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# Calcium-pH crosstalks in rat mast cells: modulation by transduction signals show non-essential role for calcium in alkaline-induced exocytosis

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**Abstract**

Alkalinization of cytosolic pH with ammonium chloride ($NH_4Cl$) was reported to be a stimulus for mast cell degranulation. This paper studied the modulatory role of drugs that target protein kinase C (PKC), adenosine 3′,5′-cyclic monophosphate (cAMP), tyrosine kinase (TyrK) and phosphatidylinositol 3-kinase ($PI_3K$) on this effect. We used Gö6976 (100 nM) and low concentrations of GF109203X (Gf) (50 nM) to inhibit calcium-dependent PKC isozymes. For calcium-independent isozymes, we used 500 nM Gf, and 10 μM rottlerin to specifically inhibit PKC δ, and chelerythrine as non-specific PKC inhibitor. Genistein (10 μM) and lavendustin A (1 μM) were used as unspecific TyrK inhibitors, and 10 nM wortmannin as a $PI_3K$ inhibitor. Chelerythrine and 50 nM Gf inhibit histamine release in the presence of external calcium. The inhibition caused by wortmannin was strictly internal calcium-dependent. cAMP-active drugs did not modify the response to $NH_4Cl$. The effect of $NH_4Cl$ on histamine release was triggered by a transient elevation on cytosolic pH, which was simultaneous to an elevation on cytosolic calcium and followed by a probable $Ca^{2+}$–$H^+$ exchange after addition of external calcium. EGTA inhibit the response to suboptimal concentrations of $NH_4Cl$, and BAPTA increased the effect of $NH_4Cl$. There is a clear relationship between $NH_4Cl$-mediated calcium release and histamine release, since those drugs that inhibit this release also inhibit $NH_4Cl$-mediated histamine release; nevertheless, $NH_4Cl$-mediated histamine release was possible in the absence of any calcium release, as shown with BAPTA. This data, in combination with the results with PKC inhibitors, suggest that calcium is not only unnecessary to trigger cell activation, but also that it may be a negative modulator of $NH_4Cl$-mediated exocytosis.


*Keywords:* Cytosolic pH; Cytosolic $Ca^{2+}$; Mast cells; Transduction signal; Alkalinization

## 1. Introduction

Mast cells are an inflammatory cellular model useful to study modulatory signals because they are easy to obtain, and their degranulation response can be quantified by measuring the release of histamine, which is stored in their granules. Although pH is generally believed to be a physico-chemical parameter to which cells have adapted to integrate all their regulatory functions, it has been recently demonstrated that, at least in mast cells, pH is not only a surrounding condition for enzymes and receptors to work, but a modulatory signal itself, for which cells have several specific mechanisms of control [1,2]. One important aspect of pH regulation is the presence of bicarbonate on the medium, since this physiological ion affects many aspects of the cell function, such as sensitivity to calcium [3,4], role of PKC [5] or regulation of pH itself [6].

It is clear therefore that pH is an important factor to bear in mind when studying the mechanism that modulates the exocytosis in mast cells. In fact, mast cells are endowed with several mechanisms to tightly regulate intracellular pH (pHi) values, either directly, such as a $Na^+$–$H^+$ exchanger [7] or a $CO_3H^-$ influx mechanism [2], or indirectly with a $Na^+$–$Ca^{2+}$ exchanger [8,9] or a $Na^+$–$K^+$ ATPase [10,11].

The role of pH is becoming evident to many aspects of the physiological economy (i.e. pH is relevant to the activation of the capsaicin-activated vanilloid receptor [12], to the activation of tumor promotion [13], and the

*Abbreviations:* PKC, protein kinase C; pHi, cytosolic pH; Gf, GF 109203X; OA, okadaic acid; CT, cholera toxin; PT, pertussis toxin; $PI_3K$, phosphatitilinositol 3 kinase

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regulation of pHi is being considered as an antineoplastic strategy [14]).

It has been established by several groups that pHi and cytosolic calcium are closely linked. In an earlier work, we studied the effect of pHi in calcium levels, and found that cytosolic alkalinization, but not intracellular calcium release, is a sufficient signal for degranulation [1]. This paper further explores the crosstalks between pH and internal signaling.

## 2. Methods

### 2.1. Chemicals

Nigericin, ethylene-glycol-bis(b-aminoethylether) *N*,*N*,*N*′,*N*′-tetracetic acid (EGTA) and rottlerine and dibutyryl cAMP were from Sigma Chemical Co.; $NH_4Cl$ and phthaldialdehyde were from Merck; Percoll® was from Pharmacia; thapsigargin, chelerythrine, Gö6976, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22,536), forskolin, H-89 and GF 109203X were from Alexis Corporation; BAPTA acetoxymethyl ester (BAPTA), 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) and FURA-2 AM were from Molecular Probes.

### 2.2. Mast cell preparation

Mast cells were obtained by lavage of pleural and peritoneal cavities of Sprague–Dawley rats ((200–400) × *g*) as described previously [15]. The composition of physiological saline solution was (mM): $Na^+$, 142.3; $K^+$, 5.94; $Ca^{2+}$, 1; $Mg^{2+}$, 1.2; $Cl^-$, 126.2; $HCO_3^-$, 22.85; $PO_4H^{2-}$, 1.2; $SO_4^{2-}$, 1.2. In all the experiments, the incubation medium was equilibrated with $CO_2$ and the final pH was adjusted to 7.4 prior to use.

The unpurified cellular suspension contained 4–8% mast cells, (1.5–2) × $10^6$ per rat. All the experiments were carried out with purified mast cells, except those studying mediator release.

### 2.3. Cell purification

Cells pooled from three rats were purified by centrifugation through 4 mL of isotonic Percoll at 400 × *g* for 10 min. Percoll was eliminated by washing three times with the medium described above at 100 × *g* for 5 min. Cell purity was always higher than 95%. Cell viability was studied by the Trypan blue exclusion test and was always higher than 97%.

