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Differential release of histamine and prostaglandin D_2 in rat peritoneal mast cells: roles of cytosolic calcium and protein tyrosine kinases

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Abstract

We studied how the release of histamine and prostaglandin D_2 (PGD_2) were connected in stimulated rat peritoneal mast cells, and to what extent these processes were controlled by the cytosolic Ca^{2+} concentration, $[Ca^{2+}]_i$, and protein tyrosine kinases. In the presence of 1 mM $CaCl_2$, the G-protein activating compound 48/80 (10 μg/ml) evoked a transient rise in $[Ca^{2+}]_i$ and a relatively high secretion of histamine, but only a low release of PGD_2. In contrast, 5 μM thapsigargin (an inhibitor of endomembrane Ca^{2+}-ATPases) and 5 μM ionomycin evoked high and prolonged rises in $[Ca^{2+}]_i$, and stimulated the cells to release relatively small amounts of histamine and high amounts of PGD_2. Stimulation of the cells with $CaCl_2$ and 10 μM ATP^{4-} gave only minor quantities of histamine and PGD_2, despite of the micromolar level of $[Ca^{2+}]_i$ reached. When $CaCl_2$ was replaced by EGTA, rises in $[Ca^{2+}]_i$ as well as release of histamine and PGD_2 were reduced with each agonist, but the preference of agonists to release more histamine or PGD_2 remained unchanged. In mast cells with depleted Ca^{2+} stores, the addition of $CaCl_2$ stimulated the store-regulated Ca^{2+} entry resulting in a prolonged rise in $[Ca^{2+}]_i$. However, simultaneous addition of compound 48/80 and $CaCl_2$ was required for release of histamine and PGD_2. In cells with full stores, PGD_2 release evoked by compound 48/80 was greatly reduced by genistein and methyl-2,5-dihydroxycinnamate, two structurally unrelated inhibitors of protein tyrosine kinases, whereas histamine secretion was not influenced by these inhibitors. Similarly, with thapsigargin or ionomycin as agonist, PGD_2 release was more sensitive to the tyrosine kinase inhibitors than histamine secretion. We conclude that in activated rat peritoneal mast cells: (i) the influx of extracellular Ca^{2+} potentiates agonist-evoked rises in $[Ca^{2+}]_i$ as well as histamine secretion and PGD_2 release; (ii) the amplitude of the $[Ca^{2+}]_i$ rise does not determine the preferential effect of agonists to release more histamine or more PGD_2; (iii) the relatively high PGD_2 release evoked by thapsigargin and ionomycin is probably due to their potency to evoke a prolonged rise in $[Ca^{2+}]_i$ and to activate protein tyrosine kinases.

Keywords: Calcium; Eicosanoid; Histamine; Mast cell; Protein tyrosine kinase; (Rat)

1. Introduction

Mast cells play important roles in processes like inflammation, anaphylaxis and asthma [1]. Activation of these cells results in the release of compounds like histamine and prostaglandin D_2 (PGD_2), which induce vasodilation, increased vascular permeability and bronchoconstriction [2,3]. Histamine and PGD_2 are released from activated mast cells by two quite different mechanisms. Histamine is stored in the intracellular secretory granules and is released into the extracellular medium by exocytosis. It is generally accepted that both elevation in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and activation of the protein kinase C pathway are important in triggering the exocytotic event [4,5]. The heterotrimeric G protein, G_{i3}, probably plays an additional role in this process [6]. On the other hand, the principle route to produce PGD_2 is most likely by activation of phospholipase A_2, a Ca^{2+}-dependent enzyme which liberates arachidonic acid from the membrane phospholipids [7,8].

Abbreviations: $[Ca^{2+}]_i$, cytosolic-free Ca^{2+} concentration; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MAP kinase, mitogen-activated protein kinase; PGD_2, prostaglandin D_2.

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The observation that stimulation of peritoneal mast cells with G protein-activating compounds (e.g. substance P or compound 48/80) and with typically Ca^{2+}-mobilizing compounds (e.g. the Ca^{2+} ionophore A23187) evokes both histamine secretion and PGD_2 release [9], may suggest that these two processes are activated by common signal transduction pathways and that elevation in $[Ca^{2+}]_i$ is part of this common mechanism. Indeed, one research group has even proposed that both pathways may coincide and PGD_2 release be largely secondary to exocytosis [10]. However, ample evidence has been presented by others that histamine secretion and PGD_2 release occur independently of each other and have to be regulated in different ways. For instance, it was found that stimulation of rat peritoneal mast cells with anti-immunoglobulin E or A23187 resulted in a relatively high PGD_2 release and a moderate histamine secretion [9,11,12], whereas stimulation with bradykinin, substance P or compound 48/80 gave a much lower PGD_2 release and increased histamine secretion [9,11]. Comparable results have been obtained with human skin mast cells [13–15].

In rat peritoneal mast cells, the release of both histamine and PGD_2 is known to be initiated after $[Ca^{2+}]_i$ is increased from basal (50–100 nM) to higher levels [12,16]. Nevertheless, the time intervals in which these two release processes take place are often different. With various agonists including compound 48/80, histamine secretion is detectable already at seconds after the elevation of $[Ca^{2+}]_i$ [16], whereas PGD_2 generation starts after a lag time of tens of seconds [9,17]. An exception appears to be the ionophore A23187, which was found to evoke a gradual and simultaneous release of both compounds that persisted several minutes [9,12]. Together, this suggests that either release process may require different types of Ca^{2+} responses which, indeed, has been reported for mast cells stimulated with A23187 [12].

