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Dual regulation of the Na^+/H^+-exchange in rat peritoneal mast cells: role of protein kinase C and calcium on pH_i regulation and histamine release

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1 The purpose of this study was to compare the actions of phorbol 12-myristate 13-acetate (PMA) and ionomycin on Na^+/H^+ exchange activation and histamine release to that of compound 48/80 in order to study the possible relationship between pH_i and secretion of histamine in rat peritoneal mast cells.

2 Resting pH_i in mast cells suspended in a bicarbonate-free physiological salt solution amounted to 6.73 ± 0.05 (mean $\pm$ s.d., $n = 52$).

3 PMA (20 nM) induced a substantial but rather slow increase in pH_i. This response was very sensitive to inhibition by staurosporine, very sensitive to inhibition by 5-(N,N-hexamethylene)amiloride (HMA), insensitive to the absence of extracellular calcium (without EGTA), and sensitive to partial depletion of intracellular calcium with EGTA.

4 Ionomycin (1 μM) induced a biphasic change in pH_i that was sensitive to inhibition by HMA, insensitive to staurosporine. In the absence of extracellular calcium using EGTA, the biphasic response disappeared, leaving only a slow, and diminished change in pH_i.

5 The effects of ionomycin and PMA on pH_i were additive.

6 Addition of the secretagogue compound 48/80 (1 $\mu g\ ml^{-1}$) increased pH_i substantially, ΔpH_i amounting to 0.29 ± 0.05 pH-units ($n = 4$). The biphasic pH_i-response was insensitive to the absence of extracellular calcium (without EGTA). The initial fast response in pH_i was, however, inhibited by HMA, not staurosporine.

7 The finding that staurosporine and HMA each inhibited approximately half of the compound 48/80-induced pH_i-response, whereas both inhibitors completely abolished the compound 48/80-induced pH_i-response seems to indicate that two independent pathways for the activation of the Na^+/H^+ exchange were stimulated by compound 48/80.

8 The histamine release induced via both PKC activation (using PMA) and calcium (using ionomycin) were much larger than the sum of each activation pathway, whereas in the absence of extracellular calcium using EGTA, the histamine release in response to PMA and ionomycin was completely abolished.

9 The compound 48/80-induced histamine release was partially sensitive to inhibition by HMA ($\approx 30\%$ inhibition) and partially sensitive to inhibition by staurosporine ($\approx 50\%$ inhibition). Preincubation with staurosporine and HMA before stimulation with compound 48/80 showed the same degree of inhibition as observed after staurosporine alone, even though this combination of drugs completely inhibited the pH_i-response. Furthermore, compound 48/80-induced histamine release was not dependent on the presence of extracellular calcium (with and without EGTA).

10 In spite of the similarities in second messenger pathways for pH_i regulation and histamine release, it is, however, not very likely that these two processes are directly related. It is, however, possible, that an increase in pH_i plays a permissive, rather than an essential role for histamine release in rat peritoneal mast cells. This hypothesis was supported by the finding that preincubation with the Na^+/H^+ exchange-inhibitor HMA inhibited 30% of the compound 48/80-induced histamine secretion.

Keywords: Mast cells; protein kinase C; calcium; Na^+/H^+ exchange; histamine secretion; exocytosis; signal transduction; pH_i

Introduction

Sodium/proton exchange activity has been found in all eukaryotic cells including mammalian cells (review: Noël & Pouysségur, 1995). The Na^+/H^+ exchanger catalyzes the electroneutral exchange of 1 H^+_{out} for 1 Na^+_{in} leading to a rise in the intracellular pH. In non-epithelial cells, the Na^+/H^+ exchange is activated by various pathways: phorbol esters (via activation of protein kinase C and hence phosphorylation of the exchanger); a rise in $[Ca^{2+}]_i$ (the Na^+/H^+ exchanger is a Ca^{2+}/calmodulin (CaM)-binding protein whose activity can be regulated by a rise in $[Ca^{2+}]_i$. This rise will shift the pH_i dependence of the exchanger toward alkaline pH values); hyperosmotic shock (increases the exchanger's sensitivity to intracellular H^+); and cell spreading (via interactions with cytoskeletal elements) (review: Noël & Pouysségur, 1995).

Changes in pH_i are important signals in a variety of cellular functions, including modification of the IP_3-induced release of sequestered intracellular Ca^{2+} in RBL-2H3 cells (Ali *et al.*, 1989) and promotion of exocytosis in chromaffin cells (Rodriguez Del Castillo *et al.*, 1992). Since protein kinase C is a stimulant for the antiporter in rat peritoneal mast cells (Alfonso *et al.*, 1994a), this study was undertaken in order to evaluate the second messenger-mediated regulation of Na^+/H^+ exchange in rat peritoneal mast cells. The actions of phorbol 12-myristate 13-acetate (PMA) and ionomycin on Na^+/H^+ exchange and histamine release were compared to that of compound 48/80 in order to study possible relations between pH_i and secretion of histamine in rat peritoneal mast cells.

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Compound 48/80 is a polycationic condensation product of N-methyl-*p*-methoxy phenylethylamine with formaldehyde, considered to act as a receptor-mimetic agonist directly at a pertussis-toxin-sensitive GTP-binding protein distal to phospholipase C (PLC) (Razin *et al.*, 1995). Thus, compound 48/80 is able to elicit a full secretory response in the absence of PLC-activity (Razin *et al.*, 1995). Stimulation by compound 48/80, however, leads to a transient increase in both protein kinase C (PKC) activity (Kurosawa & Kobayashi, 1989) and $[Ca^{2+}]_i$ (Penner, 1988). We have used the phorbol ester PMA as a membrane-permeable diacylglycerol (DAG)-analogue, since a regulation of a process by PKC is generally inferred when it is modulated by phorbol esters. There are, however, differences in the biological activities of phorbol esters and diacylglycerols, and it has been suggested that diacylglycerols and phorbol esters bind with different affinities and at different sites on PKC, and induce distinct activated conformational forms of the enzyme (Sagi-Eisenberg *et al.*, 1985; Slater *et al.*, 1994; Tuominen *et al.*, 1994). The calcium ionophore ionomycin was used in this study in order to determine the contribution of calcium on pH_i regulation. It has generally been assumed that this antibiotic directly facilitates the transport of Ca^{2+} across the plasma membrane, circumventing natural entry pathways, but a recent report has suggested that ionomycin enhances calcium influx by stimulating store-regulated cation entry and not by a direct action at the plasma membrane (Morgan & Jacob, 1994).

