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$Fc_\varepsilon RI$-mediated chloride uptake by rat mast cells: modulation by chloride transport inhibitors in relation to histamine secretion

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1 We have examined the role of extracellular chloride in the mast cell secretion process. The immunologically-directed ligand, antibody to IgE (anti-IgE) required extracellular chloride ions for optimum secretion from rat peritoneal mast cells. In contrast, replacement of extracellular chloride did not alter the mast cell secretory response to compound 48/80, calcium ionophore A23187 or substance P.

2 Anti-IgE-stimulation of mast cells evoked a significant uptake of chloride ions compared to non-stimulated cells. The magnitude of chloride uptake correlated with the magnitude of stimulated histamine secretion.

3 Compound 48/80, substance P and A23187 did not alter the rate of chloride ion uptake, although these agents caused significant histamine secretion.

4 The $Na^+/K^+/2Cl^-$ cotransport inhibitor, furosemide, reduced the rate of anti-IgE-stimulated chloride uptake at a relatively high concentration (700 μM). However, the more potent $Na^+/K^+/2Cl^-$ cotransport inhibitors, bumetanide (100 μM) and piretanide (100 μM) had no effect on the stimulated chloride uptake.

5 Furosemide inhibited anti-IgE-induced histamine secretion, bumetanide potentiated the response and piretanide had no effect. This suggests that their respective action on histamine secretion are unrelated to inhibition of the $Na^+/K^+/2Cl^-$ carrier.

6 The chloride channel blocker, 5-nitro-2-((3-phenylpropyl)-amino)-benzoic acid (NPPB), reduced both anti-IgE-stimulated chloride uptake and the corresponding histamine secretion in a dose-dependent manner. The magnitude of the inhibitory action of the drug on these two cellular processes was comparable, implying that chloride channel activity is related to the mechanism of histamine secretion.

7 It is concluded that chloride uptake has a role in the control of $Fc_\varepsilon RI$-mediated histamine secretion from rodent mast cells.

Keywords: Mast cells; chloride transport; histamine secretion; IgE receptors

Introduction

Electrophysiological studies have shown that rat mast cell activation is associated with an increase in membrane chloride conductance (Matthews *et al*., 1989; Dietrich & Lindau, 1994). Specifically, the application of external agonists and intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) induces an outwardly-rectifying current that is fully developed at 6 min post stimulus. It was proposed that the influx of chloride ions may clamp the membrane potential to a negative value and, hence, allow the sustained influx of calcium ions (Penner *et al*., 1988; Matthews *et al*., 1989). The putative chloride channel blockers, 4,4′-diisothiocyanostilbene-2,2′-disulphonate (DIDS) and 5-nitro-2-((3-phenylpropyl)-amino)-benzoic acid (NPPB), reduced the induced chloride current (Matthews *et al*., 1989; Dietrich & Lindau, 1994).

Immunological stimulation of RBL-2H3 'mucosal-like' mast cells also results in the activation of chloride channels (Romanin *et al*., 1991; Reinsprecht *et al*., 1992). NPPB inhibited both chloride channel activity and the secretory response of the cells in parallel. It was therefore suggested that the induced chloride current may be involved in stimulus-secretion coupling (Romanin *et al*., 1991). Further evidence for a functional role of chloride ions has come from a radiotracer study (Friis *et al*., 1994). Antigen-stimulation of rat peritoneal mast cells was found to cause a large increase in the rate of chloride uptake. The observed rate increase was not affected by addition of DIDS, although the $Na^+/K^+/2Cl^-$ cotransport inhibitor, furosemide, reduced the rate of chloride uptake at a relatively high concentration (700 μM).

The aim of the present study was to examine the effect of non-immunological ligands on mast cell chloride permeability and pharmacologically to characterize immunologically-induced chloride uptake in relation to the modulation of histamine secretion.

Methods

Isolation of mast cells

Male Sprague-Dawley rats (200–400 g) were used for all experiments. Rats were killed by asphyxiation under a rising concentration of CO_2, followed by cervical dislocation. Mixed peritoneal cells were obtained by injecting the abdominal cavity with a modified Tyrode solution buffered with 10 mM HEPES. After gentle massage (2 min), the abdomen was cut open along the midline and the peritoneal suspension was collected. The cells were pelleted by centrifugation ($100 \times g$, room temperature, 2.5 min), the supernatant was discarded and the cells were washed twice before either purification or direct use in secretagogue studies.

Purification of mast cells

Cell suspensions for purification were resuspended in Tyrode buffer (1 ml) containing bovine serum albumin (1 mg ml^{-1}, BSA-Tyrode buffer). A Percoll solution (4 ml) containing 9 parts Percoll and 1 part of $10 \times$ calcium-free Tyrode was mixed with the cells. The suspension was centrifuged ($150 \times g$, 4°C, 25

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min) to create a continuous Percoll gradient. Mast cells were concentrated in the lower third of the Percoll gradient. The upper fraction was aspirated and discarded. The purified mast cells were collected and washed three times in BSA-Tyrode to remove residual Percoll. Cells were resuspended in the appropriate volume of BSA-Tyrode buffer. Mast cell purity was determined by use of an improved Neubauer haemocytometer, after staining with alcian blue (0.1% w/v, 0.9% NaCl, 0.5 M HCl, 0.1% v/v Tween 20). Purity ranged from 91–100%.