In this paper, we determined the relationship between histamine secretion and PGD_2 release after the stimulation of rat peritoneal mast cells with different types of agonists, and questioned to what extent these processes were related to the amplitude and shape of the agonist-evoked elevation in $[Ca^{2+}]_i$. Therefore, we loaded mast cells with fura-2 and measured the released products after stimulation with agonists that directly or indirectly influenced intracellular Ca^{2+} homeostasis. Cells were stimulated in the presence of absence of $CaCl_2$ with compound 48/80, known to activate phospholipase C in a G protein-dependent way [18,19] and with thapsigargin, an inhibitor of the endomembrane Ca^{2+}-ATPases, which in many cell types activates the so-called store-regulated Ca^{2+} influx pathway independently of phospholipase C [20,21]. Mast cells were also stimulated with agents directly permeating the plasma membrane for Ca^{2+}, i.e. the Ca^{2+}-ionophore ionomycin and the Mg^{2+}-free form of ATP, ATP^{4-}. The latter compound opens specific pores in the plasma membrane following binding to a P_{2y}-subtype purinergic receptor [22,23]. Since in a variety of cell types protein tyrosine kinases are likely to be involved in the activation of phospholipase A_2 [24], arachidonic acid release [25,26] and eicosanoid formation [27,28], we also questioned to what extent the differential ability of agonists to release more histamine or PGD_2 may be due to the activation of these kinases. Two structurally dissimilar inhibitors of protein tyrosine kinases, genistein and methyl-2,5-dihydroxycinnamate, which have been extensively tested in a number of cell types [28–31], were used to answer this question.

Our results indicate that the store-regulated Ca^{2+} influx pathway represents a major contribution to the Ca^{2+} signal evoked by compound 48/80 and thapsigargin, and that the influx of Ca^{2+} potentiates both histamine secretion and PGD_2 release. However, we concluded that not the amplitude of the agonist-evoked Ca^{2+} response, but, instead, the duration of this response in combination with a different involvement of protein tyrosine kinases in these two release processes primarily determined the preferential release of histamine or PGD_2.

2. Materials and methods

2.1. Chemicals

Metrizamide was purchased from Nyegaard (Oslo, Norway). Bovine serum albumin, compound 48/80 (a condensation product of *N*-methyl-*p*-methoxy-phenethylamine with formaldehyde), thapsigargin and ATP^{4-} (used in the Mg^{2+}-free form) were obtained from Sigma (St. Louis, MO, USA). Heparin (Thromboliquine®) was from Organon Teknika (Boxtel, The Netherlands). Fura-2 acetoxymethyl ester and pluronic F-127 were from Molecular Probes (Eugene, OR, USA) and ionomycin was from Serva (Heidelberg, Germany). All chemicals were of analytical grade. Water was purified with a milli-Q purification unit from Millipore (Bedford, MA, USA). Methyl-2,5-dihydroxycinnamate and genistein were obtained from Sigma and freshly dissolved into dimethyl sulfoxide before each experiment.

2.2. Mast cells

Male Wistar retired breeder rats were obtained from Charles River Laboratories (Sulzfeld, Germany). Five to ten rats were used for each experiment. The rats, weighing 250 to 350 g, were anaesthetized with diethyl ether and peritoneal mast cells were obtained as described earlier [32]. Briefly, after intraperitoneal injection of lavage buffer, composed of 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, gelatin (1 mg/ml) and heparin (10 IE/ml) (pH 7.4), the abdomen was massaged and the peritoneal exudate was recovered. After centrifugation a cell sediment was obtained, which was washed twice with lavage buffer. Mast cells were separated from other

nucleated cells by centrifugation at 470 × *g* for 15 min through a 22.5% (w/v) metrizamide gradient in lavage buffer. After washing the sediment twice, contaminating erythrocytes were separated from the mast cells by centrifugation through a discontinuous 3%/9% (w/v) metrizamide gradient at 50 × *g* for 10 min. The sediment, containing the mast cells, was washed twice with buffer (pH 7.4) containing 136 mM NaCl, 2.7 mM KCl, 10 mM Hepes and 0.1% (w/v) bovine serum albumin. The cells were finally resuspended into 3 ml buffer (pH 7.4), supplemented with 5% (w/v) bovine serum albumin and 5 mM glucose in a concentration of $2 \cdot 10^6$ cells/ml. Cells were counted with a Coulter counter, model ZM (Coulter Electronics, Krefeld, Germany). Final purity of the mast cell preparations was > 99%, as determined by microscopy after staining with toluidine blue and May-Grünwald-Giemsa. Mean mast cell number obtained per rat was $8 \cdot 10^5$, sufficient to carry out 2–3 incubations.

2.3. Fura-2 fluorescence measurements

Suspended mast cells were loaded with fura-2 acetoxymethyl ester (3 μM) in the presence of the dispersing agent pluronic F-127 (1 mg/ml) under slow rotation at 20°C for 45 min, as described earlier for rat platelets [33]. These conditions were used to prevent intracellular compartmentalization of the probe. After centrifugation at 195 × *g* for 5 min, the fura-2-loaded cells were resuspended in buffer pH 7.4 in a concentration of $1 \cdot 10^5$ cells/ml. Changes in fura-2 fluorescence were measured at an emission wavelength of 500 nm, while the excitation wavelength was switched continuously between 340 and 380 nm. Fluorescence data were collected every 2 s and processed by a personal computer. Calibration values for the calculation of $[Ca^{2+}]_i$ were collected after the addition of 0.1% (w/v) Triton X-100 or digitonin in the presence of 1 mM $CaCl_2$, followed by 10 mM EGTA and 50 mM Tris-HCl (pH 8.3), according to the procedure of Grynkiewicz et al. [34]. In all experiments, we checked for possible compartmentalization of the loaded fura-2: ATP^{4-} (10 μM) in the presence of 1 mM $CaCl_2$ always evoked an immediate and high Ca^{2+} response, indicating that most of the dye was located in the cytosol.