In this paper we show that rat peritoneal mast cells have two distinct pathways for stimulating Na^+/H^+ exchange and histamine release: a PKC-mediated and a calcium-mediated pathway. The two pathways act additively/synergistically on the compound 48/80-induced pH_i-increase and histamine release. pH_i-increase and histamine release seem, however, to be only indirectly connected.

Methods

Isolation of mast cells

Male Sprague-Dawley rats weighing 455 ± 54 g (mean and s.d., $n = 39$) were used for the experiments. Rats were killed by bleeding from the carotid arteries after asphyxiation in CO_2. Mixed peritoneal cells were collected by injecting a physiological salt solution buffered with 10 mM HEPES (4,(2-hydroxyethyl)-1-piperazine ethane sulphonic acid) through a small incision in the abdominal wall. The cell suspension was removed from the peritoneal cavity and kept at 4°C. The remaining part of the cell isolation was carried out at 4°C. After centrifugation at 220 *g* for 10 min, the supernatant was discarded and the cells were resuspended in physiological salt solution and transferred to an isotonic Percoll (specific gravity = 1.117) buffered with 20 mM HEPES. A continuous gradient of Percoll was created by centrifugation at 16,000 *g* for 35 min. The mast cells were concentrated in the lower third of the gradient, whereas the peritoneal leucocytes and macrophages were located in the upper third. The mast cells were harvested and washed two times to remove the remaining Percoll. The cells were then suspended in physiological salt solution containing bovine serum albumin (BSA), 1 mg ml^{-1}, and glucose (5.6 mM), pH 7.25 at 37°C.

A coulter counter was used to count the number of cells and inspection of a stained (toluidine blue) smear was used to determine the fraction of mast cells in the cell suspension (> 97%).

Intracellular pH

The measurement of intracellular pH was performed with the fluorescence technique using the plasma membrane permeable probe 2', 7'-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethylester (BCECF-AM) as an intracellular indicator of pH (Rink *et al.*, 1982). The mast cells from one rat were divided into 4 samples of 200 μl with a cell density of 0.176 to 0.552×10^6 cells per sample (range) and loaded with 5 μM BCECF-AM by preincubation at 37°C for 30 min in a final volume of 400 μl. The samples were then washed with 10 ml of physiological salt solution (37°C). The remaining processing of the samples was carried out at 37°C. After centrifugation and resuspension in 1.9 ml of physiological salt solution containing BSA (1 mg ml^{-1}) and glucose (5.6 mM), 1.8 ml of the samples were transferred into the cuvette. Some experiments were performed in either calcium-free buffer or EGTA (1 mM) containing buffer. In these experiments, the cells were resuspended in such buffers immediately before transfer to the cuvette for pH_i-measurements.

Measurements of pH_i were performed in a ratio fluorescence system from Photon Technology International (model RF-F3001) (PTI) consisting of a computer controlled high-speed dual-wave-length filter illuminator coupled to a lens-based T-format sample compartment via a bifurcated quartz fibre optic bundle. This equipment had provisions for heating (37°C) and mixing (magnetic stirrer) the cell suspension in the fluorimeter cuvette. The scanning emission monochromator has a photon-counting photomultiplier detector. Excitations were performed at 435 nm and 490 nm and the emission was measured at 535 nm. The ratio of the fluorescence caused by excitation at 490 nm and 435 nm (490/435) was converted to pH_i by means of a calibration curve with nigericin. The calibration was performed in the presence of BSA and at 37°C. The fluorescence signals were sampled and displayed on-line every 0.1 or 0.2 s during the experiment. The converted data (pH_i) were smoothed by a smoothing buffer size of 21 points, and the data were transferred as ASCII files to math programs (Excell) in order to produce mean curves of 4 separate experiments.

Measurements of histamine release

Mast cells were incubated for 30 min at 37°C at pH_o 7.25 in physiological salt solution containing BSA (1 mg ml^{-1}) and glucose (5.6 mM), washed and resuspended in an identical solution. The mast cells were then incubated in duplicate at 37°C with the various drug combinations used in the pH_i experiments. The incubation was terminated by the addition of 1.5 ml of ice-cold physiological salt solution to the samples. After centrifugation at 220 *g* for 10 min at 4°C, the supernatants were collected for determination of the amount of histamine released. The cell pellets were suspended in 250 μl of a 0.9% NaCl solution and boiled for 3 min. After addition of 1.75 ml of physiological salt solution and centrifugation, the supernatant was collected for the determination of the remaining histamine. This was performed by the fluorometric method (Shore *et al.*, 1959) omitting the extraction procedure. The histamine release was expressed as a percentage of the total histamine content of the cells after adjustment for the spontaneous release (< 5%). Spontaneous histamine release from mast cells incubated in the absence of BSA amounted to $20 \pm 2\%$ ($n = 4$) after 10 min and $22 \pm 3.5\%$ ($n = 4$) after 30 min of incubation, whereas spontaneous histamine release in the presence of BSA amounted to $3.5 \pm 0.5\%$ ($n = 4$) after 30 min of incubation. Accordingly, all histamine release and pH_i experiments were performed in the presence of BSA.

Materials

Bovine serum albumin, compound 48/80, HEPES (4,(2-hydroxyethyl)-1-piperazine ethane sulphonic acid), nigericin, ionomycin, staurosporine, EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N'N'-tetraacetic acid) and PMA (phorbol 12-myristate 13-acetate) were supplied by Sigma Chemical Company, St. Louis, U.S.A.; Percoll by Pharmacia Fine Chemicals, Uppsala, Sweden; DMSO (dimethylsulphoxide) by Merck, Darmstadt, Germany, and BCECF-AM (2',7'-bis)2-carboxyethyl)-5-carboxyfluorescein acetoxymethylester) by Molecular Probes, Eugene, U.S.A.