Sensitization of mast cells

For studies with anti-IgE, rats were sensitized by use of third stage larvae of *Nippostrongylus brasiliensis*. The larvae (2500 per rat) were delivered by subcutaneous injection into the hind leg. Rats were used 3–5 weeks after sensitization.

Measurement of chloride uptake

Mast cell chloride uptake was determined by the method developed by Friis *et al*., (1994). Briefly, purified mast cells were resuspended in Tyrode buffer to a cell density of 0.7 to 1.3×10^6 cells ml^{-1} and preincubated in a water bath (37°C, 5 min). The cell medium contained the normal concentration of chloride ions (142 mM) together with radioactive chloride as $Na^{36}Cl$ (4.5 to 7.3 mM, specific activity 0.075 to 0.088 MBq ml^{-1}). The secretory stimulus (at $10 \times$ the required concentration) and ^{36}Cl were added simultaneously to the cells. In addition, ^{36}Cl alone was added to control cells to determine passive chloride uptake in the absence of any stimulus. For drug studies, the agent ($10 \times$ concentration) was added with the stimulus and ^{36}Cl. The incubation was terminated by transferring triplicate aliquots (100 μl) to 0.4 ml microcapped centrifuge tubes containing silicone oil (100 μl), followed by centrifugation (16 000 $\times g$, room temperature, 25 s). The tubes were frozen (−70°C) and the tips containing the cell pellets were cut off and placed in tubes containing NaOH (1 M, 50 μl). After vigorous mixing, the samples were left to digest in the dark (room temperature, 12–24 h). Scintillation counting medium (1 ml) was added and the samples were left for a further 12–24 h before being counted in a liquid scintillation spectrometer (Beckman LS 1801). All values of ^{36}Cl cellular uptake were corrected for counts at 'zero' time, as this represented ^{36}Cl trapped in the extracellular space. 'Zero' time counts were obtained by centrifugation of cell aliquots immediately after the addition of ^{36}Cl and stimulus. These samples were then processed as described above. Sample counts recorded in c.p.m. were converted into d.p.m. and the ^{36}Cl uptake (nmol/10^6 cells) was calculated by use of the specific activity of chloride in the extracellular medium.

Measurement of histamine release

For secretagogue studies, aliquots of mast cells (450 μl) were equilibrated in a water bath (37°C, 10 min) before the addition of the stimulus (50 μl, $10 \times$ the required concentration). Control cells (spontaneous release) were treated with buffer alone. The reaction was allowed to proceed for 10 min and terminated by the addition of ice-cold buffer (1 ml), followed by centrifugation (150 $\times g$, 4°C, 2.5 min). The supernatants were decanted and the residual cell pellets were resuspended in buffer (1 ml). The latter were boiled to release residual histamine and both samples were assayed by use of a fluorometric method (Shore *et al*., 1959), omitting the extraction procedure. Histamine release was expressed as a percentage of the total amount of the amine originally present in the cells. Values were corrected for the spontaneous histamine release, which ranged from 1 to 12%.

For pharmacological studies, prewarmed mast cell aliquots were added to an equilibrated buffer solution containing drug and anti-IgE (total volume 0.5 ml). Spontaneous and control anti-IgE-induced releases were determined by adding cells to buffer or buffer plus anti-IgE, respectively. The samples were incubated for 10 min and the reactions were terminated as described before. Both supernatants and cell pellets were treated with 70% v/v perchloric acid (final concentration 0.4 M) and assayed by means of the fluorometric method described by Shore *et al*.. The results are expressed in terms of the percentage inhibition occurring in the absence of the drug.

Solutions

The chloride ion requirement studies with various secretagogues were carried out in either modified Tyrode buffer or a chloride-free Tyrode buffer in which sodium isethionate (137 mM) was substituted for NaCl.

The secretagogue and pharmacological chloride uptake studies were carried out in a modified Tyrode buffer containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1, HEPES 10, $NaH_2PO_4.2H_2O$ 0.4, glucose 5.6 and BSA 1 mg ml^{-1}. The pH of the solution was adjusted to 7.4. Parallel histamine determinations on the same populations of mast cells were carried out in modified Tyrode buffer. For the antimycin A-treated cells, a modified Tyrode buffer was used with glucose omitted.

Data presentation

The data are presented as means ± s.e.mean. To compare whole sets of means in chloride uptake studies, statistical analysis was carried out by use of split plot multiple analysis of variance (MANOVA) (Norusis, 1990). The Spearman's rank correlation coefficient, *r*s, was obtained from estimates by use of the Edgeworth approximation (Ramsey, 1989). When applicable, Student's two-tailed *t* test was used to assess statistical significance.

Materials

Antimycin A, BSA, bumetanide, compound 48/80, furosemide, HEPES, ionophore A23187 and piretanide were supplied by Sigma Chemical Company, (Poole, U.K.); sodium isethionate by Fluka (Gillingham, U.K.); Percoll by Pharmacia, (Uppsala, Sweden); silicone oil (Versilube F 50) by GE Silicones (Waterford, U.S.A.); $Na^{36}Cl$ by Amersham International (Amersham, U.K.); scintillation solution (INSTA GEL) by Packard Instrument Company (High Wycombe, U.K.); substance P by Peninsula (St Helens, U.K.). NPPB was a gift from SmithKline Beecham (King of Prussia, U.S.A.). All other chemicals were of analytical grade.