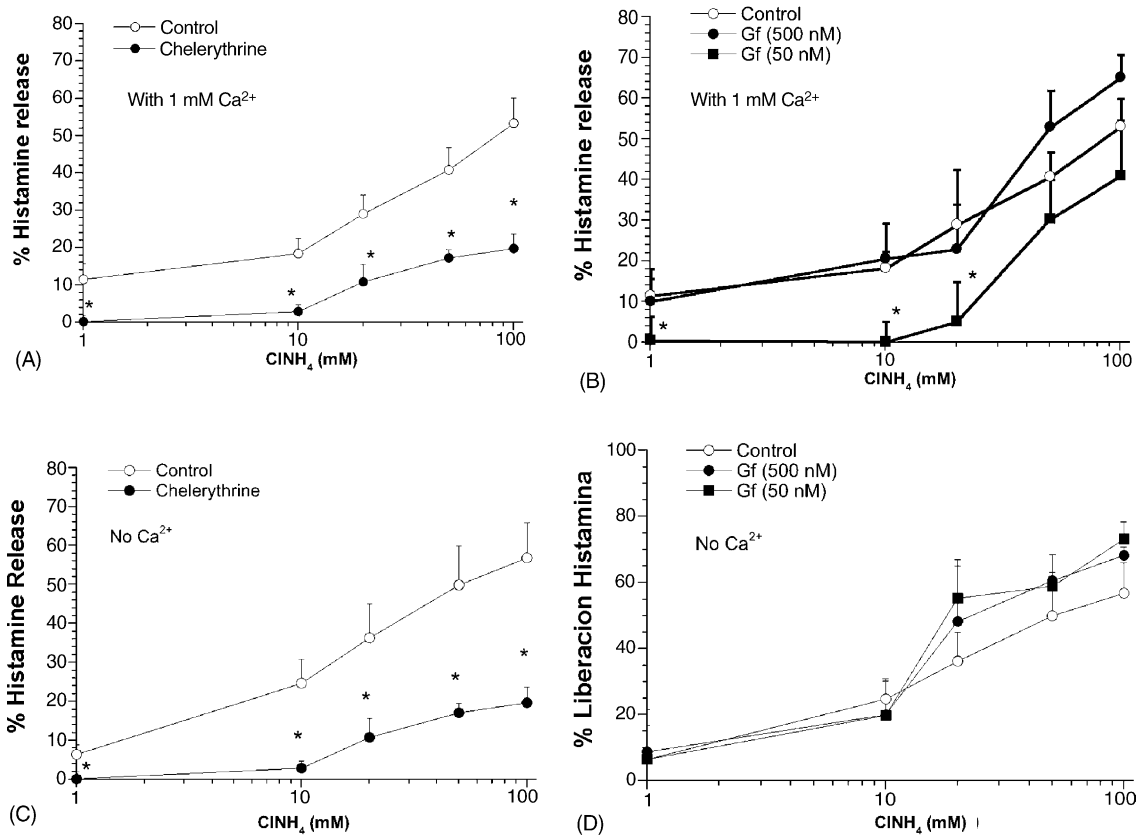


Fig. 1. Histamine release elicited by $NH_4Cl$: (A) effect of 1 μM chelerythrine in a medium with 1 mM external calcium; (B) effect of 50 and 500 nM GF109203X in a medium with 1 mM external calcium; (C) effect of 1 μM chelerythrine in a medium with no external calcium; (D) effect of 50 and 500 nM GF109203X in a medium with no external calcium. Mean ± S.E.M. of three experiments. (*) Significant differences with respect to corresponding $NH_4Cl$ control.

### 2.4. Cell incubation

Twenty-five microlitres of a freshly prepared concentrated solution of $NH_4Cl$ was added to the incubation medium to attain a final volume of 0.975 mL and preincubated. When the medium reached 37 °C, 25 μl of a cell suspension containing (1–1.5) × $10^5$ mast cells was added to each tube. Incubations were carried out in a bath at 37 °C for 10 min.

The incubations were stopped by immersing the tubes in ice-cold bath. After centrifugation at 1000 × *g* maximum for 3 min, the supernatants were collected and decanted into other tubes for histamine determination. To ensure total histamine release in pellets 0.8 mL of 0.1N HCl was added and tubes were sonicated for 60 s. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in each experiment.

### 2.5. Histamine release assay

Histamine was assayed fluorimetrically by Shore's method [16] omitting extraction procedure and modified for 96-well microtiter plate [17]. Plates were read at 355 nm excitation and 460 nm emission in a microplate fluorescence reader Labsystems Fluoroskan II. The results are expressed as a percentage of histamine released with respect to total histamine content, and corrected by spontaneous histamine release without stimulation under the same conditions. In $NH_4Cl$ experiments, histamine released was determined only in pellet because this chemical interferes with histamine fluorescence.

### 2.6. Measurement of cytosolic free calcium and intracellular pH. Image processing

Purified rat mast cells were loaded with Fura-2 AM (0.3 μM) and with 0.1 μM BCECF-AM for 10 min at 37 °C. Loaded cells were washed three times (400 × *g* per 2 min) and allowed to attach to poly-L-lysine-coated 22 mm glass coverslips for 10 min. The glass coverslips were inserted into a thermostated chamber (Life Science Resources, England) and cells were viewed with a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40X-inmersion UV-Fluor objective). The chamber was used in the open bath configuration and additions made by aspiration and addition of fresh bathing solution. Intracellular calcium concentration and pH were obtained from the images collected by quadruple excitation fluorescence with Life Science Resources (UK) equipment. The light source was a 175 W xenon lamp, and light reached the objective with optic fiber. The excitation wavelengths for Fura-2 were 340 and 380 nm, with emission at 505 nm, and for BCECF 440 and 490 nm, both for excitation, and 530 nm for emission. The calibration of the fluorescence versus intracellular calcium was made by using the method of Grynkiewicz et al. [18]. The calibration of fluorescence versus pHi was made using nigericin in $K^+$ solution as per Thomas et al. [19]. Briefly, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin for each pH value. With these values, we obtained in each experiment, a calibration curve (ratio versus pH) that was used to transform any ratio value to pH.

