Journal of Cellular Biochemistry 99:1651–1663 (2006)

PKC and cAMP Positively Modulate Alkaline-Induced Exocytosis in the Human Mast Cell Line HMC-1

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Abstract We study in HMC-1 the activation process, measured as histamine release. We know that ammonium chloride (NH_4Cl) and ionomycin release histamine, and the modulatory role of drugs targeting protein kinase C (PKC), adenosine 3′,5′-cyclic monophosphate (cAMP), tyrosine kinase (TyrK) and phosphatidylinositol 3-kinase (PI3K) on this effect. We used Gö6976 (100 nM) and low concentration of GF 109203X (GF) (50 nM) to inhibit Ca^{2+}-dependent PKC isozymes. For Ca^{2+}-independent isozymes, we used 500 nM GF and 10 μM rottlerin (specifically inhibits PKCδ). Phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) was used to stimulate PKC, and genistein (10 μM) and lavendustin A (1 μM) as unspecific TyrK inhibitors. STI571 10 μM was used to specifically inhibit the activity of Kit, the receptor for stem cell factor, and 10 nM wortmannin as a PI3K inhibitor. Activation of PKC with PMA enhances histamine release in response to NH_4Cl and ionomycin. PMA increases NH_4Cl-induced alkalinization and ionomycin-induced Ca^{2+} entry. Inhibition of PKCδ strongly inhibits Ca^{2+} entry elicited by ionomycin, but failed to modify histamine release. The effect of cAMP-active drugs was explored with the adenylate cyclase activator forskolin (30 μM), the inhibitor SQ22,536 (1 μM), the cAMP analog dibutyryl cAMP (200 μM), and the PKA blocker H89 (1 μM). Forskolin and dibutyryl cAMP do increase NH_4Cl-induced alkalinization, and potentiate histamine release elicited by this compound. Our data indicates that alkaline-induced exocytosis is modulated by PKC and cAMP, suggesting that pH could be a modulatory signal itself. J. Cell. Biochem. 99: 1651–1663, 2006.

Key words: mast cells; HMC-1 cells; cytosolic pH; cytosolic calcium; ammonium chloride; ionomycin; calcium pools; histamine release

Mast cells are an inflammatory model of non-excitable cells suitable to study the intracellular signaling pathways that modulate secretion. Therefore, mast cell lines are commonly used in studies of mast cell biology. Up to now, two human mast cell lines have been described, LAD 1/2 and HMC-1 cells [Butterfield et al., 1988; Kirshenbaum et al., 2003], but only the second has been widely used and characterized in the last years. We have previously described the activation process in HMC-1 cells, measured as histamine release. We have demonstrated that exocytosis can be triggered in this cellular model with just an intracellular alkalinization and no Ca^{2+} increase [Pernas-Sueiras et al., 2005b]. We have also reported that the NH_4Cl-induced intracellular alkalinization negatively modulates cytosolic Ca^{2+} levels, probably by activation of Ca^{2+} efflux through the plasma membrane Ca^{2+}/H^+-ATPase [Pernas-Sueiras et al., 2005a]. In the present study, we explore how different kinase pathways modulate histamine release elicited by two well-known stimuli such as the Ca^{2+}-ionophore ionomycin and NH_4Cl, two drugs that we have used for our previous works, and that have also been reported to release histamine in different cellular models [Hsu and MacGlashan, 1996; Teofoli et al., 1999; Alfonso et al., 2005; Pernas-Sueiras et al., 2005b].

PKC is a family of ser/thr kinases with different isoforms, usually classified depending on their sensitivity to Ca^{2+} and phorbol esters: (A) Ca^{2+}-dependent isozymes (α, β, and γ) that are activated by PMA; (B) Ca^{2+}-independent isozymes (δ, ϵ, η, μ, and θ) also activated by PMA; and (C) atypical isozymes (ζ, λ, and ι), which are Ca^{2+}-independent and

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Received 16 February 2006; Accepted 1 May 2006
DOI 10.1002/jcb.21009

PMA-unresponsive. Several differences have been described between different types of mast cells concerning the role of PKC on degranulation. In RBL-2H3 cells, PKC β and δ seem to be the isoforms responsible for exocytosis, although these cells are also endowed with the isoforms α, ϵ, and ζ. [Ozawa et al., 1993a,b; Chang et al., 1997]. PMA alone does not induce exocytosis in RBL-2H3 cells, in opposition to what is observed in serosal rat mast cells [Beaven et al., 1987; Wolfe et al., 1996]. In a previous work we have found that activation of PKC with PMA alone does not activate histamine release in HMC-1 cells [Pernas-Sueiras et al., 2005a]. In mast cells, PKC plays a primordial role on degranulation, since it has been reported to increase exocytosis elicited by stimuli such as thapsigargin (TG) or Ca^{2+} ionophores. On the other hand, it has also been described an inhibitory effect of PKC on compound 48/80-induced histamine release in rat mast cells [Katakami et al., 1984; Heiman and Crews, 1985; White and Zembryki, 1989; Chakravarty et al., 1990; Botana et al., 1992; Alfonso et al., 1994b].