5-(N,N-hexamethylene)amiloride (HMA), PMA and staurosporine were dissolved in DMSO (stock solutions: 5 mM, 5 mM and 1 mM, respectively) and then diluted in physiological salt solution. The final concentrations of DMSO (in v/v) were 0.2%, 0.02% and 0.025% for HMA, PMA and staurosporine, respectively. These concentrations of DMSO did not affect measurements of pH_i and histamine release *per se*. Ionomycin was dissolved in 96% ethanol (stock solution 3 mM) and diluted in physiological salt solution. The final concentration of ethanol was 0.03% (v/v). This concentration of ethanol did not affect measurements of pH_i and histamine release *per se*.

Solutions

The physiological salt solution contained (in mM): NaCl 141.9, KCl 4.73, $MgSO_4$ 1.18, $CaCl_2$ 1.02, HEPES 10.0, glucose 5.6, bovine serum albumin 1 mg ml^{-1}, pH 7.25 (37°C).

Data presentation

The data are presented as mean curves or means ± s.e. mean with the number of observations stated as (n). The mean curves are produced from 4 independent experiments performed on different days and upon mast cells from different rats. Student's t test (unpaired, two-tailed) was used when applicable in order to calculate statistical significance (P; 5% or 1% level).

Results

Phorbol 12-myristate 13-acetate (PMA)

Resting pH_i in mast cells suspended in a bicarbonate-free physiological salt solution amounted to 6.73 ± 0.05 (mean ± s.d., n = 52). Addition of the phorbol ester PMA (20 nM) resulted in an increase in pH_i (ΔpH_i) amounting to + 0.06 ± 0.02 pH-units (n = 4, Figure 1a), and a subsequent addition of the Na^+/H^+ exchange inhibitor HMA (5-(N,N-hexamethylene)amiloride) (10 μM) inhibited a large part of this response (ΔpH_i: −0.04 ± 0.02 pH-units, n = 4, Figure 1a). A parallel set of experiments showed that the PMA-induced increase in pH_i was completely unaffected by the addition of vehicle in place of HMA (results not shown). The increase in pH_i after stimulation by PMA was detectable after a lag period of 46 ± 4 s (n = 16). HMA alone did not have any effect on pH_i, resting pH_i being largely unaffected (Figure 1b). When HMA was added before stimulation by PMA, the PMA-induced increase in pH_i was, however, completely abolished (Figure 1b).

Similar results were obtained by the combinations of phorbol ester and the protein kinase C (PKC) inhibitor staurosporine. Thus, staurosporine (250 nM) decreased the PMA-induced increase in pH_i (ΔpH_i: −0.04 ± 0.01 pH-units, n = 4, Figure 2a). Staurosporine alone did not have any effect on pH_i, resting pH_i being largely unaffected (Figure 2b), but when staurosporine was added to the cell suspension before stimulation by PMA, the response was completely abolished (Figure 2b).

Compound 48/80

Addition of the secretagogue compound 48/80 in a high concentration (1 μg ml^{-1}) increased pH_i substantially, ΔpH_i amounting to 0.29 ± 0.05 pH-units (n = 4, Figure 3A(a)). This response consisted of two phases: one rapid steep increase in pH_i followed by a slower and larger pH_i increase. When mast cells were stimulated by compound 48/80 in a low concentration (0.02 μg ml^{-1}), the stimulation-induced increase in pH_i only amounted to 0.10 ± 0.02 pH-units (n = 4, Figure 3A(b)), and the initial rapid steep increase in pH_i had disappeared (Figure 3A(b)).

When the PKC-inhibitor staurosporine (250 nM) was added 5 min before stimulation with the high concentration of compound 48/80 (1 μg ml^{-1}), the resulting ΔpH_i-value amounted to + 0.16 ± 0.02 (n = 4, Figure 3B(a)), whereas the pH_i-response after stimulation with the low concentration of compound 48/80 (0.02 μg ml^{-1}) in the presence of staurosporine only amounted to 0.06 ± 0.02 pH-units (n = 4, Figure 3B(b)).

When the amiloride-analogue HMA (10 μM) was added 3 min before stimulation with the high concentration of compound 48/80 (1 μg ml^{-1}), the resulting ΔpH_i-value amounted to + 0.11 ± 0.01 (n = 4, Figure 3C). It was noted, however, that when HMA was added to the cell suspension before stimulation by compound 48/80, then the steep initial rise in pH_i was completely absent and the compound 48/80-induced increase in pH_i was detected only after a lag period of 109 ± 11 s (n = 4, Figure 3C), whereas when staurosporine was added to the cell suspension before stimulation by compound 48/80, the steep initial rise in pH_i was present (Figure 3B(a)). In addition, stimulation (by either compound 48/80 or PMA) in the presence of HMA leads to a small, but transient fall in pH_i (Figure 1b and 3C).

When both staurosporine and HMA were added to the cell suspension before stimulation with the high concentration of compound 48/80 (1 μg ml^{-1}), the stimulation-induced increase in pH_i was completely inhibited (ΔpH_i after compound 48/80 amounted to + 0.01 ± 0.01 pH-units, n = 4, Figure 3D). Using the same experimental protocol with compound 48/80 in a low concentration (0.02 μg ml^{-1}) revealed similar results (data not shown).