### 2.7. Statistical analysis

Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± S.E.M.

## 3. Results

Mast cells do have calcium-dependent PKC isozymes (α, β), and calcium-independent PKC isozymes (δ, ε and the atypical ζ, which does not translocate to the membrane

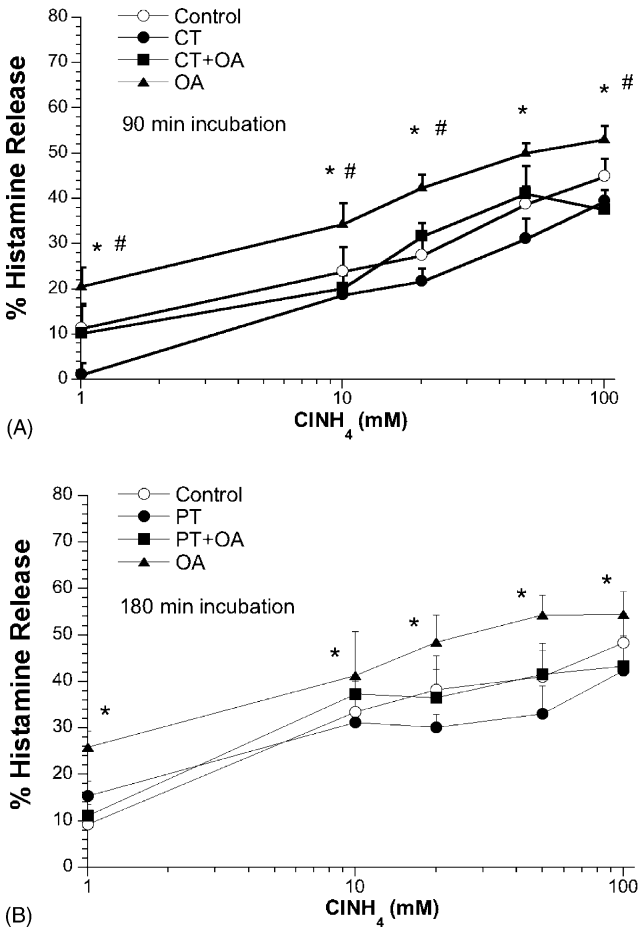


Fig. 2. Histamine release elicited by $NH_4Cl$: (A) effect of 10 ng/mL cholera toxin (CT) and 1 μM okadaic acid (OA) in a medium with 1 mM external calcium; (B) effect of 50 ng/mL pertussis toxin (PT) and 1 μM okadaic acid in a medium with 1 mM external calcium. Significant differences with respect to (*) $NH_4Cl$ control and (#) cholera toxin plus okadaic acid. Mean ± S.E.M. of three experiments.

by antigen or the phorbol ester PMA) [20]. We have used chelerythrine as non-specific PKC inhibitor, Gö6976 (100 nM) and low concentrations of GF 109203X (50 nM) to inhibit calcium-dependent isozymes. And in order to inhibit calcium-independent isozymes, we used high concentrations (500 nM) of Gf, and rottlerin (10 μM) to specifically inhibit PKC δ, which is important to mast cells [5].

In a previous paper, alkalinization of cytosolic pH with $NH_4Cl$ was reported to be a stimulus for mast cell degranulation [1]. Fig. 1 shows the effect of PKC modulation in histamine release from mast cells activated with $NH_4Cl$. Of all the compounds tested (not shown phorbol ester PMA (100 ng/mL), Gö6976 (100 nM) and rottlerine (10 μM)), only the unspecific PKC inhibitor chelerythrine (1 μM) does clearly inhibit histamine release elicited under these conditions (Fig. 1A). Gf shows a dual effect, at large doses (500 nM) does not show any effect, but at low concentrations (50 nM), a clear inhibition can be observed (Fig. 1B).

Calcium is important to modulate PKC isoforms, since some of them are calcium-independent. For this reason, we have repeated the same experiments in the absence of external calcium (Fig. 1C–D). Under these conditions, only chelerythrine shows the same effect, and, interestingly, the calcium-dependent component of Gf inhibition completely disappears.

The activation of PKC triggers a phosphorylation sequence of events. In order to determine the role of phosphorylation on mechanisms both activated by PKC or by protein kinase A (PKA) (cAMP activated), we used 1 μM okadaic acid to inhibit cytosolic phosphatases and cause hyperphosphorylation in the cell [21,22], in cells incubated with pertussis toxin or with cholera toxin, which modulate G proteins and increase cAMP levels by 120 and 365%, respectively [23]. Fig. 2 shows that the response to $NH_4Cl$ is insensitive to the effect of both cholera (10 ng/mL) (Fig. 2A) or pertussis (50 ng/mL) (Fig. 2B) toxin. On the other hand, OA induces an increase on the response of mast cells to $NH_4Cl$ not shown in the presence of toxins.