2.4. Mast cell activation

Fura-2-loaded cells ($2.5 \cdot 10^5$ cells/2.5 ml) were activated in plastic cuvettes at a stirring rate of 150 rpm at 37°C. Agonists were added from 1000-fold stock solutions in doses required to induce maximal Ca^{2+} responses at this cell concentration. Calcium measurements were performed continuously or at certain time intervals, as indicated. Samples of 500 μl were withdrawn from the incubations, immediately layered onto 200 μl of silicon oil ($d = 1.049$) and centrifuged in an Eppendorf centrifuge at 15 000 × *g* for 10 s. The supernatants were collected for the determination of histamine or PGD_2, and were stored at −70°C until analysis. Samples for analysis of PGD_2 were methoximated directly after the experiment, according to the instructions of the kit. Total releasable histamine was measured by solubilization of a subsample of the cells with 0.1% (w/v) Triton X-100 and centrifugation of residual cell material at 15 000 × *g* for 10 s. The degrees of histamine secretion and PGD_2 release were corrected for the amounts of histamine and PGD_2 present in the supernatant of unstimulated cells, which values were constant within each experiment and attributed to < 7% of total releasable histamine and < 2 ng $PGD_2/10^6$ cells, respectively. The lower detection limits were 0.3% of total releasable histamine and 1 ng $PGD_2/10^6$ cells.

2.5. Store-depleted mast cells

Mast cells with depleted Ca^{2+} stores were prepared for some experiments. To this end, the cells were loaded with fura-2 as described above, except that ionomycin (5 μM), thapsigargin (100 nM) and EGTA (0.2 mM) were added 15 min after the addition of the fura-2 acetoxymethyl ester. These additions did not interfere with the uptake or deesterification of the fura-2 ester and resulted in a depletion of the intracellular store content of 85 ± 5% and in a reduction of the releasable histamine content of 15 ± 5% (mean ± S.D., $n = 4$). After 30 min of incubation, the cells were centrifuged at 195 × *g* for 5 min, washed with buffer (pH 7.4) and resuspended at a concentration of $1 \cdot 10^6$ cells/ml. The content of the internal Ca^{2+} stores was estimated from the maximal increase in $[Ca^{2+}]_i$ evoked by ionomycin (5 μM) in the presence of EGTA (1 mM). Because of the low dose of thapsigargin used during the store depletion procedure, the mast cells still responded well to higher doses and to compound 48/80.

2.6. Determination of PGD_2 and histamine

PGD_2 was measured with an enzyme-linked immunoassay kit purchased from Cayman Chemical (Ann Arbor, MI, USA). Histamine was determined by a HPLC-method described earlier [35]. Briefly, after the addition of the internal standard 1-methyl histamine, samples were purified and concentrated on carboxylic acid cation exchange columns. Histamine and 1-methyl histamine were eluted with 1 M HCl. After drying under nitrogen at 40°C, the samples were redissolved into 500 μl of a 40 mM sodium borate buffer (pH 9.1) and the samples were derivatized with 500 μl of a freshly prepared fluorescamine solution (0.2 mg/ml acetonitrile). The derivatives were dried under nitrogen and redissolved into 1 ml of the HPLC elution phase consisting of water/acetonitrile/methanol/14.8 M phosphoric acid (750:150:100:2, v/v), brought at pH 6.87 with NH_4OH. Separation was performed at 20°C on a reversed phase Inertsil ODS-2 column (200 × 3 mm internal diameter, 5 μm particles) from Chrompack (Middel-

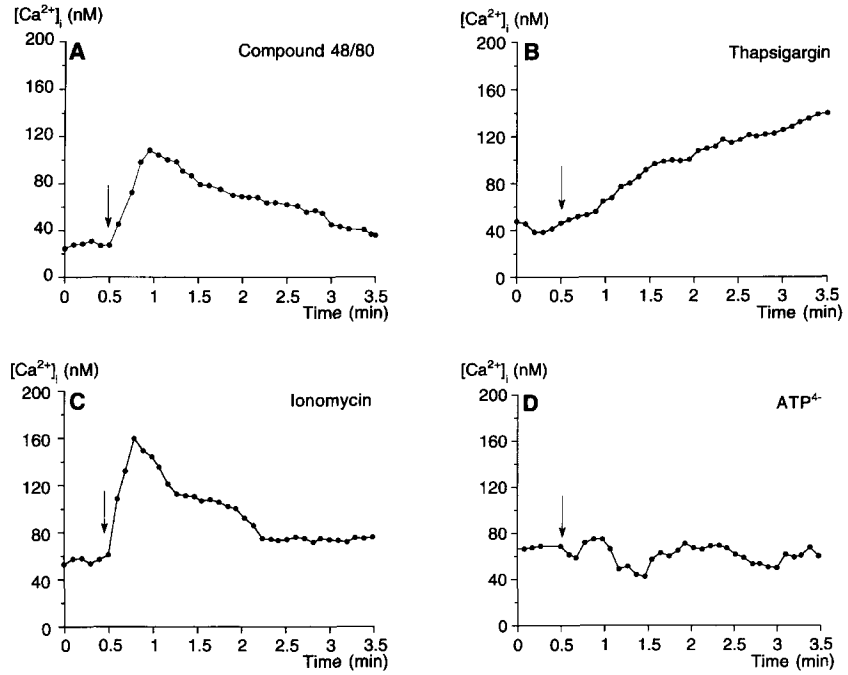

Fig. 1. Calcium responses of mast cells in the presence of EGTA. Fura-2-loaded mast cells were stimulated with (A) compound 48/80 (10 μg/ml), (B) thapsigargin (5 μM), (C) ionomycin (5 μM) or (D) ATP^{4-} (10 μM) in the presence of 1 mM EGTA. Tracings are shown of changes in $[Ca^{2+}]_i$, representative for six experiments. Arrows indicate the addition of agonists.

burg, The Netherlands). The injection volume was 20 μl and flow rate was 0.4 ml/min. Fluorescence was detected at excitation and emission wavelengths of 360 and 440 nm, respectively. Samples were kept in an auto-injector in the dark at 4°C. Data were processed as described before [36].