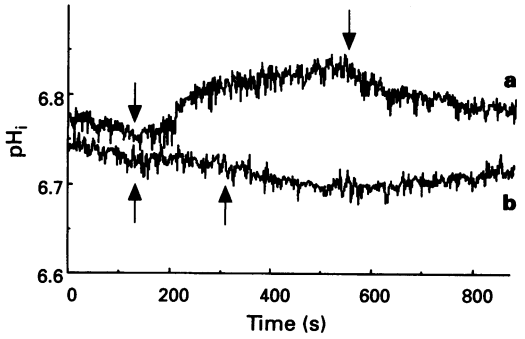

Figure 1 Effect of phorbol 12-myristate 13-acetate (PMA) on intracellular pH in rat peritoneal mast cells. (a) PMA (20 nM) was added to the cell suspension at 120 s (arrow), and 5-(N,N-hexamethylene)amiloride (HMA) (10 μM) was added at 550 s (arrow). (b) Effect of HMA on the PMA-induced pH_i increase. HMA (10 μM) was added to the cell suspension at 120 s (arrow), and PMA (20 nM) was added at 300 s (arrow). The data were sampled at a frequency of 10 Hz, and the traces shown are the mean of 4 independent experiments.

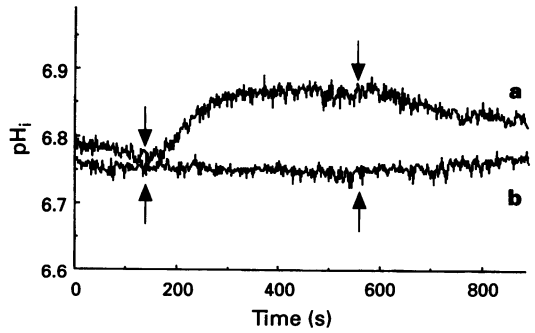

Figure 2 Effect of phorbol 12-myristate 13-acetate (PMA) on intracellular pH in rat peritoneal mast cells. (a) PMA (20 nM) was added to the cell suspension at 120 s (arrow), and staurosporine (250 nM) was added at 550 s (arrow). (b) Effect of staurosporine on the PMA-induced pH_i increase. Staurosporine (250 nM) was added to the cell suspension at 120 s (arrow), and PMA (20 nM) was added at 550 s (arrow). The data were sampled at a frequency of 10 Hz, and the traces shown are the mean of 4 independent experiments.

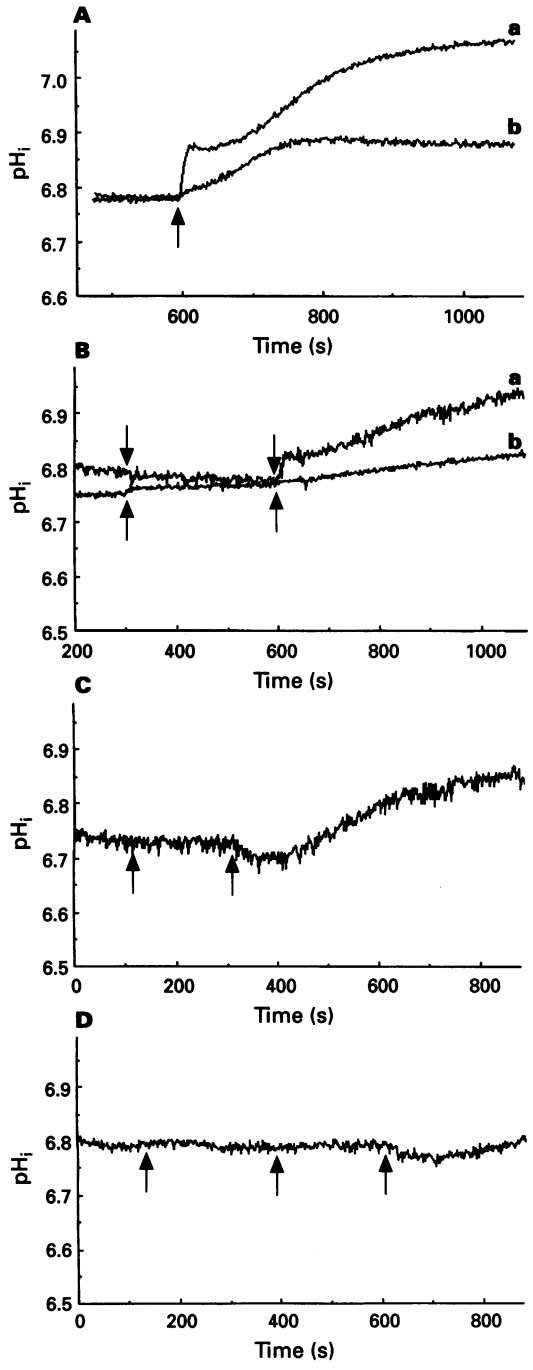

Figure 3 (A) Effect of compound 48/80 on intracellular pH in rat peritoneal mast cells. (a) Compound 48/80 (1 $\mu g\,ml^{-1}$) was added to the cell suspension at 600 s (arrow). (b) Compound 48/80 (0.02 $\mu g\,ml^{-1}$) was added to the cell suspension at 600 s (arrow). (B) Effect of staurosporine on the compound 48/80-induced pH_i increase. (a) Staurosporine (250 nM) was added to the cell suspension at 300 s (arrow), then compound 48/80 (1 $\mu g\,ml^{-1}$) was added at 600 s (arrow). (b) Staurosporine (250 nM) was added to the cell suspension at 300 s (arrow), then compound 48/80 (0.02 $\mu g\,ml^{-1}$) was added at 600 s (arrow). (C) Effect of 5-(N,N-hexamethylene)amiloride (HMA) on the compound 48/80-induced pH_i increase. HMA (10 μM) was added to the cell suspension at 120 s (arrow), then compound 48/80 (1 $\mu g\,ml^{-1}$) was added at 300 s (arrow). (D) Effect of staurosporine and HMA on the compound 48/80-induced pH_i increase. Staurosporine (250 nM) was added to the cell suspension at 120 s (arrow), then HMA (10 μM) at 400 s (arrow), and finally compound 48/80 (1 $\mu g\,ml^{-1}$) added at 600 s (arrow). In (A, B, C and D) the data were sampled at a frequency of 10 Hz, and the traces shown are the mean of 4 independent experiments.