Since the effect of PKC on $NH_4Cl$-activated mast cells is clearly calcium-dependent, we studied the effect of calcium in other signals. Of all the drugs used (10 μM genistein and 1 μM lavendustin A as unspecific TyrK inhibitors, and 10 nM wortmannin, a $PI_3K$ inhibitor [24]), genistein did not show any effect (not shown). As Fig. 3 shows, lavendustin A significantly inhibits the release of histamine only in the presence of calcium (Fig. 3A and B), in a similar fashion to 50 nM GF 109203X (Fig. 1B). Wortmannin inhibits the response to

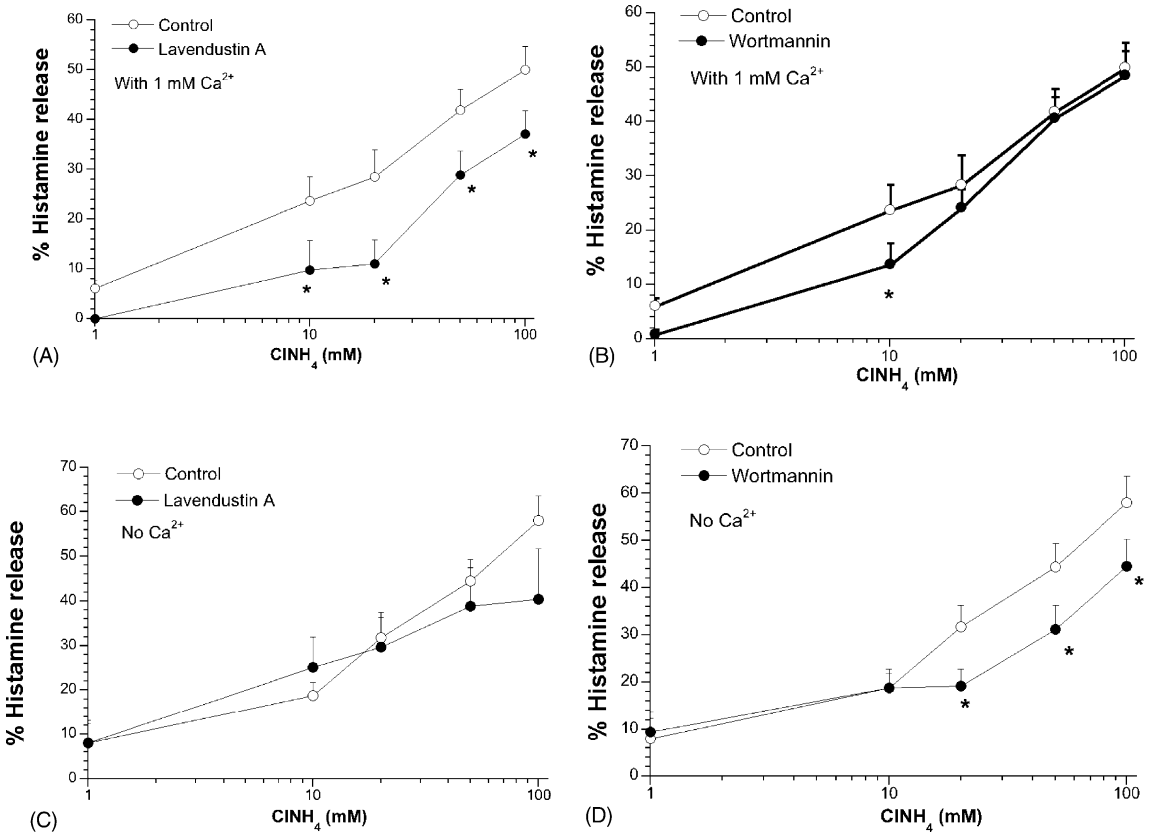


Fig. 3. Histamine release elicited by $NH_4Cl$: (A) effect of 1 μM lavendustin A in a medium with 1 mM external calcium; (B) effect of 10 nM wortmannin in a medium with 1 mM external calcium; (C) effect of 1 μM lavendustin A in a medium with no external calcium; (D) effect of 10 nM wortmannin in a medium with no external calcium. Mean ± S.E.M. of three experiments. (*) Significant differences with respect to corresponding $NH_4Cl$ control.

$NH_4Cl$ but in a $Ca^{2+}$-free medium, while lavendustin A shows no effect (Fig. 3B and D).

We explored the effect of cAMP-active drugs with the adenylate cyclase activator forskolin (30 μM), the adenylate cyclase inhibitor SQ22536 (1 μM), the cAMP analog dibutyryl cAMP (200 μM), and the PKA blocker H89 (1 μM). No modulation of the response to $NH_4Cl$ was observed (not shown), in agreement with data shown in Fig. 2.

The effect of calcium on the response to $NH_4Cl$ is shown in Fig. 4. The effect of $NH_4Cl$ as mast cell stimulus is not dependent on the presence of external calcium (Fig. 4A), neither it is sensitive to the addition of calcium in cell previously stimulated in calcium-free conditions (Fig. 4B). In the presence of the external calcium chelator EGTA, the response is slightly reduced (22%) with a maximum stimulus, but it is clearly inhibited at suboptimal $NH_4Cl$ concentrations (by 58%) (Fig. 4C). Unexpectedly, the chelation of internal calcium with BAPTA has the opposite effect to external EGTA (Fig. 4D), since a 23 and 33% increase in histamine release is observed with 50 and 20 mM $NH_4Cl$, respectively.

In order to understand the crosstalks between calcium, cytosolic pH and histamine release, we studied calcium and pHi profiles with those compounds that did induce a change on the cell response to $NH_4Cl$. The procedure used was to incubate the cells with the drug in calcium-free conditions, and then add external calcium to have a measurement of calcium influx under the influence of the drug, while measuring simultaneously cytosolic pH.

Fig. 5 shows cytosolic calcium and pH in control and $NH_4Cl$-treated cells. The addition of $NH_4Cl$ slightly increases cytosolic calcium (Fig. 5A) and generates a transient alkaline peak (Fig. 5C); the maximum alkaline pH values are again restored after addition of external calcium. In the presence of 2.5 mM EGTA, which prevents any resting external calcium to enter the cell, the calcium profile is as expected (no increase after addition of external calcium, Fig. 5B), but the profile of cytosolic pH in the complete absence of external calcium (with EGTA) matches the data obtained in the $NH_4Cl$ control (Fig. 5D) before the addition of calcium. Nevertheless, pHi alkalinization after the addition of external calcium is completely inhibited.