2.7. Statistical analysis

To evaluate the effects of inhibitors, data were compared to those of control incubations, using the variance analysis of Bonferoni [37].

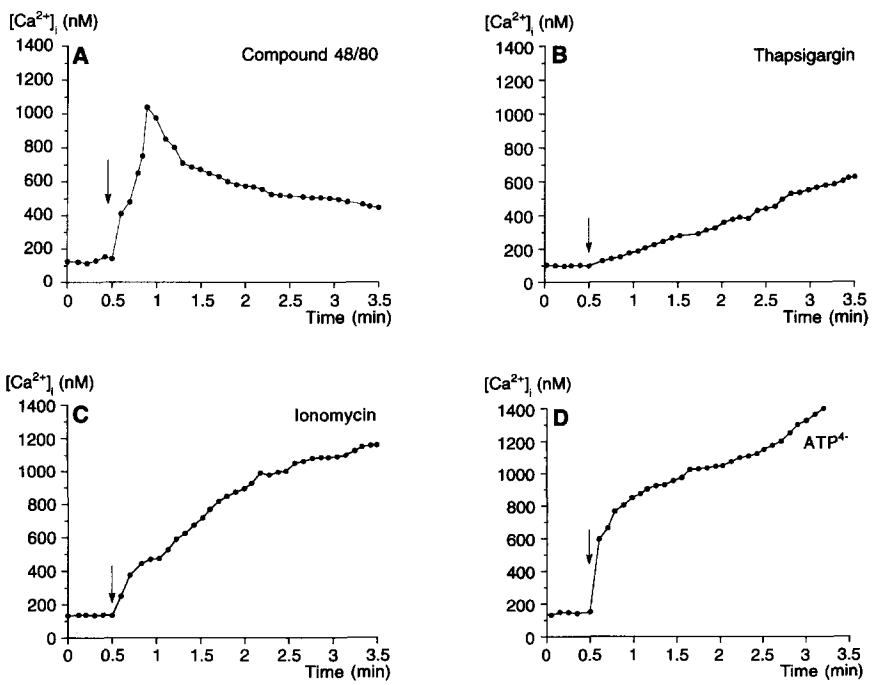

Fig. 2. Calcium responses of mast cells in the presence of $CaCl_2$. Fura-2-loaded mast cells were stimulated with (A) compound 48/80 (10 μg/ml), (B) thapsigargin (5 μM), (C) ionomycin (5 μM) or (D) ATP^{4-} (10 μM) in the presence of 1 mM $CaCl_2$. Tracings are shown of changes in $[Ca^{2+}]_i$, representative for six experiments. Note the different scale of the *Y*-axis in comparison with Fig. 1.

3. Results

3.1. Calcium responses of rat peritoneal mast cells

Fura-2-loaded mast cells were stimulated with non-lytic doses of various agonists in the presence of EGTA or $CaCl_2$, and changes in $[Ca^{2+}]_i$ were measured by spectrofluorometry. Agonists were added in doses evoking maximal Ca^{2+} responses at a cell concentration of $1 \cdot 10^5$/ml. Calcium responses with 1 mM EGTA were considered to reflect the release of Ca^{2+} from internal Ca^{2+} stores into the cytosol, and the responses in the presence of 1 mM $CaCl_2$ both internal Ca^{2+} mobilization and influx of Ca^{2+} from the extracellular medium. Fig. 1 shows typical time courses of cells stimulated in the presence of EGTA. Compound 48/80 (10 μg/ml) gave a rapid and transient increase in $[Ca^{2+}]_i$ (Fig. 1A), probably caused by Ins(1,4,5)-P_3-evoked depletion of the intracellular Ca^{2+} stores [18,19]. Stimulation with thapsigargin (5 μM) resulted in a slow and prolonged rise in $[Ca^{2+}]_i$ (Fig. 1B), pointing to gradual depletion of the Ca^{2+} stores by inhibition of the thapsigargin-sensitive Ca^{2+}-ATPases in the store membranes. Stimulation with the Ca^{2+} ionophore ionomycin (5 μM) resulted in an increase in $[Ca^{2+}]_i$ which was transient in four out of six experiments (Fig. 1C) and was not reversed in the two other experiments. The Mg^{2+}-free ATP^{4-} (10 μM) gave no significant Ca^{2+} response in the presence of EGTA (Fig. 1D), indicating that ATP^{4-} at this concentration did not evoke store depletion.

In the presence of $CaCl_2$, stimulation of the cells with compound 48/80 (10 μg/ml) resulted in a transient increase in $[Ca^{2+}]_i$ with a similar shape but of almost ten-fold higher amplitude than the transient seen in the absence of extracellular Ca^{2+} (Fig. 2A). Similarly, thapsigargin induced comparably shaped Ca^{2+} signals in the presence (Fig. 2B) and absence (Fig. 1B) of $CaCl_2$, although the level of $[Ca^{2+}]_i$ reached was much higher when $CaCl_2$ was present. Stimulation of the cells with ionomycin and $CaCl_2$ resulted in a micromolar $[Ca^{2+}]_i$ which remained high for at least 3 min. (Fig. 2C). ATP^{4-} in combination with $CaCl_2$ gave an immediate and high increase in $[Ca^{2+}]_i$ (Fig. 2D), indicating that this agonist promoted the influx of Ca^{2+}, despite its inability to evoke store depletion (Fig. 1D).