Results

Extracellular chloride requirement for histamine release

The omission of extracellular chloride reduced the anti-IgE secretory response compared to that observed in the presence of the anion. MANOVA treatment of all concentrations tested indicated that this reduction was statistically significant; $F_{10,1} = 5.26$, $P = 0.045$ (Figure 1a). In contrast, substitution of extracellular chloride with sodium isethionate had no effect on A23187-induced secretion at concentrations of 0.03 to 0.5 μM (Figure 1b). At the higher concentrations of 1.0 and 1.3 μM, A23187-induced histamine release was actually enhanced in the chloride-free buffer compared to the 142 mM chloride-containing buffer. Omission of extracellular chloride had no effect on compound 48/80- or substance P-induced secretion (Figure 1c and d, respectively).

Chloride uptake

Stimulation of rat mast cells with anti-IgE induced a significant and rapid uptake of extracellular chloride ions com-

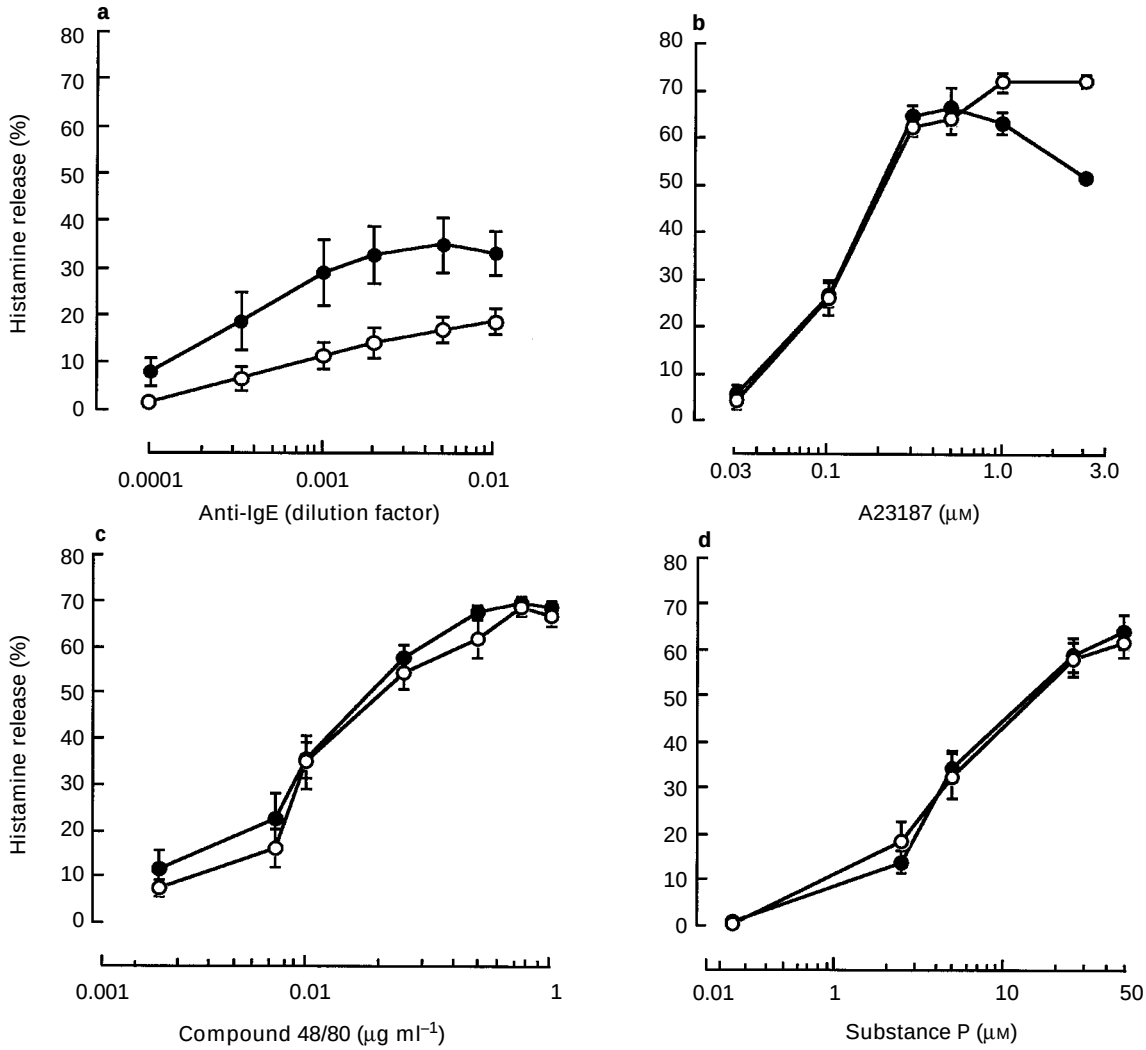

Figure 1 Concentration-response relationship for histamine secretion from rat mast cells at 10 min in the presence of extracellular chloride (●) and absence of extracellular chloride (○) stimulated with anti-IgE (a), A23187 (b), compound 48/80 (c) and substance P (d). Each point is the mean from six experiments; vertical lines show s.e.mean.