Calcium

The calcium ionophore ionomycin (1 μM) increased pH_i by + 0.06 ± 0.01 pH-units and this increase was completely reversed by a subsequent addition of HMA (Figure 4a, $n=4$). The reverse experimental protocol, i.e. HMA added to the cell suspension 5 min before stimulation by ionomycin, revealed similar inhibitory results (data not shown). The response after addition of ionomycin alone consisted of two phases: one rapid steep increase in pH_i followed by a slower pH_i increase.

The ionomycin-induced pH_i increase was largely unaffected by staurosporine added to the cell suspension 5 min before stimulation by ionomycin. The pH_i increase under these experimental conditions amounted to + 0.04 ± 0.01 pH-units (Figure 4b, $n=4$).

The consecutive addition of ionomycin and PMA revealed an additive effect on pH_i. Thus, when PMA was added to ionomycin-stimulated cells, intracellular pH was increased by a further + 0.07 ± 0.02 pH-units ($n=4$, Figure 5a). This additive effect on pH_i was preserved, albeit reduced (by 40%), even under completely calcium-free conditions using EGTA (1 mM) ($n=4$, Figure 5b). Under these experimental conditions, the steep initial rise in pH_i was abolished, and the increase in pH_i was observed only after a lag period of 42 ± 10 s ($n=4$).

In the absence of extracellular calcium without EGTA (free extracellular calcium concentration around 10–50 μM), resting pH_i amounted to 6.66 ± 0.02 pH-units ($n=8$). Compared to the response in the presence of extracellular calcium (Figure 6a), the response in pH_i after stimulation by compound 48/80 in the absence of extracellular calcium without EGTA was faster and slightly larger (Figure 6a). In contrast, the response in pH_i after stimulation by PMA (20 nM) was unaffected by the presence or absence (without EGTA) of extracellular calcium (Figure 6b).

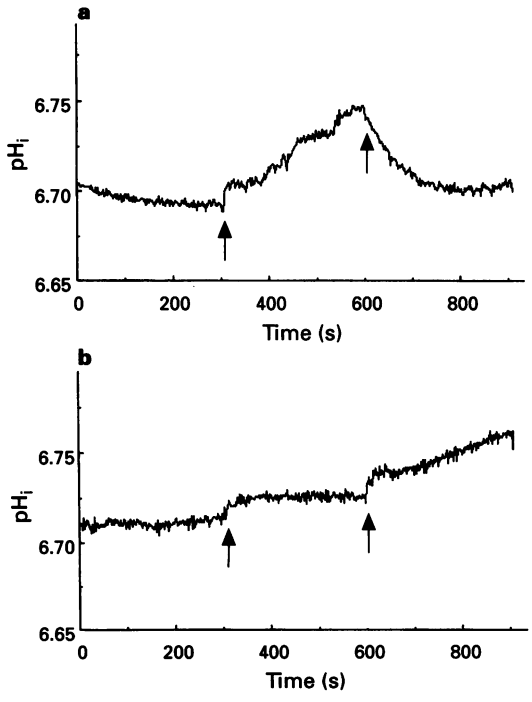

Figure 4 (a) Effect of ionomycin on intracellular pH in rat peritoneal mast cells. Ionomycin (1 μM) was added to the cell suspension at 300 s (arrow) followed by an addition of 5-(N,N-hexamethylene)amiloride (HMA) (10 μM) at 600 s (arrow). (b) Effect of staurosporine on the ionomycin-induced pH_i increase. Staurosporine (250 nM) was added to the cell suspension at 300 s (arrow) followed by the addition of ionomycin (1 μM) at 600 s (arrow). The data were sampled at a frequency of 5 Hz, and the traces shown are the mean of 4 independent experiments.

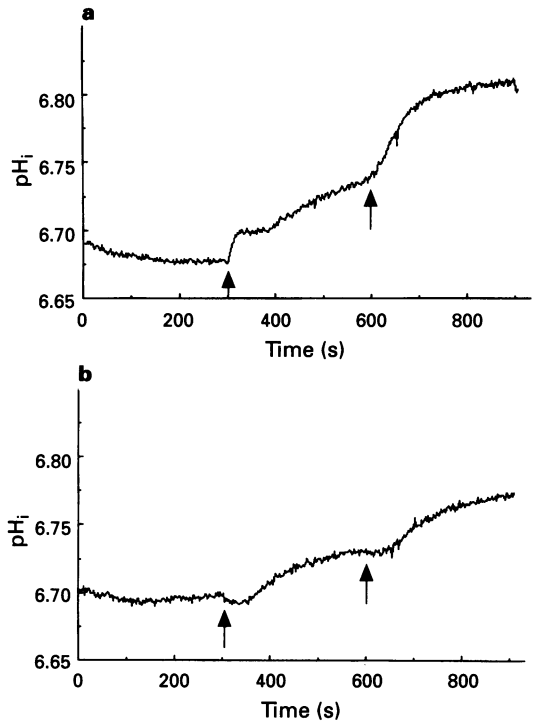

Figure 5 (a) Effect of ionomycin on intracellular pH in rat peritoneal mast cells. Ionomycin (1 μM) was added to the cell suspension at 300 s (arrow) followed by the addition of phorbol 12-myristate 13-acetate (PMA) (20 nM) at 600 s (arrow). (b) Effect of ionomycin on intracellular pH in rat peritoneal mast cells incubated in a physiological salt solution containing EGTA (1 mM). Ionomycin (1 μM) was added to the cell suspension at 300 s (arrow) followed by the addition of PMA (20 nM) at 600 s (arrow). The data were sampled at a frequency of 5 Hz, and the traces shown are the mean of 4 independent experiments.