So far, the results suggested that the depletion of the intracellular Ca^{2+} stores by compound 48/80 and thapsigargin was accompanied by a considerable influx of Ca^{2+} from the extracellular medium. Additional support that these agonists opened divalent cation channels in the plasma membrane came from experiments, where the quenching of fura-2 fluorescence caused by the agonist-evoked entry of Mn^{2+} was measured (data not shown). Furthermore, the Ca^{2+} entry blocker $NiCl_2$ (5 mM) appeared to inhibit the $CaCl_2$-dependent part of the Ca^{2+} responses evoked by both compound 48/80 and thapsigargin (data not shown).

3.2. Calcium responses, histamine secretion and PGD_2 release

Using fura-2-loaded mast cells, we compared the rises in $[Ca^{2+}]_i$ with the potency of the agonists to release histamine and PGD_2 under conditions where Ca^{2+} influx was present or absent. Therefore, the cells were incubated at 37°C under stirring and, at 2 and 10 min after the addition of agonists, $[Ca^{2+}]_i$ was measured after which samples were taken from the incubation mixture for the determination of extracellular histamine and PGD_2. The results are shown in Table 1. When extracellular Ca^{2+} was chelated with EGTA, stimulation with compound 48/80 (10 μg/ml) resulted in a relatively high histamine secretion (20% of total releasable histamine after 10 min of activation), but in a low PGD_2 release (below the detection limit of 1 ng/10^6 cells). Stimulation with thapsigargin (5 μM) and EGTA resulted in a somewhat lower histamine secretion (15% of total histamine), whereas the PGD_2 release was relatively high (10 ng/10^6 cells). In the

Table 1
Effects of various agonists on $[Ca^{2+}]_i$, histamine secretion and PGD_2 release

Agonist	Addition	Increase in $[Ca^{2+}]_i$ (nM)		Histamine secretion (% of total)		PGD_2 release (ng/10^6 cells)	
		2 min	10 min	2 min	10 min	2 min	10 min
Compound 48/80	$CaCl_2$	496 ± 57	275 ± 37	68 ± 5	72 ± 5	4 ± 1	7 ± 1
	EGTA	30 ± 2	5 ± 3	17 ± 4	20 ± 4	< 1	< 1
Thapsigargin	$CaCl_2$	460 ± 140	1156 ± 314	15 ± 2	18 ± 2	13 ± 2	15 ± 2
	EGTA	71 ± 20	90 ± 17	5 ± 1	15 ± 2	9 ± 2	10 ± 3
Ionomycin	$CaCl_2$	691 ± 215	869 ± 579	9 ± 3	13 ± 3	22 ± 3	30 ± 2
	EGTA	33 ± 11	29 ± 7	< 0.3	< 0.3	< 1	< 1
ATP^{4-}	$CaCl_2$	896 ± 117	1420 ± 210	5 ± 1	4 ± 2	< 1	< 1
	EGTA	11 ± 11	12 ± 12	< 0.3	< 0.3	< 1	< 1

Mast cells were stimulated with compound 48/80 (10 μg/ml), thapsigargin (5 μM), ionomycin (5 μM) or ATP^{4-} (10 μM) in the presence of 1 mM $CaCl_2$ or 1 mM EGTA, as indicated. Agonist-evoked increase in $[Ca^{2+}]_i$, secretion of histamine (% of total releasable histamine) and release of PGD_2 (ng released/10^6 cells) were determined at 2 and 10 min after the onset of activation. Detection limits were for histamine secretion 0.3% and for PGD_2 release 1 ng/10^6 cells. Data given are mean values ± S.E. ($n = 4$).

Table 2
Rises in $[Ca^{2+}]_i$, histamine secretion and PGD_2 release in store-depleted mast cells

Addition	Increase in $[Ca^{2+}]_i$ (nM)		Histamine secretion (% of total)		PGD_2 release (ng/10^6 cells)	
	2 min	10 min	2 min	10 min	2 min	10 min
$CaCl_2$	325 ± 94	309 ± 123	2 ± 1	2 ± 1	4 ± 1	5 ± 1
Compound 48/80	24 ± 5 *	38 ± 4 *	< 0.3	< 0.3	< 1	< 1
$CaCl_2$ + compound 48/80	613 ± 68	392 ± 53	59 ± 3	56 ± 4	38 ± 14	44 ± 17

Fura-2-loaded mast cells were prepared that were depleted in Ca^{2+} stores, as described in Section 2. The responses evoked by the addition of $CaCl_2$ (1 mM) and/or compound 48/80 (10 μg/ml) were determined after 2 and 10 min. Data are presented as indicated in Table 1. Results are mean values ± S.E. ($n = 3$).
* Control incubations without compound 48/80 showed an equal rise in $[Ca^{2+}]_i$.

presence of $CaCl_2$, the release of both histamine and PGD_2 appeared to be potentiated, when compared to the EGTA-containing incubations. Compound 48/80 with $CaCl_2$ evoked, again, a relatively high histamine secretion (72% of total histamine) and low PGD_2 release (7 ng/10^6 cells); thapsigargin with $CaCl_2$, again, was less efficient in evoking the release of histamine (18% of total) than of PGD_2 (15 ng/10^6 cells). Apparently, with either agonist histamine secretion and eicosanoid formation were potentiated by the higher levels of $[Ca^{2+}]_i$ reached by the entry of extracellular Ca^{2+}. This conclusion was supported by the observation that the addition of $NiCl_2$ significantly reduced the release of the mast cell products induced by compound 48/80 and $CaCl_2$ (data not shown).

In the presence of EGTA, ionomycin (5 μM) and ATP^{4-} (10 μM) were unable to evoke significant formation of histamine or PGD_2 (Table 1). With $CaCl_2$, however, ionomycin did promote the release of relatively low amounts of histamine (13% of total releasable histamine) and high amounts of PGD_2 (30 ng/10^6 cells). In contrast, ATP^{4-} gave only a marginal secretion of histamine (4% of total) and no PGD_2 release, despite the high, micromolar level of $[Ca^{2+}]_i$ reached under this condition.