PKA is also a ser/thr kinase, which is activated by increased cAMP cytosolic levels. There are data reporting some relationship between cAMP and intracellular Ca^{2+} in different cellular models [Yoshii et al., 1988], and in general, histamine release is correlated with increased intracellular Ca^{2+} and decreased cAMP levels [Izushi and Tasaka, 1989; Botana and MacGlashan, 1994; Takei and Endo, 1994]. However, up to now, no specific substrates for PKA have been described in mast cells.

TyrK have been considered as an important step for the cell response to different receptor-mediated stimuli, including FcϵRI, since it has been reported that TyrK inhibitors might inhibit immunological activation in mast cells [Kawakami et al., 1992]. An important TyrK present in HMC-1 cells is the receptor for stem cell factor, Kit. The activity of this receptor is very important in this cellular model, since different activating mutations in the *c-kit* proto-oncogene (that codifies the Kit receptor) account for a stem cell factor-independent growth of HMC-1 cells.

In the present study, we study the influence of several kinase pathways on histamine release elicited by ionomycin and NH_4Cl in HMC-1 cells.

METHODS

Chemicals

Ammonium chloride (NH_4Cl) was from Panreac (Barcelona, Spain); rottlerin, ionomycin, Gö6976, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22,536), forskolin, H89, GF 109203X, genistein and lavendustin A were from Alexis Corporation (Läufelfingen, Switzerland); 2,7-bis (carboxyethyl)-5(6)carboxy-fluorescein-acetoxymethylester (BCECF-AM) and FURA-2 AM were from Molecular Probes (Leiden, The Netherlands); Phorbol 12-myristate 13-acetate (PMA), wortmannin and dibutyryl cAMP were from Sigma-Aldrich (Madrid, Spain). STI571 was provided by Dr. Luis Escribano Mora (Servicio de Hematología, Hospital Ramón y Cajal, Madrid).

Cell Cultures

HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove′s modified Dulbecco′s medium (IMDM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were expanded weekly.

Cell Preparation

For histamine release assays, cells were centrifuged (1,500 rpm, 5 min, 4°C) and washed twice with saline solution (1,000 rpm, 5 min, 4°C). The composition of this solution was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2; SO_4^{2-} 1.2; glucose 1 g/L.

For Ca^{2+} and pH measurements cells were treated in the same conditions, but washed in saline solution plus 0.1% bovine serum albumin (BSA).

The incubation medium was equilibrated with CO_2 prior to use, in all the experiments the pH (7.4) was maintained constant by bubbling CO_2 during the experiment. All experiments were carried out at least three times, by duplicate, both for histamine release assays and also Ca^{2+} and pH measurements.

Cell Incubation

Freshly prepared concentrated solution (6.25 μl) of each drug were added to the incubation medium to attain a final volume of 150 μl and preincubated. When the medium reached 37°C, 100 μl of a cell suspension with an

approximate density of $1.5–2 \times 10^6$ cells/ml were 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 a cold bath. After centrifugation at 2,300 rpm for 5 min, the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in every experiment.

Histamine Release Assays

Histamine release was tested with a spectrofluorometer, (Fluoroskan II, Labsystems, Barcelona, Spain) both in pellet (non-released histamine) and supernatants (released histamine) according to Shore's method [Shore, 1971]. However, 0.1% orthophthaldialdehyde was employed, and also trichloroacetic acid (7%) was used to avoid protein interferences in the histamine release determination. To ensure total histamine, pellets were sonicated for 60 s in 0.2 ml of 0.1 N HCl. In all the NH_4Cl experiments, histamine released was measured only in pellets, because this compound interferes with fluorescent complex. Results shown are expressed as the percentage of histamine released from the total histamine content.