pared to unstimulated cells (MANOVA; $F_{20,1}=37.6$, $P<0.001$) (Figure 2a). The corresponding histamine secretion for unstimulated versus stimulated cells was $8.6\pm1.2\%$ and $25.5\pm2.4\%$, respectively. Further analysis of the data at 10 min showed that the magnitude of anti-IgE-induced chloride uptake correlated significantly with the stimulated histamine secretion (Spearman's rank correlation coefficient $rs=0.617$, $P<0.005$) (Figure 2b). Examination of early timepoints showed that chloride uptake increased steadily up to 10 min (Figure 2c). The corresponding anti-IgE-induced histamine releases at 1, 2, 4, 6, 8 and 10 min were 10.5 ± 3.7, 10.9 ± 4, 17.7 ± 5.6, 21.2 ± 5.7, 22.3 ± 5.2 and $22.9\pm5.1\%$, respectively. The metabolic inhibitor, antimycin A (1 μM), reduced both anti-IgE-stimulated chloride uptake (MANOVA; $F_{10,1}=8.04$, $P=0.018$) and histamine release (from $22.4\pm2.1\%$ to $3.9\pm1.1\%$) (Figure 2d).

Figures 3a,b and c demonstrates that ionophore A23187, compound 48/80 and substance P had no effect on chloride uptake, even though the associated histamine secretion at 4 min was comparable to that induced by anti-IgE ($36.6\pm8.1\%$, $24.0\pm3.8\%$ and $29.3\pm3.2\%$, respectively). Early timepoints up to 4 min were examined initially because it has been established that these agents have faster kinetics of secretion compared to anti-IgE. Examination of the 10 min (Figure 3d) and 30 min timepoints (data not shown) also showed that these secretagogues did not elicit appreciable uptake compared to unstimulated cells.

Effect of furosemide, bumetanide and piretanide on chloride uptake and histamine release

The $Na^+/K^+/2Cl^-$ cotransport inhibitor, furosemide, significantly reduced anti-IgE-stimulated chloride uptake into rat mast cells at 700 μM ($F_{10,1}=8.74$, $P=0.015$) (Figure 4a). The drug also inhibited (by $45.2\pm4.9\%$) anti-IgE-induced histamine secretion in the same experiments. However, at a lower concentration (50 μM), furosemide had no effect on the stimulated chloride uptake (Figure 4b), although this concentration inhibited anti-IgE-induced histamine secretion by $16.2\pm1.7\%$. Furthermore, the more potent $Na^+/K^+/2Cl^-$ cotransport inhibitors, bumetanide (100 μM) and piretanide (100 μM) did not alter the anti-IgE-stimulated chloride uptake (Figure 4c and d, respectively). Piretanide had no effect on anti-IgE-induced histamine secretion, while bumetanide enhanced (by $49.9\pm17.3\%$) anti-IgE-stimulated secretion.

Effect of NPPB on chloride uptake and histamine release

As the $Na^+/K^+/2Cl^-$ cotransport inhibitors did not appreciably affect anti-IgE-induced chloride uptake, studies were conducted with the chloride channel blocker, NPPB. At 10 μM, NPPB inhibited both anti-IgE-stimulated chloride uptake (Figure 5a) and the corresponding histamine secretion ($51.5\pm11.9\%$). Further investigation at 4 min demonstrated