Taken together, these results indicate that stimulation of the mast cells with compound 48/80 favoured the release of histamine, whereas stimulation with the Ca^{2+} mobilizers thapsigargin and ionomycin resulted in a more efficient release of PGD_2. ATP^{4-} (10 μM) was a rather ineffective inducer for either process. Since the rises in $[Ca^{2+}]_i$ obtained with thapsigargin and ionomycin were more prolonged than the rises with compound 48/80 (Table 1 and Fig. 2), it is tempting to suggest that the relatively high PGD_2 release seen with the Ca^{2+} mobilizers is related to the long duration of the Ca^{2+} signal. On the other hand,

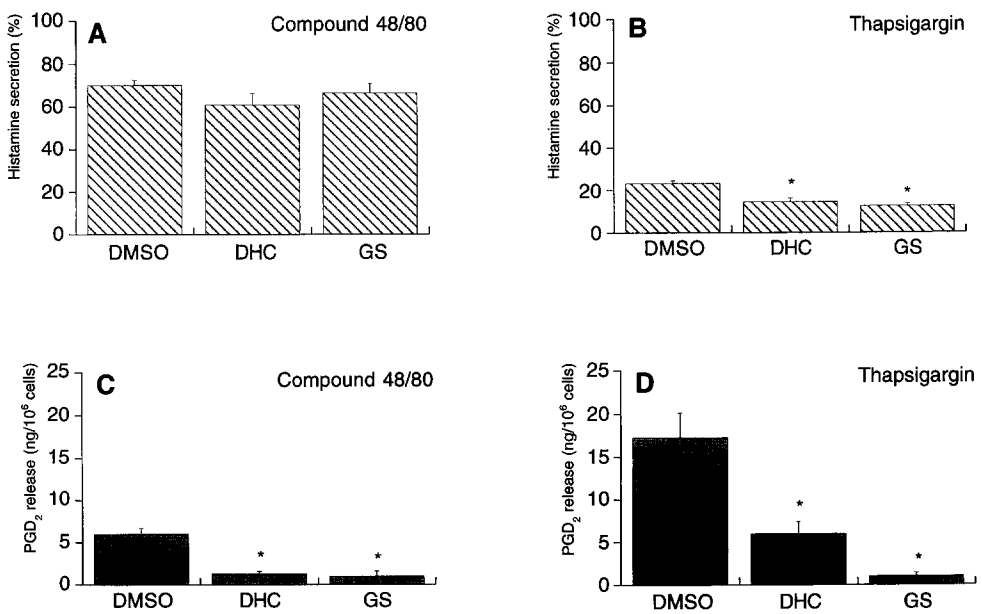

Fig. 3. Effects of protein tyrosine kinase inhibitors on the histamine secretion and PGD_2 release by stimulated mast cells. Mast cells were pre-incubated with 0.2% (v/v) dimethyl sulfoxide (DMSO, vehicle control), with 5 μM methyl-2,5-dihydroxycinnamate (DHC) or with 100 μM genistein (GS) during 45 min. The cells were subsequently stimulated with 10 μg/ml compound 48/80 or with 5 μM thapsigargin in the presence of 1 mM $CaCl_2$. The release of histamine and PGD_2 from the cells was determined at 10 min after activation. Data (means ± S.E., $n = 4$) represent histamine secretion (A, B) and PGD_2 release (C, D), expressed as the percentage of total releasable histamine and ng PGD_2/10^6 cells, respectively. Asterisks indicate statistically significant differences ($P < 0.05$) compared to control cells.

the results do not suggest that the amplitude of the $[Ca^{2+}]_i$ signal may determine the preferential action of the agonists to release more histamine or PGD_2 since, in general, this preference was the same in the presence and absence of extracellular $CaCl_2$, where Ca^{2+} influx did or did not amplify the signal.

3.3. Histamine secretion and PGD_2 release by store-depleted mast cells

To further investigate the significance of Ca^{2+} influx for histamine secretion and PGD_2 release, fura-2-loaded mast cells were prepared with depleted Ca^{2+} stores (see Section 2). Stimulation of these store-depleted cells with ionomycin in the presence of EGTA resulted in a maximal increase in $[Ca^{2+}]_i$ which was only $15 \pm 5\%$ (mean $\pm$ S.E., $n = 3$) of the increase seen in untreated control cells, indicating that most of the intracellular Ca^{2+} was removed by the depletion procedure. Determination of the histamine content in the store-depleted cells learned that no more than $15 \pm 5\%$ of the total releasable histamine was lost by the procedure.

When 1 mM $CaCl_2$ was added to the store-depleted mast cells, this resulted in an immediate and prolonged increase in $[Ca^{2+}]_i$ of about 300 nM (Table 2), due to the influx of extracellular Ca^{2+} through the store-regulated entry pathway. Using control cells with full Ca^{2+} stores, the addition of $CaCl_2$ resulted in a $[Ca^{2+}]_i$ rise of only 10 nM. Despite the high Ca^{2+} response in the store-depleted cells, $CaCl_2$ addition resulted in only a marginal release of histamine and PGD_2 (Table 2). Apparently, the influx of Ca^{2+} and the resulting increase in $[Ca^{2+}]_i$ were insufficient to promote substantial release of these compounds. When compound 48/80 was added to the store-depleted cells, no Ca^{2+} signal was detected and neither histamine nor PGD_2 were released (Table 2). However, when compound 48/80 was added with $CaCl_2$, a sustained increase in $[Ca^{2+}]_i$ was accompanied by a high histamine secretion (56% of total releasable histamine) and high PGD_2 release (44 ng/10^6 cells).