Measurement of Cytosolic Free Ca^{2+} and Intracellular pH

HMC-1 cells were loaded with FURA-2 AM (0.2 μM) and BCECF-AM (0.05 μM) at 37°C, for 10 min. After this time, loaded cells were washed with saline solution (2,300 rpm, 5 min, 4°C). Cells were allowed to attach to 22-mm glass coverslips treated with poly-L-lysine, and the coverslips were inserted into a thermostated chamber (Life Sciences Resources, UK). Cells were viewed using a Nikon Diaphot 200 microscope equipped with epifluorescence optics (Nikon 40x—immersion UV—Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Intracellular pH and cytosolic Ca^{2+} concentration were obtained from the images collected by a fluorescence equipment (Life Sciences Resources, UK). The light source was a 175 W xenon lamp, and the different wavelengths used were selected with filters. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm; for BCECF-AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration of the fluorescence values versus intracellular Ca^{2+} was made according to the method of Grynkiewicz [Grynkiewicz et al., 1985]. The calibration of the fluorescence values versus pH was made as per [Thomas et al., 1979]. In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^+ solution, for each pH value.

Statistical Analysis

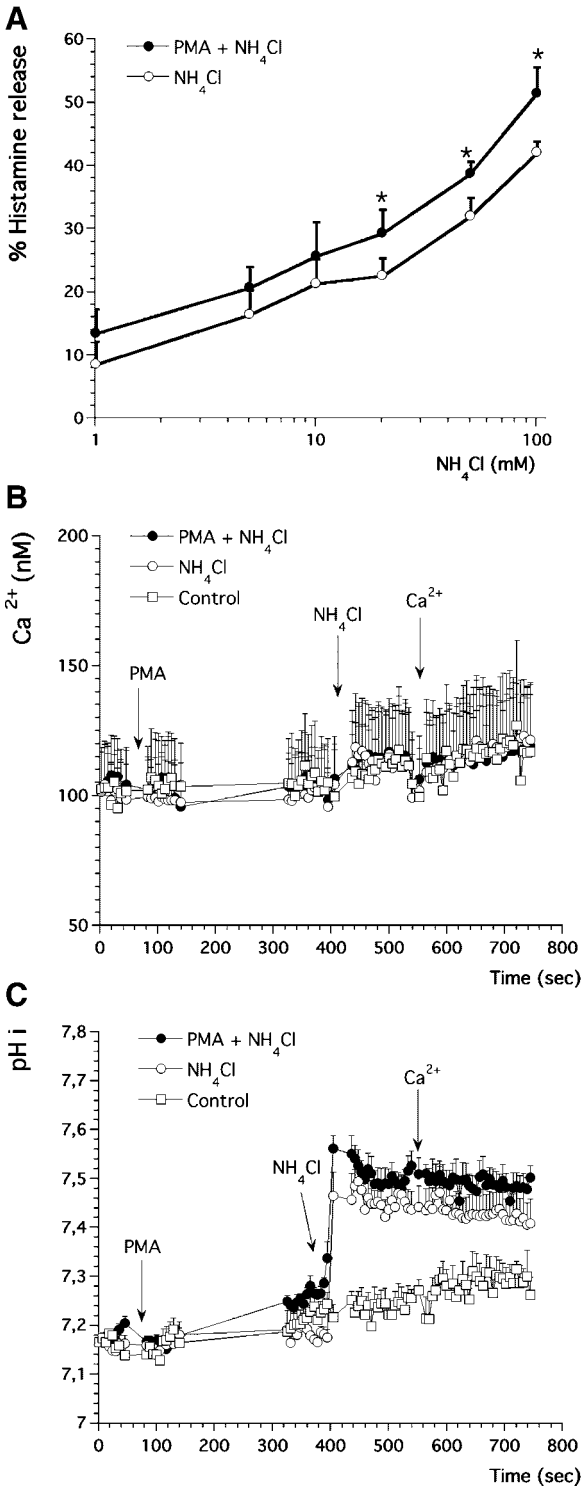
Results were analyzed using the Student's *t*-test for unpaired data. A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM.