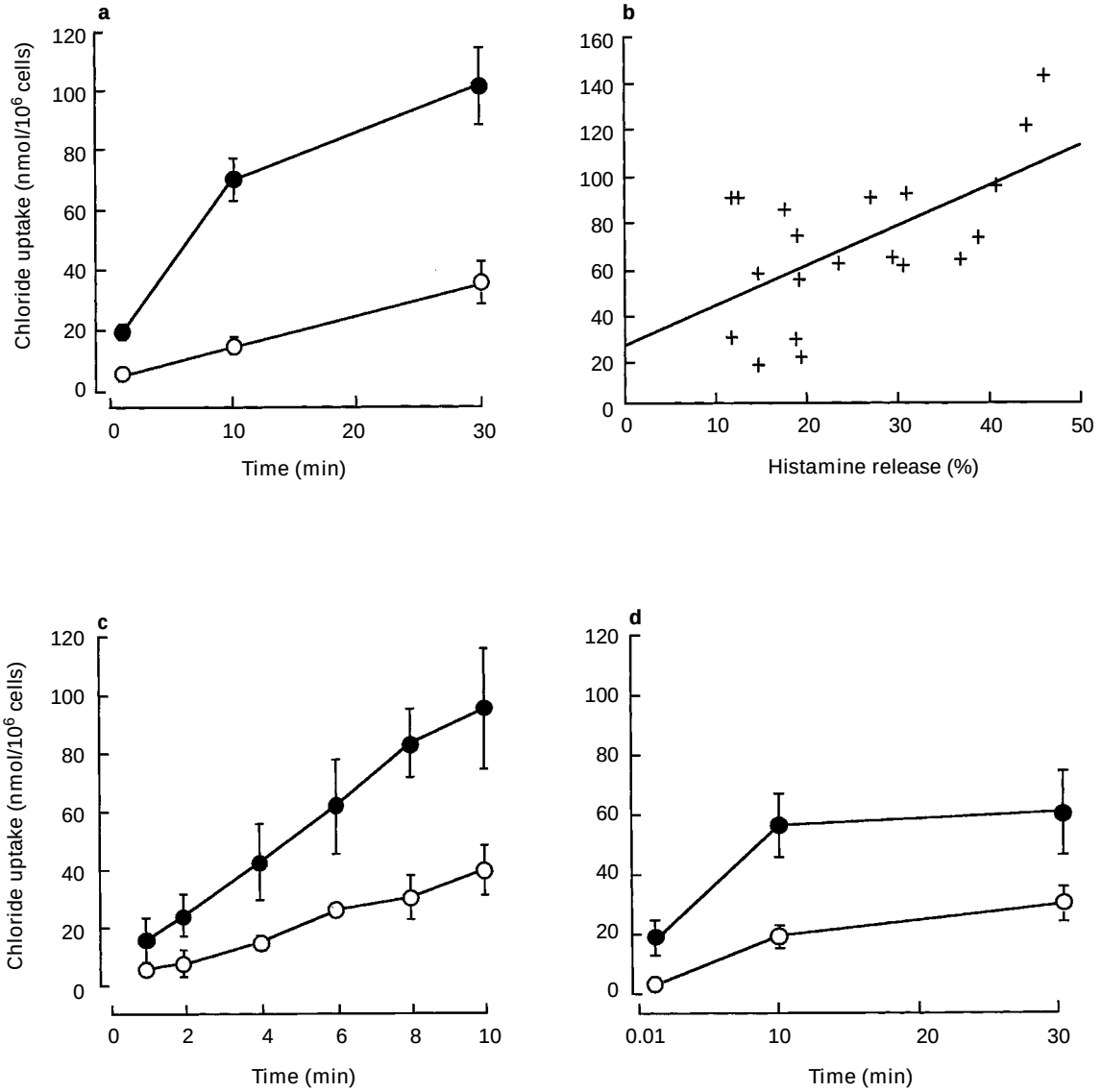

Figure 2 (a) Chloride uptake by rat mast cells. Unstimulated cells (○), cells stimulated with anti-IgE (1/100 dilution) (●). Each point is the mean from twenty experiments; vertical lines show s.e.mean. (b) Correlation diagram of anti-IgE-induced (1/100 dilution) chloride uptake and histamine release by rat mast cells at 10 min. (c) Early time course of chloride uptake by rat mast cells. Unstimulated cells (○), cells stimulated with anti-IgE (1/100 dilution) (●). Each point is the mean from three experiments; vertical lines show s.e.mean. (d) Effect of antimycin A (1 μM) on anti-IgE-induced (1/100 dilution) chloride uptake by rat mast cells. Cells stimulated with anti-IgE (●), cells stimulated with anti-IgE in the presence of antimycin A (○). Each point is the mean from four experiments; vertical lines show s.e.mean.

that NPPB dose-dependently reduced anti-IgE-stimulated chloride uptake (Figure 5b). The inhibitions of the control anti-IgE-stimulated uptake at drug concentrations of 5, 10 and 20 μM were 7.9 ± 6.8, 38.8 ± 11.8 and 62.5 ± 4.8%, respectively. These values were comparable to the inhibitory effect of the drug on anti-IgE-induced histamine release in the same experiments. Thus, the inhibition values for concentrations of 5, 10 and 20 μM were 6.3 ± 3.0, 37.3 ± 9.6, 72.2 ± 6.9%, respectively.

Discussion

A number of studies have shown that mast cell activation is accompanied by an inward movement of chloride ions (Matthews *et al*., 1989; Dietrich & Lindau, 1994; Friis *et al*., 1994). In particular, the Friis *et al*. study demonstrated that antigen stimulation of rat peritoneal mast cells evoked a rapid and large increase of chloride uptake into the cells. They also showed that antigen-induced histamine secretion was reduced when extracellular chloride was replaced with either isethionate or gluconate ions. Similarly, we have found that anti-IgE stimulation results in a rapid uptake of chloride ions compared to control cells and that histamine secretion is suppressed in the absence of extracellular chloride. However, the rate of chloride uptake induced by these two immunological stimuli appears to differ. Antigen-stimulated chloride uptake was near maximal 1 min post stimulus while anti-IgE-stimulated chloride was slower and near maximal at 10 min. This difference may reflect the dissimilar kinetics of histamine secretion exhibited by these two immunological stimuli. Antigen-induced secretion is essentially complete within 1 min of cell stimulation, while anti-IgE-stimulated histamine release is slower and increases over a 10 min incubation period (White & Pearce, 1981).

The magnitude of anti-IgE-induced secretion that we observed was found significantly to correlate with the magnitude of chloride uptake, suggesting that secretion is coupled to the increased chloride permeability of the membrane. Our