3.4. Effects of protein tyrosine kinase inhibitors on the release of histamine and PGD_2 by mast cells

We studied the role of protein tyrosine kinases in the release of histamine and PGD_2 from mast cells by using two structurally unrelated inhibitors of these signalling components, methyl-2,5-dihydroxycinnamate and genistein. After pre-incubation with these inhibitors, the mast cells were stimulated with compound 48/80 or thapsigargin in the presence of $CaCl_2$. The histamine secretion induced by compound 48/80 appeared not to be influenced by either inhibitor (Fig. 3A), whereas the PGD_2 release by this agonist was considerably reduced (Fig. 3C). With thapsigargin as agonist, histamine secretion was slightly influenced by both methyl-2,5-dihydroxycinnamate and genistein (Fig. 3B), in contrast to the PGD_2 release which, again, was potently inhibited (Fig. 3D). Similar effects of these tyrosine kinase inhibitors were found, when the cells were stimulated with ionomycin instead of thapsigargin (data not shown).

4. Discussion

4.1. Role of store-regulated Ca^{2+} influx in histamine and PGD_2 release

Activation of rat peritoneal mast cells with the G-protein activating compound 48/80 and the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin resulted in Ca^{2+} signals, which consisted of a relatively small component representing mobilization of Ca^{2+} from internal stores and a larger component representing influx of Ca^{2+} from the extracellular medium (Figs. 1 and 2). A body of data suggested that the influx with these agonists amplified the rises in $[Ca^{2+}]_i$ evoked by store depletion and occurred through the store-regulated influx pathway: (i) both with compound 48/80 and the endomembrane-active thapsigargin, the $[Ca^{2+}]_i$ rises were of similar shape in the presence and absence of $CaCl_2$, notwithstanding the higher amplitude reached with $CaCl_2$ (Figs. 1 and 2); (ii) when extracellular Ca^{2+} was present, only the $CaCl_2$-dependent part of $[Ca^{2+}]_i$ rises was suppressed by the Ca^{2+} entry blocker Ni^{2+}; (iii) in mast cells with depleted Ca^{2+} stores, addition of $CaCl_2$ was sufficient to evoke an immediate and high increase in $[Ca^{2+}]_i$ (Table 2), as predicted by the store-regulated pathway. In many other cells types in which this influx pathway is identified, including the rat mastocytoma cell line RBL-2H3 [38–41], similarly, store-regulated influx is known to be initiated by G protein-mediated activation of phospholipase C and by inhibition of thapsigargin-sensitive Ca^{2+}-ATPases in the store membranes. In peritoneal mast cells, this influx pathway has already been detected in electrophysiological studies, where specific inward Ca^{2+} currents were measured following depletion of the Ca^{2+} stores [42,43]. The mechanism responsible for this type of Ca^{2+} influx is still unclear, although some proposals have been made, such as the release of an intermediary messenger other than Ca^{2+} from the store compartments [38,44].

Our data indicate that the relatively high levels of $[Ca^{2+}]_i$ caused by entry of external Ca^{2+} potentiated the secretion of both histamine and PGD_2 from the mast cells (Table 1). On the other hand, there were several activation conditions, where a considerable increase in $[Ca^{2+}]_i$ was not accompanied by an appreciable release of histamine or PGD_2. For instance, stimulation of the mast cells with 10 μM ATP^{4-} and $CaCl_2$ resulted in only marginal histamine secretion and no detectable PGD_2 release despite a micromolar rise in $[Ca^{2+}]_i$ (Table 1). Furthermore, the addition of $CaCl_2$ to store-depleted cells resulted in a

prolonged increase in $[Ca^{2+}]_i$ of about 300 nM, but not in the release of histamine or PGD_2 (Table 2). Typically, in both cases, the Ca^{2+} responses of the mast cells consisted entirely of Ca^{2+} influx, whereas under conditions where significant amounts of histamine and PGD_2 were released, i.e., in mast cells activated with compound 48/80, thapsigargin or ionomycin, Ca^{2+} influx was always accompanied by store depletion. Thus, these results suggest (intracellular signalling associated with) the influx of Ca^{2+} potentiates the release of histamine and PGD_2, but that the influx alone is insufficient to trigger these events. Whereas the additional factors required for exocytosis most likely include activation of protein kinase C [4,5] and/or of the newly identified G protein, G_{i3} [6], the signalling factors required for eicosanoid formation are still less clear (see below). So far, our data suggest that at least one of these factors is generated upstream or downstream of store depletion, but not downstream of Ca^{2+} influx.

4.2. Differential release of histamine and PGD_2 by activated mast cells

Mast cell-activating agonists may be divided into two types: agonists that are more efficient in evoking histamine secretion and agonists favouring PGD_2 release. We found that compound 48/80, known to act by stimulation of G proteins, is a relatively good histamine releaser, whereas thapsigargin and ionomycin stronger promoted PGD_2 release (Table 1). This preferential action appeared to be independent of the presence or absence of external $CaCl_2$ and, thus, of the amplitude of the agonist-evoked rise in $[Ca^{2+}]_i$. Since compound 48/80 evoked a Ca^{2+} signal that was shorter than that evoked by thapsigargin and ionomycin, it is possible that especially the duration, but not the amplitude, of the rise in $[Ca^{2+}]_i$ is a decisive factor in promoting PGD_2 release. These results are consistent with earlier observations showing that G protein-activating compounds like substance P, compound 48/80 and bradykinin evoke in mast cells a relatively high histamine secretion [9,11], whereas Ca^{2+} ionophore A23187 and anti-immunoglobulin E were better generators of PGD_2 [9,11,12,15]. Kawabe et al. [12] have provided good evidence that PGD_2, once produced, is rapidly released from the mast cells. This observation could be confirmed (data not shown). According to the presented data, it was difficult to classify ATP^{4-}, since this agent was hardly capable to release histamine or PGD_2 at a concentration of 10 μM, sufficient to elevate $[Ca^{2+}]_i$ substantially (Table 1).