RESULTS

Different intracellular signaling pathways were checked to address their possible influence on the exocytosis process, activated by NH_4Cl and ionomycin, in HMC-1 cells. Figure 1 shows the results obtained in experiments carried out with the PKC activator PMA (100 ng/ml) and NH_4Cl. Figure 1A shows that preincubation of the cells with PMA significantly increases NH_4Cl-induced histamine release, reaching maximum response values of over 50% for PMA-treated cells. For the three highest concentrations of NH_4Cl, the average PMA-induced enhancement of histamine release is approximately 22%. In the same conditions, no effect on cytosolic Ca^{2+} was observed in these cells, since preincubation of the cells with PMA prior to addition of NH_4Cl showed no effect of this drug on Ca^{2+} levels (Fig. 1B). However, and still in the same conditions, PKC activation with PMA prior to addition of NH_4Cl does increase NH_4Cl-induced alkalinization, as shown in Figure 1C. After the addition of NH_4Cl to the cells, intracellular pH immediately increases to reach a maximum peak, and then values remain elevated over control cells during all the experiment. PMA-treated cells showed an initial peak that is significantly higher (0.1 pH units) when compared to cells only treated with NH_4Cl. Values obtained for PMA-treated cells remain higher during all the experiment, although differences with cells treated only with NH_4Cl are not significant after the initial moments.

We next studied the effect on histamine release of different compounds that have been

widely used as PKC inhibitors. In order to inhibit Ca^{2+}-dependent isozymes, we used 100 nM Gö6976 [Martiny-Baron et al., 1993; Qatsha et al., 1993; Behrens et al., 1999] and also 50 nM GF [Toullec et al., 1991; Alfonso et al., 2005]: in both cases, preincubation of HMC-1 cells with the inhibitor did not show any effect on the later NH_4Cl-induced histamine release (Table I). For Ca^{2+}-independent PKC isoforms, we used 500 nM GF, that also failed to inhibit NH_4Cl-induced exocytosis (Table I). Since PKCδ has been proposed as an important step for mast cells degranulation [Vilarino et al., 2001; Cho et al., 2004], we have also included rottlerin in our study, to specifically inhibit this PKC isoform [Gschwendt et al., 1994; Alfonso et al., 2005]. This compound showed no effect on NH_4Cl-induced histamine release (Table I).



In previous studies carried out with rat mast cells, our group has observed that inhibition of TyrK or PI3K had a negative effect on the response of cells to NH_4Cl [Alfonso et al., 2005]. We thus checked the effect of two unspecific TyrK inhibitors (such as 10 μM genistein and 1 μM lavendustin A), STI571 (10 μM), that inhibits the kinase activity of the Kit receptor present in HMC-1 cells [Buchdunger et al., 2000; Akin et al., 2003], and also 10 nM wortmannin, that was found to impair NH_4Cl-induced exocytosis in rat mast cells [Alfonso et al., 2005]. All these compounds failed to cause any change on NH_4Cl-induced histamine release in HMC-1 cells, after a 10 min preincubation with the inhibitor prior to addition of the stimulus (Table I).

Finally, we studied how PKA and cAMP might affect the action of NH_4Cl on HMC-1 cells, since a relationship between histamine release and cAMP levels has been proposed by several authors [Alm, 1984; Izushi and Tasaka, 1989; Botana and MacGlashan, 1994; Takei and Endo, 1994]. Preincubation of the cells with the adenylate cyclase activator forskolin (30 μM) resulted in a significant enhancement of the histamine release triggered by NH_4Cl (Fig. 2A). For all the NH_4Cl concentrations checked, an important potentiation elicited by forskolin was observed, always increasing response over 25%

Fig. 1. Effect of PMA plus NH_4Cl on histamine release, cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells. **A**: Dose-response of histamine released in the presence of NH_4Cl in saline solution. Cells were preincubated (closed circles) or not (open circles) with 100 ng/ml PMA. Significant differences are marked with an asterisk. **B**: Cytosolic Ca^{2+} profile in cells stimulated with 100 ng/ml PMA plus 50 mM NH_4Cl. First arrow indicates the addition of PMA, second indicates the addition of NH_4Cl, and third indicates that extracellular Ca^{2+} is restored. **C**: Intracellular pH profile in cells subject to the protocol described in (B). Mean ± SEM of three experiments (approximately 45 cells/single experiment).

TABLE I. Summary of Effects of Different Kinase Pathways on NH_4Cl-Induced Histamine Release

Kinase family	Drug	Effect on kinase	Effect on histamine release
PKC	PMA 100 ng/ml	Unspecific activation	Increases release
	Gö6976 100 nM	Inhibition of Ca^{2+}-dependent isozymes	No effect
	GF102903X 50 nM	Inhibition of Ca^{2+}-dependent isozymes	No effect
	GF102903X 500 nM	Inhibition of Ca^{2+}-independent isozymes	No effect
	Rottlerin 10 μM	Inhibition of PKCδ	No effect
TyrK	Genistein 10 μM	Unspecific inhibition	No effect
	Lavendustin 1 μM	Unspecific inhibition	No effect
	STI571 10 μM	Inhibition of Kit receptor	No effect
PI3K	Wortmannin 10 nM	Unspecific inhibition	No effect