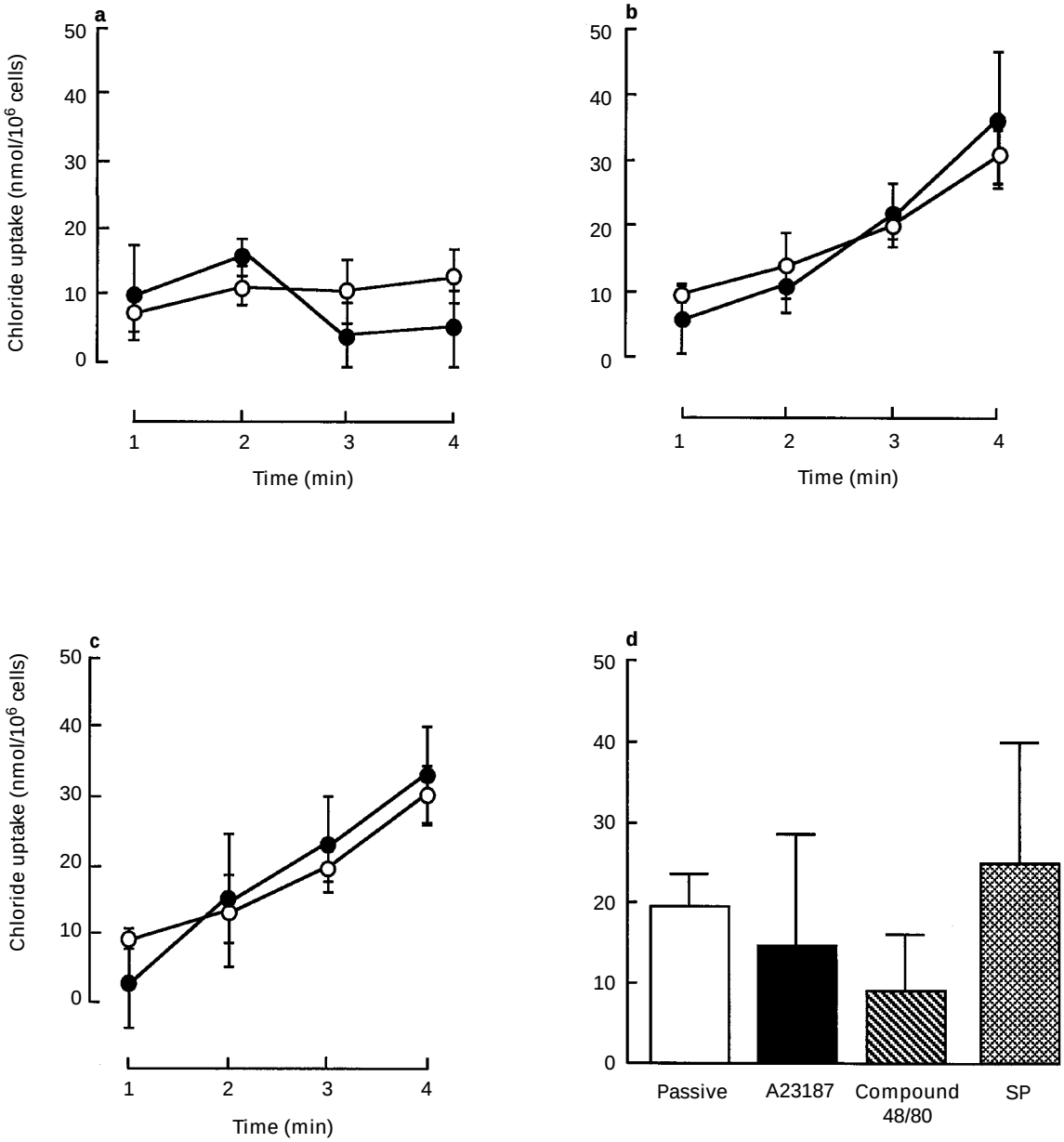

Figure 3 (a) Chloride uptake by rat mast cells up to 4 min in the absence (○) or presence (●) of A23187 (0.1 μM). Each point is the mean from five experiments; vertical lines show s.e.mean. (b) Chloride uptake by rat mast cells up to 4 min in the absence (○) or presence (●) of compound 48/80 (0.1–0.2 μg ml^{-1}). Each point is the mean from five experiments; vertical lines show s.e.mean. (c) Chloride uptake by rat mast cells up to 4 min in the absence (○) or presence (●) of substance P (20 μM). Each point is the mean from five experiments; vertical lines show s.e.mean. (d) Effect of A23187 (0.1 μM), compound 48/80 (0.2 μg ml^{-1}) and substance P (20 μM) on chloride uptake at 10 min compared to passive chloride uptake. Each column is the mean ± s.e.mean from four experiments.

study also showed that antimycin A-pretreatment of rat peritoneal mast cells suppressed anti-IgE-stimulated chloride uptake as well as histamine secretion. This result was surprising because, theoretically, movement of chloride into cells via channels or electrically neutral cotransporters should be independent of cellular production of ATP. However, a lymphocyte channel activated by osmotic stress has been shown to be dependent upon the presence of ATP (Lewis *et al*., 1993). Thus it is possible that the chloride uptake observed in our study is regulated by ATP at some point in the signal transduction pathway. Alternatively, reducing intracellular ATP could alter the resting membrane potential with the consequent change in conductances of one or more ion channels. Hence, the reduced chloride uptake observed in the presence of antimycin A could reflect a change in membrane potential.

In contrast to anti-IgE, we have found that compound 48/80, substance P and A23187 did not cause an increase in chloride uptake into mast cells, even though the magnitude of histamine release was comparable to that of anti-IgE. These findings are consistent with the extracellular chloride replacement studies which showed that non-immunologically-induced histamine secretion was unaffected by the omission of extracellular chloride. Our results therefore suggest that the chloride uptake phenomenon is associated with $Fc_\varepsilon RI$ aggregation because compound 48/80 and substance P are thought to trigger mast cell secretion by activating G-proteins (Mousli *et al*., 1991), while A23187 initiates mast cell secretion by directly elevating intracellular calcium (Foreman *et al*., 1973).