When $CaCl_2$ was added to store-depleted mast cells, this resulted in the release of only minor amounts of histamine and PGD_2, despite the persistently high level of $[Ca^{2+}]_i$ reached. However, when $CaCl_2$ was added in combination with compound 48/80, the prolonged Ca^{2+} signal was accompanied by a high release of histamine as well as PGD_2 (Table 2). This partially contrasts with the low formation of PGD_2 and high secretion of histamine, when mast cells with full Ca^{2+} stores were activated by compound 48/80 in the presence of $CaCl_2$ (Table 1). The histamine secretion is probably caused by the activation of protein kinase C by compound 48/80 [4,5]. It is tempting to suggest that the high PGD_2 release may be a consequence of store depletion in combination with a prolonged Ca^{2+} signal: compound 48/80 allowed the store-depleted cells to re-empty the Ca^{2+} stores, once these were filled by the store-regulated influx pathway evoked by the addition of $CaCl_2$. Similar activation conditions were present, when cells with full stores were stimulated with thapsigargin or ionomycin in the presence of $CaCl_2$.

4.3. Roles of protein tyrosine kinases in the release of histamine and PGD_2

We found that genistein and methyl-2,5-dihydroxycinnamate, two well-known inhibitors of protein tyrosine kinases, suppressed PGD_2 formation much better than histamine secretion in cells that were activated with ionomycin (not shown), compound 48/80 and thapsigargin (Fig. 3). Although we do not have measured protein phosphorylation patterns directly, this suggests that tyrosine kinases are more prominently involved in the reactions leading to eicosanoid formation than in those mediating exocytosis. There is recent evidence that protein tyrosine kinases are, indeed, involved in eicosanoid formation. For instance, from experiments with the same or similar tyrosine kinase inhibitors, it has been shown that tyrosine phosphorylation events contributed to the production of prostaglandin E_2 by murine macrophages [27] and to the mobilization of arachidonic acid in platelets [25] and in mast cells that were activated with stem cell factor [26].

Typically, genistein and methyl-2,5-dihydroxycinnamate strongly influenced the relative amounts of histamine and PGD_2 that were released by the mast cells in response to thapsigargin: in the presence of the tyrosine kinase inhibitors thapsigargin changed from a good PGD_2 releaser to a relatively better histamine releaser (Fig. 3). In several other cell systems, including rat mastocytoma RBL-2H3 cells, it has been demonstrated that compounds like thapsigargin, ionomycin and A23187 are potent inducers of the phosphorylation at tyrosine of a whole series of proteins [28,31,45–47]. Thus, it is possible that the property of these Ca^{2+}-mobilizing compounds to preferentially evoke PGD_2 release is related to their effect to stimulate protein tyrosine kinases.

Studies with RBL-2H3 cells have shown that the secretion of histamine evoked by A23187, thapsigargin and anti-immunoglobulin E is modulated by tyrosine kinase or phosphatase inhibitors, suggesting these enzymes have also a regulatory function in ionophore- and antigen-evoked exocytosis [45,47]. In agreement with this, we found that in rat mast cells histamine secretion evoked by thapsigar-

gin and ionomycin was slightly reduced by protein tyrosine kinase inhibition, in contrast to the secretion evoked by compound 48/80 which was not influenced (Fig. 3). The latter effect is well compatible with the evidence that signalling pathways involving protein tyrosine phosphorylation in RBL-2H3 cells act largely independently of the activation by protein kinase C [48].

The cytoplasmic, 85-kDa phospholipase A_2 is considered to mediate agonist-stimulated release of arachidonic acid in many cell types [49,50]. Recent data indicate that the activation of p85 phospholipase A_2 requires, besides elevation in $[Ca^{2+}]_i$, a phosphorylation step which is putatively catalyzed by mitogen-associated protein kinases (MAP kinases) [50,51]. MAP kinases belong to a family of serine/threonine kinases which can be activated by phosphorylation of their tyrosine and threonine residues and may serve as convergence points in intracellular signal transduction [52]. It is known that arachidonic acid mobilizing agents activate MAP kinases and cause increased phosphorylation of p85 phospholipase A_2 ([25,50] and refs. therein). For instance, in rat RBL-2H3 cells activated with A23187 or thapsigargin [45] and in cultured mouse mast cells activated with anti-immunoglobulin E or stem cell factor [53], MAP kinases or related enzymes appeared to be phosphorylated on tyrosine. Taken together, these findings lead to the conclusion that the potency of such agonists to favour the release of PGD_2 in mast cells might be a consequence of their ability to phosphorylate and activate MAP kinases and/or phospholipase A_2. This is a subject of further investigation.

It has recently been proposed that besides phospholipase A_2 also a pathway involving phospholipase D may contribute to arachidonic acid liberation and eicosanoid formation in mast cells and RBL-2H3 cells [26,54,55]. In our experiments, we did not consider the involvement of either pathway in the release of PGD_2. Interestingly, however, it was found by these authors that protein tyrosine kinases may also participate in the signalling events of the phospholipase D pathway.

Taken together, our results indicate that in rat peritoneal mast cells the store-regulated influx of Ca^{2+} forms a major contribution to the agonist-evoked increase in $[Ca^{2+}]_i$ and is a potentiating factor in the release of histamine as well as PGD_2. However, the Ca^{2+} influx as such appears to be insufficient to initiate these release processes. Three factors may contribute to a high release of PGD_2 by mast cell agonists: a relatively prolonged Ca^{2+} signal, the process of store depletion and the phosphorylation at tyrosine of proteins involved in the phospholipase A_2-mediated signal transduction cascade. It has been reported that in human platelets, activated with Ca^{2+} ionophore or thapsigargin, tyrosine phosphorylation and dephosphorylation are controlled by the cytosolic and stored Ca^{2+} concentrations [46]. Hence, also in mast cells these three factors may not be mutually independent. A next task will be to unravel their interplay in the control of PGD_2 release.

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