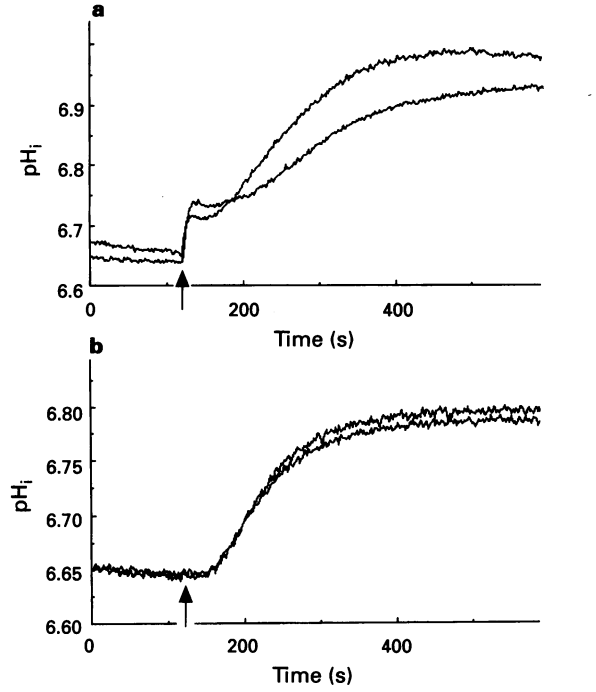

Figure 6 (a) Effect of extracellular calcium on the compound 48/80-induced pH_i increase. Compound 48/80 (1 $\mu g\,ml^{-1}$) was added to the cell suspension at 120 s (arrow) in the absence (upper curve at 600 s) or presence (lower curve at 600 s) of extracellular calcium. (b) Effect of extracellular calcium on the phorbol-12-myristate 13-acetate (PMA)-induced pH_i increase. PMA (20 nM) was added to the cell suspension at 120 s (arrow) in the absence (lower curve at 600 s) or presence (upper curve at 600 s) of extracellular calcium. The data were sampled at a frequency of 5 Hz, and the traces shown are the mean of 4 independent experiments.

Histamine release

Every combination of drugs used in the pH_i-experiments was also evaluated in histamine release experiments in order to investigate any relation between pH_i and histamine release. The drugs were added to the cell suspension in the same order and time interval as in the pH_i-experiments. PMA (20 nM) only resulted in a very small (and non-significant) histamine release (1–2% above basal release; $n=4$, $P>0.05$) and furthermore, this response was unaffected by the calcium content of the incubation buffer (Table 1). The Na^+/H^+ exchange inhibitor HMA and the PKC inhibitor staurosporine did not affect basal histamine release *per se* (Table 1), and the effect of these inhibitors on the PMA-induced histamine release was not significant, due to the small release after stimulation by PMA alone (Table 1; $n=4$, $P>0.05$).

The histamine release induced by the secretagogue compound 48/80 was large and unaffected by the absence or presence of extracellular calcium (Table 1, $n=4$, $P<0.01$). The compound 48/80-induced histamine secretion was greatly reduced by the PKC-inhibitor staurosporine (51% reduction, Table 1, $n=4$, $P<0.01$), whereas the response was less inhibited by preincubation with HMA (30% reduction, Table 1, $n=4$, $P<0.05$). Preincubation with staurosporine and HMA before stimulation with compound 48/80 showed the same degree of inhibition as observed after staurosporine alone (50% reduction, Table 1, $n=4$, $P<0.01$), even though this combination of drugs almost completely inhibited the pH_i-response.

The calcium ionophore ionomycin (1 μM) induced a small and non-significant histamine release amounting to $4.1\pm2.3\%$ (Table 1, $n=4$, $P>0.05$). This response was not affected by the absence of extracellular calcium using EGTA (Table 1, $n=4$, $P>0.05$). The effect of ionomycin was not due to indirect activation of PKC by calcium, since the ionomycin-induced response was unaffected by preincubation with staurosporine (Table 1, $n=4$, $P>0.05$). The effect of ionomycin was, however, dependent upon incubation time, and when the cells were incubated with ionomycin for 10 min, the histamine release was more than doubled (Table 1, $n=4$, $P<0.05$).

The histamine release was greatly potentiated by the combined activation of ionomycin and PMA. Thus, when PMA and ionomycin were added alone, the histamine release amounted to 1.2 and 10.0%, respectively, whereas the combination of drugs resulted in a histamine release amounting to 52% (Table 1, $n=4$, $P<0.01$). This potentiation was even more pronounced when the cells were incubated for longer periods (Table 1). The synergistic effect of ionomycin and PMA was, however, completely abolished in the absence of extracellular calcium with EGTA (1 mM). Under these experimental conditions, the histamine release was reduced to 2.3% (Table 1).

Histamine release after stimulation by a low concentration of compound 48/80 (0.02 $\mu g\,ml^{-1}$) was small, and not significantly different from basal release (Table 1, $n=4$).

Discussion

Intracellular pH

The intracellular pH of mast cells has previously been determined to be 6.8 and 6.9 (Tasaka *et al.*, 1987; Strukova *et al.*, 1992, respectively), values close to the pH_i value measured in this study (6.7). The slightly lower value in our study is probably due to a lower pH of the extracellular buffer

Table 1 The effect of the drugs used in the pH_i experiments on histamine release from rat peritoneal mast cells

Drug(s)	*Histamine release* (%)
PMA (20 nM)	1.2 ± 1.7
PMA (20 nM) in calcium-free buffer	1.1 ± 1.2
PMA (20 nM) in EGTA buffer (1 mM)	1.4 ± 0.5
HMA (10 μM)	0.02 ± 0.03
Staurosporine (250 nM)	0.07 ± 0.13
PMA + HMA	2.0 ± 0.3
HMA + PMA	2.3 ± 0.5
Staurosporine + PMA	0.5 ± 0.3
PMA + staurosporine	2.2 ± 0.5
Compound 48/80 (1 μg ml^{-1})	74.0 ± 14.2
Compound 48/80 (1 μg ml^{-1}) in calcium-free buffer	68.1 ± 14.2
Compound 48/80 (1 μg ml^{-1}) in EGTA buffer (1 mM)	68.5 ± 18.0
HMA + compound 48/80 (1 μg ml^{-1})	52.1 ± 7.2
Staurosporine + compound 48/80 (1 μg ml^{-1})	36.3 ± 5.8
Staurosporine + HMA + compound 48/80 (1 μg ml^{-1})	37.2 ± 4.4
Compound 48/80 (0.02 μg ml^{-1})	0.98 ± 1.12
HMA + compound 48/80 (0.02 μg ml^{-1})	2.38 ± 1.31
Staurosporine + compound 48/80 (0.02 μg ml^{-1})	0.38 ± 0.62
Staurosporine + HMA + compound 48/80 (0.02 μg ml^{-1})	0.25 ± 0.24
Ionomycin (5 min) (1 μM)	4.1 ± 2.3
Ionomycin (5 min) in EGTA buffer (1 mM)	1.6 ± 1.8
Staurosporine + ionomycin (5 min)	3.7 ± 4.6
Ionomycin (10 min) (1 μM)	10.0 ± 3.7
Ionomycin (10 min) + PMA	52.4 ± 11.0
Ionomycin (15 min) + PMA	74.2 ± 10.7
Ionomycin (20 min) + PMA	83.6 ± 10.5
Ionomycin (10 min) + PMA in EGTA buffer (1 mM)	2.3 ± 2.2