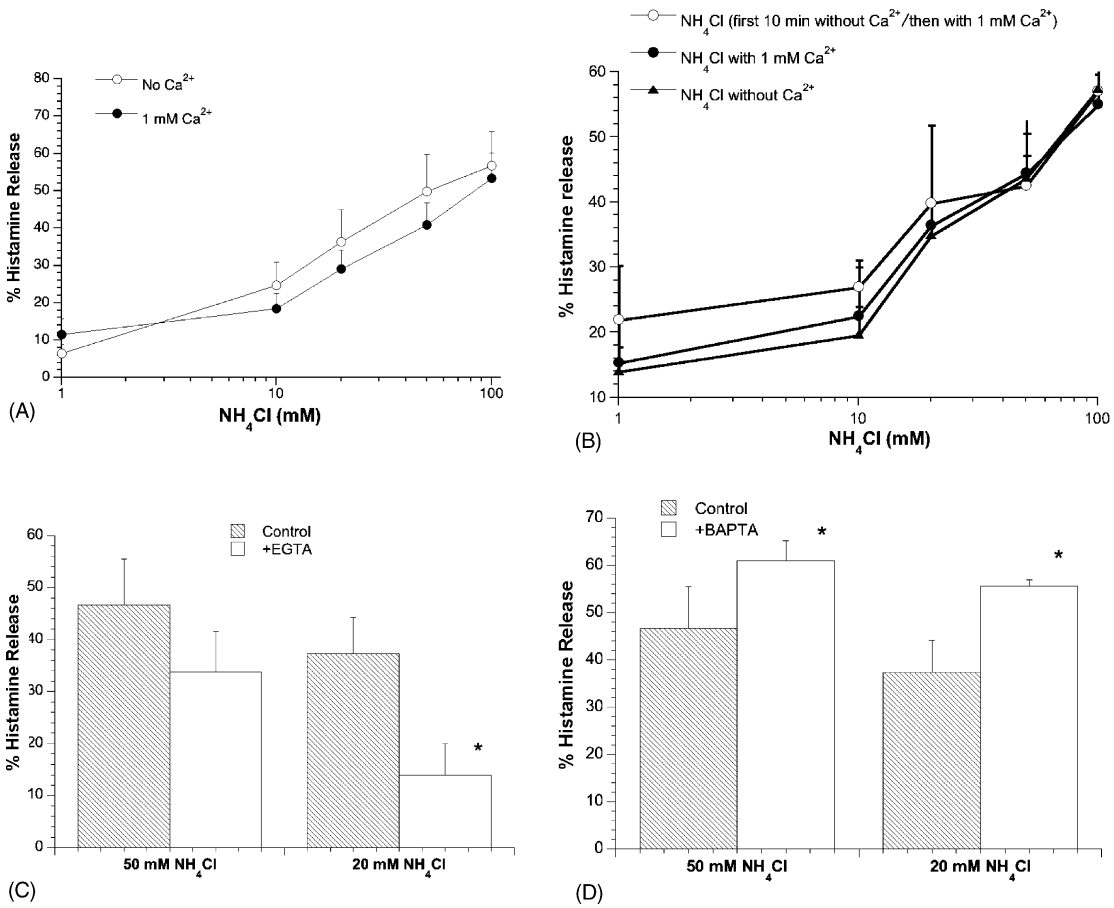


Fig. 4. (A) Effect of external calcium on histamine release elicited by $NH_4Cl$; (B) effect of addition of calcium on histamine release elicited by $NH_4Cl$; (C) effect of 2.5 mM EGTA on histamine release elicited by $NH_4Cl$; (D) effect of 30 μM BAPTA on histamine release elicited by $NH_4Cl$. Mean ± S.E.M. of three experiments. (*) Significant differences with respect to corresponding $NH_4Cl$ control.

