, Min HK and Rhee SG, IgE-induced tyrosine phosphorylation of phospholipase Cγ1 in rat basophilic leukemia cells. *J Biol Chem* **266:** 24237–24240, 1991.
8. Ali H, Maeyama K, Sagi-Eisenberg R and Beaven MA, Antigen and thapsigargin promote influx of Ca^{2+} in rat basophilic RBL-2H3 cells by ostensibly similar mechanisms that allow filling of inositol 1,4,5-trisphosphate-sensitive and mitochondrial Ca^{2+} stores. *Biochem J* **304:** 431–440, 1994.
9. Fewtrell C, Ca^{2+} oscillations in non-excitable cells. *Ann Rev Physiol* **55:** 427–454, 1993.
10. Fasolato C, Innocenti B and Pozzan T, Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? *Trends Pharmacol Sci* **15:** 77–83, 1994.
11. Hamawy MM, Mergenhagen SE and Siraganian Rp, Cell adherence to fibronectin and the aggregation of the high affinity immunoglobulin E receptor synergistically regulate tyrosine phosphorylation of 105–115 kDa proteins. *J Biol Chem* **268:** 5227–5233, 1993.
12. Hamawy MM, Oliver C, Mergenhagen SE and Siraganian Rp, Adherence of rat basophilic leukemia (RBL-2H3) cells to fibronectin-coated surfaces enhances secretion. *J Immunol* **149:** 615–621, 1992.
13. Bominaar AA and van Haastert PJM, Phospholipase C activity in dictyostelium discoideum using endogenous non-radioactive phosphatidylinositol 4,5-bisphosphate as substrate. *Enzymol* **238:** 207–218, 1994.
14. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
15. Lin PY, Wiggan GA, Welton AF and Gilfillan AM, Differential effects of propranolol on the IgE-dependent, or calcium ionophore-stimulated, phosphoinositide hydrolysis and calcium mobilization in a mast (RBL-2H3) cell line. *Biochem Pharmacol* **41:** 1941–1948, 1991.
16. Thastrup O, Cullen PJ, Drøbak BK, Hanley MR and Dawson AP, Thapsigargin, a tumor promotor, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATpase. *Proc Natl Acad Sci USA* **87:** 2466–2470, 1990.
17. Franzius D, Hoth M and Penner R, Non-specific effect of calcium entry antagonists in mast cells. *Pflüg Arch* **428:** 433–438, 1994.
18. Yu K, Lyall R, Jariwala N, Zilberstein A, and Haimovich J, Antigen- and ionophore-induced signal transduction in rat basophilic leukemia cells involves protein tyrosine phosphorylation. *J Biol Chem* **266**, 22564–22568, 1991.
19. Santini F and Beaven MA, Tyrosine phosphorylation of a mitogen-activated protein kinase-like protein occurs at a late step in exocytosis. *J Biol Chem* **268:** 22716–22722, 1993.
20. Hamawy MM, Mergenhagen SE and Siraganian RP, Tyrosine phosphorylation of $pp125^{FAK}$ by the aggregation of high affinity immunoglobulin E receptors requires cell adherence. *J Biol Chem* **268:** 6851–6854, 1993.
21. Fischer MJE, Paulussen JJC, Kok-van Esterik JAE, van der Heijden VS, de Mol NJ and Janssen LHM, Effects of the anti-allergics astemizole and norastemizole on FcεRI receptor-mediated signal transduction processes. *Eur J Pharmacol* **322**: 97–105, 1997.
22. Teshima R, Ikebuchi H, Sawada J, Furuno T, Nakanishi M and Terao T, Effects of herbimycin A and ST638 on Fcε receptor-mediated histamine release and Ca^{2+} signals in rat basophilic leukemia (RBL-2H3) cells. *Biochim Biophys Acta* **1221:** 37–46, 1994.
23. Berlin RD and Preston SF, Okadaic acid uncouples calcium entry from depletion of intracellular stores. *Cell Calcium* **14:** 379–386, 1993.
24. Parekh AB and Penner R, Depletion-activated calcium current is inhibited by protein kinase in RBL-2H3 cells. *Proc Natl Acad Sci USA* **92:** 7907–7911, 1995.
25. Berridge MJ, Capacitative calcium entry. *Biochem J* **312**, 1–11, 1995.
26. Lee K-M, Toscas K and Villereal ML, Inhibition of bradykinin- and thapsigargin-induced Ca^{2+} entry by tyrosine kinase inhibitors. *J Biol Chem* **268:** 9945–9948, 1993.
27. Mohr FC and Fewtrell C, Depolarization of rat basophilic leukemia cells inhibits calcium uptake and exocytosis. *J Cell Biol* **104**, 783–792, 1987.