The drugs were added to the cell suspension in the same order and time interval as in the pH_i experiments. Incubation time for cells stimulated with ionomycin is indicated in the table. Results are presented as mean ± s.d., averaged from 4 individual experiments. PMA = phorbol 12-myristate 13-acetate; HMA = 5-(N,N-hexamethylene)amiloride.

(amounting to 7.25, 37°C). We have used a nominally bicarbonate-free buffer, because in the presence of bicarbonate, other pH_i regulating systems such as the Cl^-/HCO_3^- exchanger may modify the pH_i changes that are mediated via the Na^+/H^+ exchanger.

In our study, PMA induced a substantial but rather slow increase in pH_i (lag period 46 s). This response was very sensitive to inhibition by staurosporine, very sensitive to inhibition by HMA, insensitive to the absence of extracellular calcium (without EGTA), and sensitive to partial depletion of intracellular calcium with EGTA. Therefore, we conclude that PMA exerts its effect on pH_i via PKC-mediated activation of Na^+/H^+ exchange independently of extracellular calcium. The sensitivity to partial depletion of intracellular calcium by EGTA could be due to a calcium requirement that is retained after phorbol ester-induced insertion of PKC (Kazanietz *et al.*, 1992).

Ionomycin induced a biphasic change in pH_i: a small, rapid increase followed by a rather slow and larger increase in pH_i. The ionomycin-induced increase in pH_i was sensitive to inhibition by HMA, insensitive to staurosporine, and in the absence of extracellular calcium by use of EGTA, the biphasic response disappeared, leaving only a slow, and diminished change in pH_i. It has been shown by Morgan & Jacob (1994) that in fura-2 loaded ECV304 cells ionomycin elicits a saturable biphasic change in intracellular Ca^{2+} concentration, where the initial phase represents mobilization of intracellular stores and the sustained component represents Ca^{2+} influx. It is therefore possible that the initial rapid increase in pH_i induced by ionomycin (or compound 48/80) is somehow regulated by the mobilization of intracellular calcium rather than an influx of extracellular calcium. Thus, experiments with compound 48/80 have shown that the initial fast response in pH_i is maintained in the absence of extracellular calcium (without EGTA), and, in addition, that the initial ionomycin-induced rise in pH_i disappears only after partial depletion of intracellular stores by use of EGTA. Furthermore, experiments with compound 48/80 have shown that this calcium-activated fast increase in pH_i is inhibitable by HMA, not staurosporine, indicating a calcium-mediated activation of Na^+/H^+ exchange independent of PKC. The ionomycin-induced increase in pH_i is not due to a simple exchange of $Ca^{2+}{}_{in}$ for $2H^+{}_{out}$ leading to the non-specific extrusion of protons, since the increase in pH_i was abolished in the presence of the Na^+/H^+ exchange inhibitor HMA. Furthermore, it is not very likely that the stimulation-induced fast initial rise in pH_i is due to release of BCECF trapped in the secretory granula since this steep increase in pH_i was abolished in compound 48/80 stimulated mast cells preincubated with HMA. These cells responded with a histamine release amounting to 52%. In addition, ionomycin added to the cell suspension for 5 min resulted in a full pH_i response with a fast initial rise in pH_i, whereas the histamine secretion was very small (4%). Therefore, we conclude that ionomycin-induced Ca^{2+} transients exert their effect on pH_i via activation of the Na^+/H^+ exchange transport protein independent of PKC.

The additive effects of ionomycin and PMA further support the independent pathways for Na^+/H^+ exchange activation by the two drugs. In a human U937 cell line, a similar additive effect of calcium and PKC on Na^+/H^+ exchange has been described (Alvarez *et al.*, 1989), whereas others have shown partial additivity between these two pathways in acidified cells only (Törnquist & Tashjian, 1992; Graham & Tashjian, 1992).

We have compared the effects of PMA and ionomycin on Na^+/H^+ exchange activation to that of compound 48/80. In our study, compound 48/80 in a high concentration induced a substantial and biphasic change in pH_i, whereas compound 48/80 in a low concentration induced a smaller, slow and monophasic change in pH_i. The compound 48/80-induced increase in pH_i was partially sensitive to inhibition by staurosporine, partially sensitive to inhibition by HMA, and very sensitive to

inhibition when both drugs were applied, independent of secretagogue concentration. Furthermore, the compound 48/80-induced increase in pH_i was insensitive to the absence of extracellular calcium (without EGTA). The finding that staurosporine and HMA each inhibited approximately half of the compound 48/80-induced pH_i-response, whereas both inhibitors completely abolished the compound 48/80-induced pH_i-response seems to indicate that two independent pathways for the activation of the Na^+/H^+ exchange are stimulated by compound 48/80. When these results are compared to the results obtained from experiments using PMA and ionomycin, it seems reasonable to suggest that the two independent pathways mediated by compound 48/80 are comprised of a PKC-mediated and a calcium-mediated pathway, and that these two pathways act additively on the compound 48/80-induced pH_i-increase. The fact that the stimulation-induced increase in $[Ca^{2+}]_i$ precedes the activation of PKC (Penner, 1988; Kurosawa & Kobayashi, 1989) explains why cells preincubated with HMA (which inhibits the fast initial increase in pH_i) respond to compound 48/80 only after a rather long lag period. Differences in the biological activities of phorbol esters and diacylglycerols (Sagi-Eisenberg *et al.*, 1985; Slater *et al.*, 1994; Tuominen *et al.*, 1994) can probably explain why the compound 48/80-induced increase in pH_i was less sensitive to inhibition by staurosporine than expected from experiments using PMA.