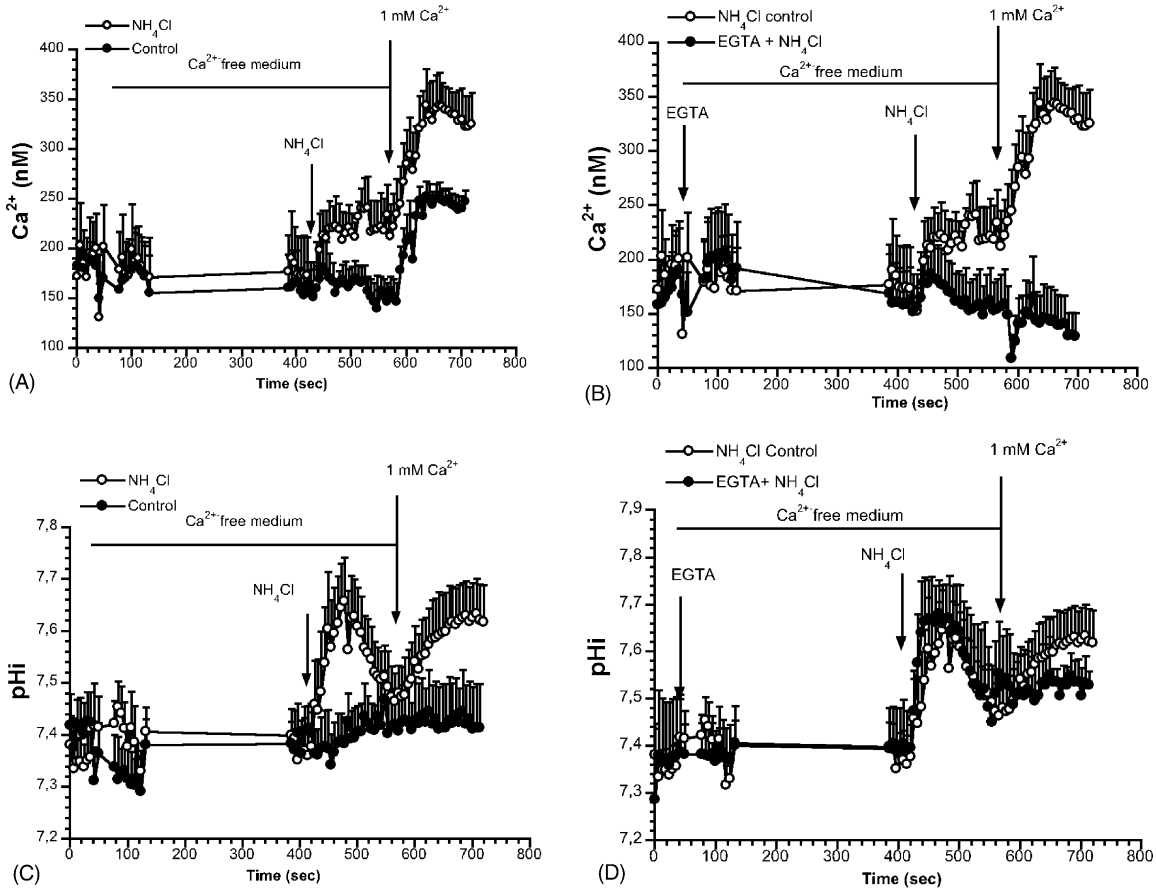


Fig. 5. Effect of addition of 1 mM $Ca^{2+}$ on cytosolic calcium (A and B) and pHi (C and D) levels, in control and 2.5 mM EGTA-treated rat mast cells after addition of 20 mM $NH_4Cl$ in a $Ca^{2+}$-free medium. Mean ± S.E.M. of three experiments.

Fig. 6 shows the effect of PKC inhibition on $[Ca^{2+}]_i$ and $[H^+]_i$ levels. Unspecific PKC inhibition with chelerythrine (1 μM) does change the profile of $[Ca^{2+}]_i$ with respect to the control shown in Fig. 6A, the effect being clear after addition of $NH_4Cl$, since there is no increase in cytosolic calcium. After addition of 1 mM external $Ca^{2+}$, the increase is similar to the control (Fig. 5A) in the absence of $NH_4Cl$. Therefore, chelerythrine inhibits the release of internal calcium induced by $NH_4Cl$. On the other hand, the change in $[H^+]_i$ profile is striking. While in control cells $[H^+]_i$ shows two clear peaks, before and after calcium addition, in the presence of chelerythrine both peaks change to a flat line at maximum alkaline values (Fig. 6B), while abolishing the transient pHi peak observed in $NH_4Cl$ control. The selective inhibition of calcium-dependent PKC isozymes with 50 nM Gf109203X, which did inhibit mast cell exocytosis (Fig. 1B), does show a modification pattern of $[Ca^{2+}]_i$ as observed with chelerythrine, but no change of $[H^+]_i$ with respect to the control (Fig. 6C and D). Therefore, Gf109203X inhibits the release of internal calcium induced by $NH_4Cl$, but it does not modify pHi profile.

Fig. 7 shows the effect of wortmannin, a drug that did show an inhibitory effect on histamine release in the absence of external calcium (Fig. 3D), on $[Ca^{2+}]_i$ and $[H^+]_i$ levels. As for chelerythrine (Fig. 6A and B), wortmannin does modify the profile of calcium with respect to the control (Fig. 7A), and causes a change of the transient pHi peak observed in $NH_4Cl$ control to a constant alkaline line at maximum pHi values (Fig. 7B).

## 4. Discussion

This paper is important because it clearly indicates that calcium is a secondary signal and that a change in cytosolic pH is a signal by itself to activate the cell. $NH_4Cl$ is a unique stimulus to mast cells because the underlying mechanism that triggers the release of histamine is a change to alkaline values in cytosolic pH [1]. This change in internal pH activates the release of internal calcium, and the exocytosis process. On the other hand, just the release of internal calcium with thapsigargin is insufficient to trigger cell activation. This paper explores the mechanisms that modulate the stimulatory activity of $NH_4Cl$.

The effect of $NH_4Cl$ is independent of external calcium, and histamine release levels are unaffected by either the presence or absence of this ion. Since cytosolic pH is regulated by the $Na^+$–$H^+$ exchanger, which recovers the cell from acid loads thereby alkalinizing the cytosol, we