The $Na^+/K^+/2Cl^-$ cotransport inhibitor, furosemide, lowered anti-IgE-induced chloride uptake into mast cells at the

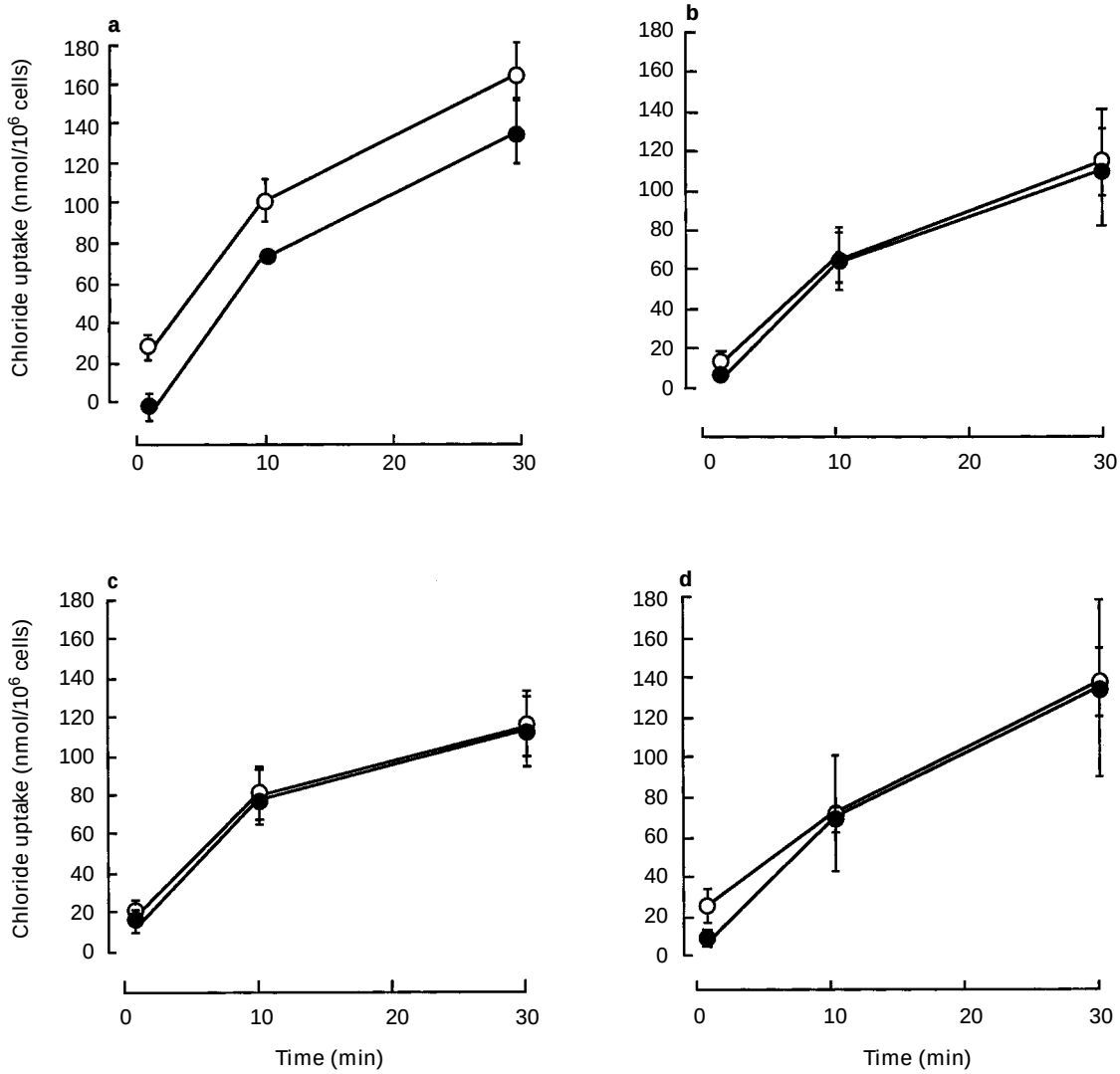

Figure 4 (a) Effect of furosemide (700 μM) on the rate of chloride uptake by rat mast cells stimulated with anti-IgE (1/100 dilution). Cells stimulated with anti-IgE (○), cells stimulated with anti-IgE in the presence of furosemide (●). Each point is the mean from five experiments. (b) Effect of furosemide (50 μM) on the rate of chloride uptake by rat mast cells stimulated with anti-IgE (1/100 dilution). Cells stimulated with anti-IgE (○), cells stimulated with anti-IgE in the presence of furosemide (●). Each point is the mean from four experiments. (c) Effect of bumetanide (100 μM) on the rate of chloride uptake by rat mast cells stimulated with anti-IgE (1/100 dilution). Cells stimulated with anti-IgE (○), cells stimulated with anti-IgE in the presence of bumetanide (●). Each point is the mean from five experiments. (d) Effect of piretanide (100 μM) on the rate of chloride uptake by rat mast cells stimulated with anti-IgE (1/100 dilution). Cells stimulated with anti-IgE (○), cells stimulated with anti-IgE in the presence of piretanide (●). Each point is the mean from five experiments. Vertical lines show s.e.mean.