Histamine release

The histamine release induced via both PKC activation (using PMA) and calcium (using ionomycin) was much larger than the sum of each activation pathway, whereas in the absence of extracellular calcium by use of EGTA, the response to PMA and ionomycin was completely abolished. The synergistic effect of phorbol ester and calcium ionophore has been described as early as 1984 (Katakami *et al.*, 1984), and a number of studies have investigated this phenomenon further (Kurosawa & Kobayashi, 1989; Chakravarty, 1990; 1992; Chakravarty *et al.*, 1990; Koopmann & Jackson, 1990; Grosman, 1992; Izushi & Tasaka, 1992). Then, in 1994, Choi and coworkers were able to show that in rat basophilic RBL-2H3 cells, phorbol ester alone induced phosphorylation of myosin light chains by PKC exclusively, but failed to induce secretion until accompanied by low concentrations of the calcium ionophore A23187, which activates myosin light chain kinase.

We have also compared the PMA- and ionomycin-induced activation of histamine secretion to that of compound 48/80. The compound 48/80-induced histamine release was partially sensitive to inhibition by HMA (30% inhibition) and partially sensitive to inhibition by staurosporine (50% inhibition). Preincubation with staurosporine and HMA before stimulation with compound 48/80 showed the same degree of inhibition as observed after staurosporine alone, even though this combination of drugs almost completely inhibited the pH_i-response. Furthermore, the compound 48/80-induced histamine release was completely insensitive to the absence of extracellular calcium (with and without EGTA). We have previously shown, however, that compound 48/80-induced histamine release is dependent upon intracellular calcium, since the compound 48/80-induced histamine release amounted to less than 10% if the cells were preincubated for longer periods (60 min) in calcium free media (Johansen *et al.*, 1990). Similar to our findings, it has been shown that staurosporine inhibits histamine release in parallel with inhibition of PKC activity by approximately 50% in rat peritoneal mast cells (Kurosawa & Kobayashi, 1989), whereas in another study, the mast cell response to compound 48/80 was not very sensitive to staurosporine (Grosman, 1992). In addition, it was shown that compound 48/80-induced histamine release is unaffected by preincubation with amiloride (Linnebjerg *et al.*, 1989).

Taken together, our results suggest that compound 48/80-induced histamine release is mediated by two pathways: a PKC-mediated and a calcium-mediated (from internal store(s)), and that these two pathways act synergistically on compound 48/80-induced histamine release.

pH_i regulation and histamine release

In spite of the similarities in second messenger pathways for pH_i regulation and histamine release, it is, however, not very likely that these two processes are directly related. Thus, the ionomycin + PMA-induced histamine release was completely inhibited by the EGTA buffer, whereas the pH_i increase was only diminished. Furthermore, compound 48/80-induced pH_i increase was completely abolished by staurosporine and HMA, but histamine release was not. It has been suggested that one of the roles of PKC is to alkalinize the cytosol to favour calcium release in rat peritoneal mast cells (Alfonso *et al.*, 1994b). Similarly, it has previously been shown that the hydrolysis of inositol phospholipids in combination with an increase in $[Ca^{2+}]_i$ and pH_i probably accounts for the major stimulatory signals for secretion in RBL-2H3 cells (Ali *et al.*, 1989). Finally, alkalinization *per se* induces calcium mobilization in the T-lymphocyte cell line Jurkat, and this mobilization is not due to an increase in IP_3 formation but rather a sensitization of the IP_3/Ca^{2+}-release system (Guse *et al.*, 1994). It is, therefore, possible, that an increase in pH_i plays a permissive, rather than an essential role for histamine release in rat peritoneal mast cells. This hypothesis is supported by the finding that preincubation with the Na^+/H^+ exchange-inhibitor HMA inhibited 30% of the compound 48/80-induced histamine secretion.

Conclusions

Taken together, our results suggest that activation of the Na^+/H^+ exchange mediated via stimulation of PKC by PMA is independent of extracellular Ca^{2+}, whereas the maximum ionomycin-induced rise in pH_i requires not only influx of extracellular Ca^{2+}, but also release of sequestered intracellular Ca^{2+}, and, in addition, that the ionomycin-induced increase in pH_i is independent of PKC.

Comparison of PMA- and ionomycin-induced activation of Na^+/H^+ exchange to that of compound 48/80 has shown that rat peritoneal mast cells have two distinct pathways for the activation of the Na^+/H^+ exchange. These two independent pathways activated by compound 48/80 are comprised of a PKC-mediated and a calcium-mediated pathway acting additively on compound 48/80-induced pH_i-increase. Thus, PMA and ionomycin could be used to reproduce compound 48/80-induced signals for the activation of Na^+/H^+ exchange.

In addition, comparison of PMA- and ionomycin-induced activation of histamine release to that of compound 48/80 has shown that rat peritoneal mast cells have two distinct pathways for stimulation of histamine release. These two independent pathways activated by compound 48/80 are comprised of a PKC-mediated and a calcium-mediated pathway acting synergistically on compound 48/80-induced histamine release. Thus, PMA and ionomycin could be used to reproduce compound 48/80-induced signals for histamine secretion.

In spite of the similarities in second messenger pathways for pH_i regulation and histamine release, it is not very likely that these two processes are directly related. It is, however, possible that an increase in pH_i plays a permissive, rather than an essential role for histamine release in rat peritoneal mast cells.

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