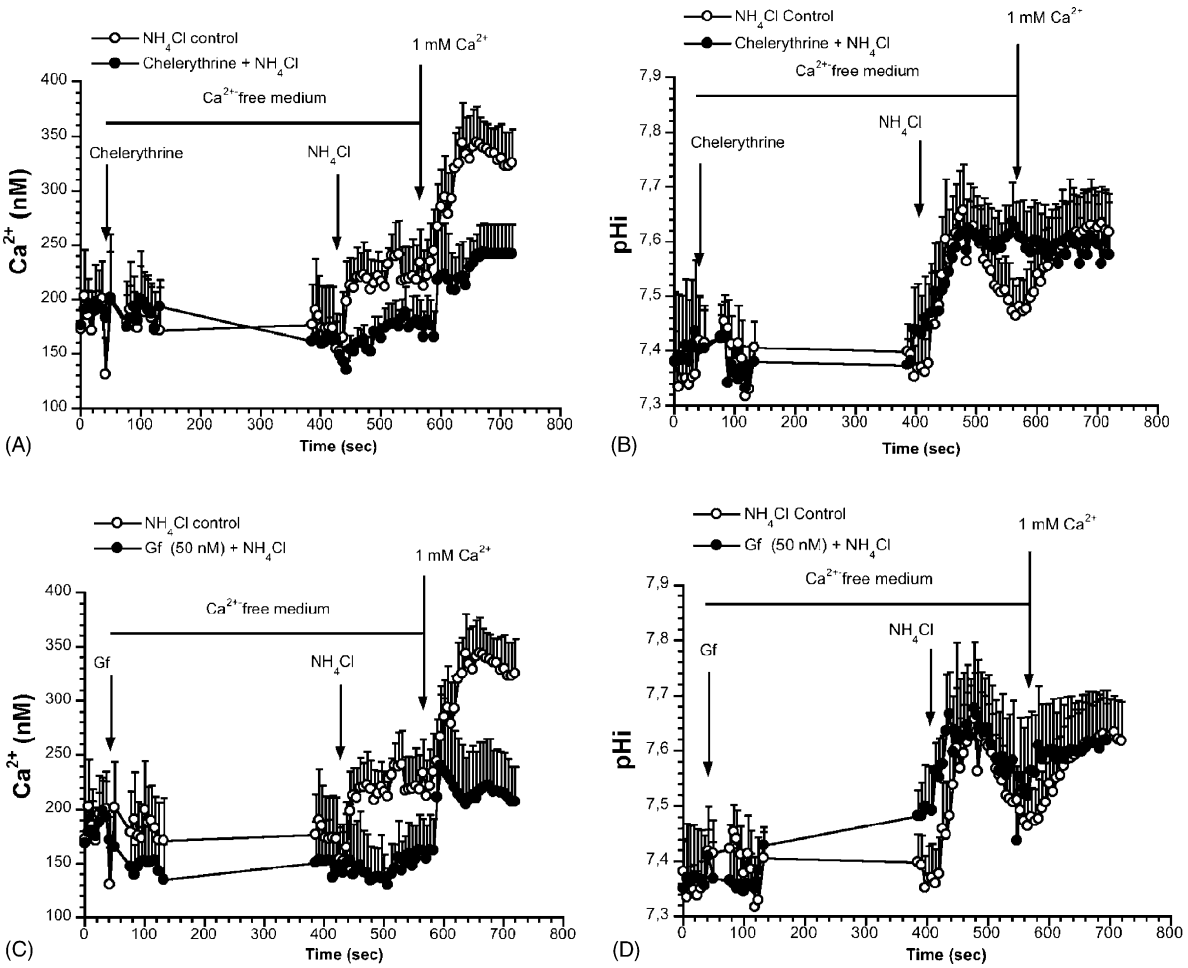


Fig. 6. Effect of addition of 1 mM $Ca^{2+}$ on cytosolic calcium (A and C) and pHi (B and D) levels, in 1 μM chelerythrine-treated and 50 nM GF109203X-treated rat mast cells after addition of 20 mM $NH_4Cl$ in a $Ca^{2+}$-free medium. Mean ± S.E.M. of three experiments.

studied the role of PKC, which enhances the activity of the exchanger [7,25–27], on $NH_4Cl$-mediated histamine release. The inhibition of PKC with the unspecific drug chelerythrine or the calcium-dependent drug GF 109203X (50 nM) did show a decrease of histamine release. Since the only direct relationship of PKC to pHi is through the $Na^+$–$H^+$ exchanger, it seems feasible to assume that $NH_4Cl$-mediated effect includes a role for this exchanger. Also, the modulatory role of PKC on $NH_4Cl$-mediated response is sustained by the calcium-dependent isozymes.

The slight increase that OA induces on $NH_4Cl$-mediated exocytosis is also attributable to the activation that OA induces in the $Na^+$–$H^+$ exchanger [28,29]. Therefore, the activation of the $Na^+$–$H^+$ exchanger by increasing the phosphorylation with OA, a phosphatase PP2A and PP1 inhibitor [30], or the blockage of its activation with $Ca^{2+}$-dependent PKC inhibitors suggest that the exchanger is an important modulator of mast cell $NH_4Cl$-mediated exocytosis. The total lack of effect of cAMP-active drugs indicates that the cAMP-PKA system is not important to $NH_4Cl$-mediated exocytosis.

Nevertheless, we cannot rule out the possible effect of the drugs on $NH_4Cl$-mediated response through other mechanisms independent of $H^+$ exchangers. This is supported by the fact the tyrosine kinase inhibitors also inhibit $NH_4Cl$-mediated exocytosis, which indicates that tyrosine phosphorylation is either relevant to signals activated by pHi alkalinization, or that a regulator of pHi is modulated by these enzymes. This effect is external calcium-dependent, again suggesting a role for plasma membrane components, since internal calcium does not sustain the inhibition. On the other hand, $NH_4Cl$-mediated response is independent of external calcium, hence suggesting that the role of the calcium-dependent PKC isozymes is to negatively modulate the activation, but also that it is not necessary for the process.