relatively high concentration of 700 μM. Friis *et al*., (1994) have previously shown that furosemide slows antigen-induced chloride uptake into mast cells. However, when we reduced the concentration of furosemide to 50 μM, the drug had no effect on the stimulated chloride uptake even though there was significant inhibition of histamine release. Typically, furosemide inhibits the $Na^+/K^+/2Cl^-$ cotransporter at concentrations of approximately 3 μM (Schlatter *et al*., 1983), therefore, the $Na^+/K^+/2Cl^-$ carrier is probably not involved in anti-IgE-stimulated chloride uptake into mast cells. Our observations that the more potent $Na^+/K^+/2Cl^-$ cotransport inhibitors, bumetanide and piretanide, had no effect on anti-IgE-stimulated chloride further supports this conclusion. Previous studies on the rat lacrimal gland (Evans *et al*., 1986) and the frog cornea (Patarca *et al*., 1983) have shown that furosemide inhibits chloride channel activity at 1000 μM. This may therefore account for the attenuation by furosemide of anti-IgE-stimulated chloride uptake at 700 μM. It is also unlikely that the $Na^+/K^+/2Cl^-$ cotransporter is involved in anti-IgE-stimulated histamine secretion because the three inhibitors had distinct modulatory actions; furosemide inhibited the stimulated histamine release, bumetanide potentiated secretion while piretanide had no effect.

Electrophysiological recordings on stimulated rat peritoneal mast cells have shown that the chloride channel blocker, NPPB, inhibits chloride channel activity at 10 μM (Matthews *et al*., 1989). We have shown that NPPB dose-dependently (5–20 μM) inhibits anti-IgE-stimulated chloride uptake into mast cells and this suggests that chloride uptake occurs through chloride channels. In addition, NPPB inhibited anti-IgE-induced histamine secretion and this effect may be a consequence of the channel blocking action of the drug. In support of this, NPPB-induced attenuation of chloride uptake correlated with the magnitude of inhibition of histamine release. However, a study on insulinoma cells has shown that NPPB (50 μM) blocked oxidative phosphorylation by acting on mitochondrial anion channels (de Weille & Lazdunski, 1990). Within this context, an agent that uncouples oxidative phosphorylation would non-specifically inhibit both stimulated histamine secretion and chloride uptake. This explanation of the possible mode of action of NPPB may not apply to our studies because we used lower concentrations of the drug. In addition, we have

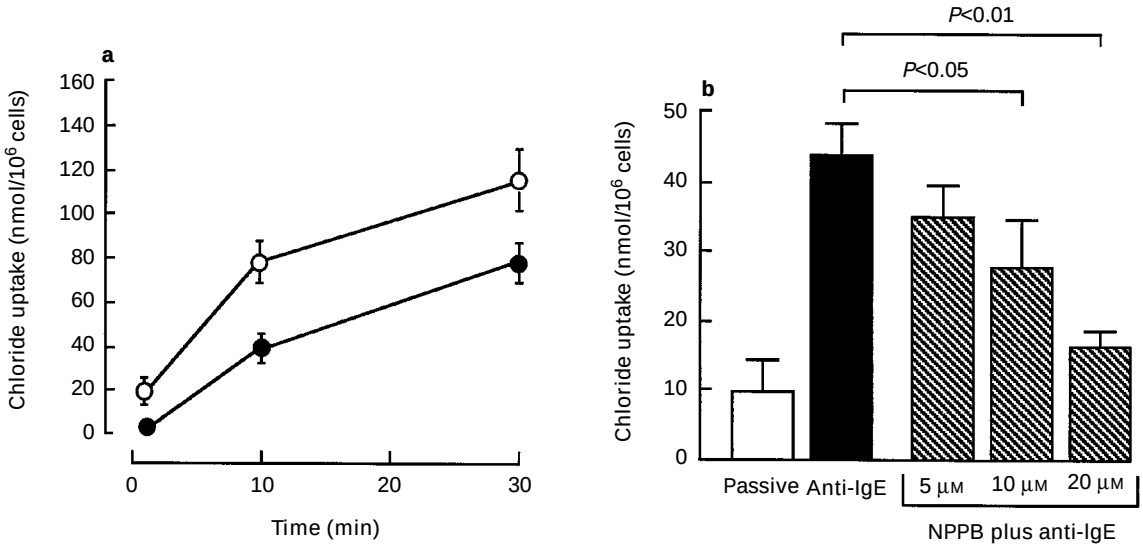

Figure 5 (a) Effect of NPPB (10 μM) on the rate of chloride uptake by rat mast cells stimulated with anti-IgE (1/100 dilution). Cells stimulated with anti-IgE (○), cells stimulated with anti-IgE in the presence of NPPB (●). Each point is the mean from six experiments; vertical lines show s.e.mean. (b) Concentration-response effect of NPPB on anti-IgE-stimulated chloride uptake by rat mast cells at 4 min. The open column represents passive chloride uptake. Each column is the mean ± s.e.mean from four experiments.

found that NPPB has little effect on concanavalin A-stimulated histamine release from mouse peritoneal mast cells and anti-IgE-induced histamine secretion from human basophils (unpublished observations). These findings argue against NPPB having a non-specific inhibitory action on oxidative phosphorylation.

In conclusion, we have identified a chloride uptake pathway that is unique to $Fc_{\varepsilon}RI$ activation in rat peritoneal mast cells. The inward movement of chloride ions significantly correlates with the magnitude of histamine release and, hence, chloride uptake may potentially contribute to the $Fc_{\varepsilon}RI$ signal transduction.

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