On the other hand, the inhibition of $PI_3K$ is internal calcium-dependent. In mast cells, $PI_3K$ induces PLCγ activation [31], and an increase of antigen-induced response and $Ca^{2+}$ entry [31,32]. However, the $PI_3K$-induced increase of the $Ca^{2+}$ entry seems to be independent of depletion of intracellular $Ca^{2+}$ stores in this cellular model [31]. Although the GPCR-derived signals from $PI_3K$ have not been identified [33], the results suggest that the effect of wortmannin in $NH_4Cl$-mediated exocytosis takes place by a mechanism solely dependent on internal cal-

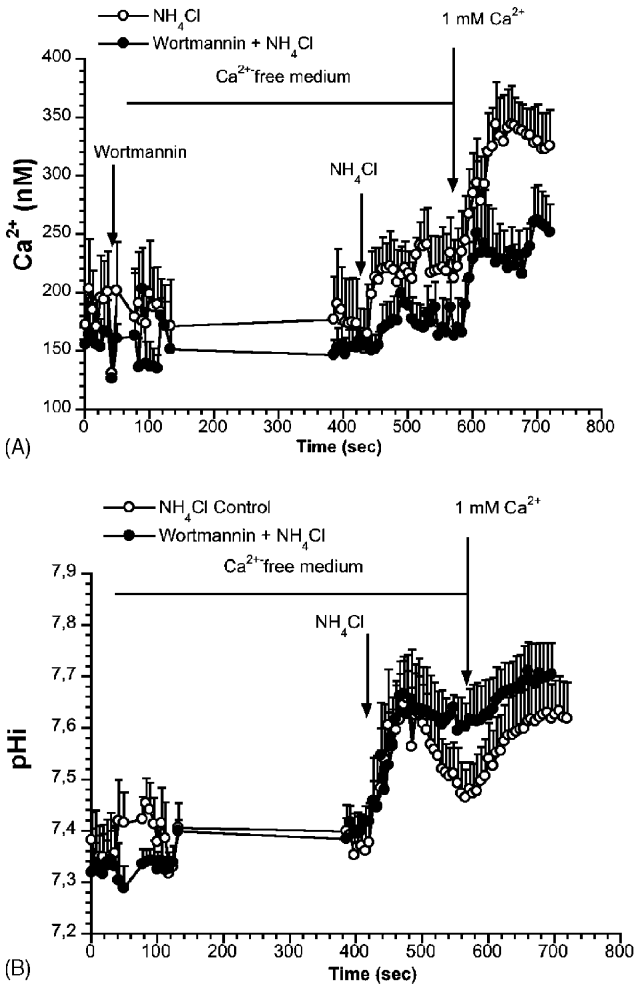


Fig. 7. Effect of addition of 1 mM $Ca^{2+}$ on cytosolic calcium (A) and pHi (B) levels, in 10 nM wortmannin-treated rat mast cells after addition of 20 mM $NH_4Cl$ in a $Ca^{2+}$-free medium. Mean ± S.E.M. of three experiments.

cium. The observation that EGTA inhibits the response to suboptimal concentrations of $NH_4Cl$, and that BAPTA increases the effect of $NH_4Cl$ suggests that calcium is not only unnecessary to trigger cell activation, but also that it may be a negative modulator of $NH_4Cl$-mediated exocytosis. This further supports the conclusion shown above that calcium-dependent PKC isozymes negatively modulate the activation.

Cytosolic [$Ca^{2+}$] and [pHi] were studied in the absence of calcium, in order to see the effect of $NH_4Cl$ on internal calcium reservoirs. The posterior addition of 1 mM external calcium provides insights about the regulation of calcium influx on the plasma membrane. The data show that the chelation of internal calcium changes $NH_4Cl$-mediated histamine release. This is rather important, since even in the complete absence of calcium changes (Fig. 5B) $NH_4Cl$ is able to induce histamine release. Therefore, the relevance of the release of internal calcium caused by $NH_4Cl$ is unclear. Those drugs that inhibit the release of histamine induced by $NH_4Cl$ do ablate this internal calcium release. This pattern is further supported by the fact that those drugs that do not inhibit $NH_4Cl$-mediated histamine release (i.e. cAMP-drugs) do not change $NH_4Cl$-mediated calcium release. Nevertheless, the fact that $NH_4Cl$-mediated release can still take place in the absence of any change in cytosolic calcium, indicates that the release of calcium is a process that takes place downstream $NH_4Cl$-activation, and is modulated by kinase signals. The role therefore of this modulation is unknown.

The second aspect to discuss is the pH signal itself. The effect of $NH_4Cl$ on histamine release is triggered by a transient elevation on cytosolic pH, which is simultaneous to an elevation on cytosolic calcium. As demonstrated elsewhere [1], the elevation of calcium, with no change in pHi is an insufficient signal for degranulation. The results show also that external calcium is irrelevant for this activatory mechanism. The initial pHi peak is modulated by those drugs that inhibit the release of histamine elicited by $NH_4Cl$. Nevertheless, at this point we can not establish if the profile of this pHi peak is relevant or not to the activatory mechanism of $NH_4Cl$, since Gf does barely modify the profile of pHi, but it does show a clear calcium-dependent inhibition of histamine release. Although much remains to be studied, it is clear that pHi can be considered a signal by itself, and that this signal shows a higher priority than calcium.

On the other hand, EGTA ablates the second alkalinization that takes place after addition of external calcium (Fig. 5D), and this matches the lower release of histamine in the presence of EGTA. It is not clear the role of this second alkaline peak after addition of external calcium, but it is related to the optimal cell response. In fact, those drugs that eliminate this second alkalinization (chelerythrine, wortmannin) also inhibit $NH_4Cl$-mediated histamine release. There is a clear relationship between $NH_4Cl$-mediated calcium release and histamine release, since those drugs that inhibit this release also inhibit $NH_4Cl$-mediated histamine release. The mechanism to this effect remains to be investigated.

The most logical explanation to this second alkaline peak, which is caused after addition of external calcium, is the activation of a $Ca^{2+}$–$H^+$ exchange ($Ca^{2+}$ influx, $H^+$ efflux). The pathways that are affected remain to be investigated. The first alkaline peak, which shows a clear and sharp profile, seems to be connected to the activation mechanism. The recovery of initial pHi values on this first pHi peak can be caused by a $Na^+$-independent $Cl^-$–$CO_3H^-$ mechanism, which mediates acid loads to the cells [2,34]. The results suggest that alkaline pHi is a signal modulated by many mechanisms, but still we do not know the exact target to alkaline pHi dependent activation.

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