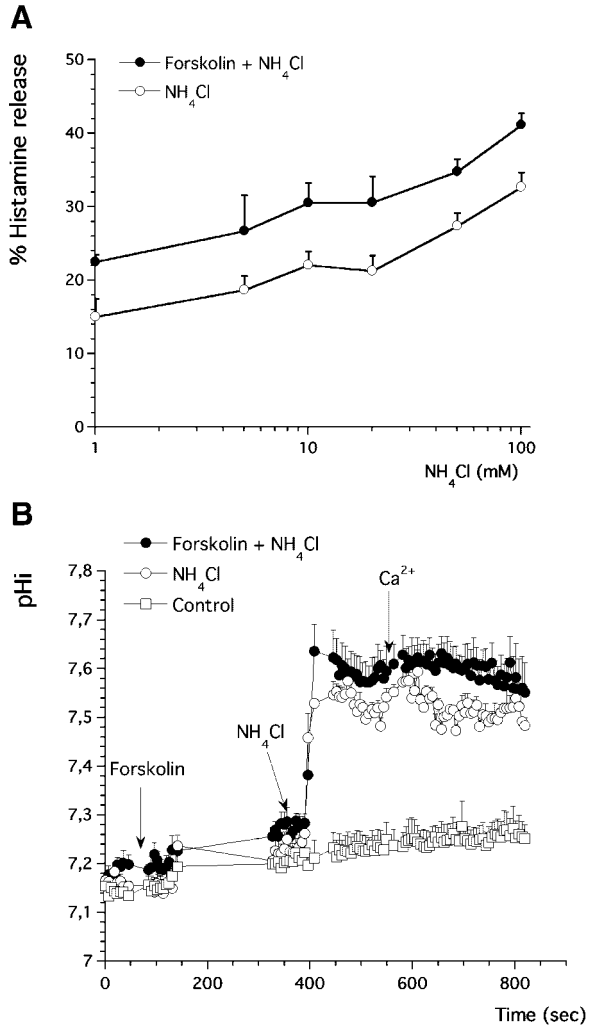


Fig. 2. Effect of forskolin plus NH_4Cl on histamine release and intracellular pH in HMC-1 cells. **A**: Dose-response of histamine released in the presence of NH_4Cl in saline solution. Cells were preincubated (closed circles) or not (open circles) with 30 μM forskolin. **B**: Intracellular pH profile in cells stimulated with 30 μM forskolin plus 50 mM NH_4Cl. First arrow indicates the addition of forskolin, second indicates the addition of NH_4Cl, and third indicates that extracellular Ca^{2+} is restored. Mean ± SEM of four experiments (approximately 45 cells/single experiment).

when compared with cells only treated with NH_4Cl. Average of increases for the different concentrations is approximately 38%. As shown in Figure 2B, this increased histamine release in forskolin-treated cells matches with an increase on intracellular alkalinization induced by NH_4Cl. After the addition of this drug to the cells, intracellular pH immediately increases reaching at first a peak, and then values remain elevated over control cells during all the experiment. Cells only treated with NH_4Cl reached a significantly lower peak (about 0.1 pH units less) than those that have been preincubated with forskolin. After this initial moment, values also remain more elevated in forskolin-treated cells than in cells only treated with NH_4Cl. In order to confirm this positive modulation observed in Figure 2, we carried out experiments with the cAMP analog dibutyryl cAMP (200 μM): results are shown in Figure 3. As per forskolin, preincubation of HMC-1 cells with dibutyryl cAMP resulted in an increase of both histamine release (Fig. 3A) and intracellular alkalinization induced by NH_4Cl (Fig. 3B). For all the NH_4Cl concentrations tested, preincubation of the cells with dibutyryl cAMP resulted in an enhanced histamine release (average of increase for all the concentrations is approximately 34%). This effect matches with a potentiation of NH_4Cl-induced intracellular alkalinization: pH profile in cells preincubated with dibutyryl cAMP (Fig. 3B) is similar to that presented in Figure 2B, with an increased initial peak (about 0.1 pH units higher) for dibutyryl cAMP-treated cells. It is important to mention that control experiments carried out with forskolin or dibutyryl cAMP alone showed no changes on intracellular pH due to these compounds.

On the other hand, in experiments carried out with HMC-1 cells, the PKA blocker H89 (1 μM)

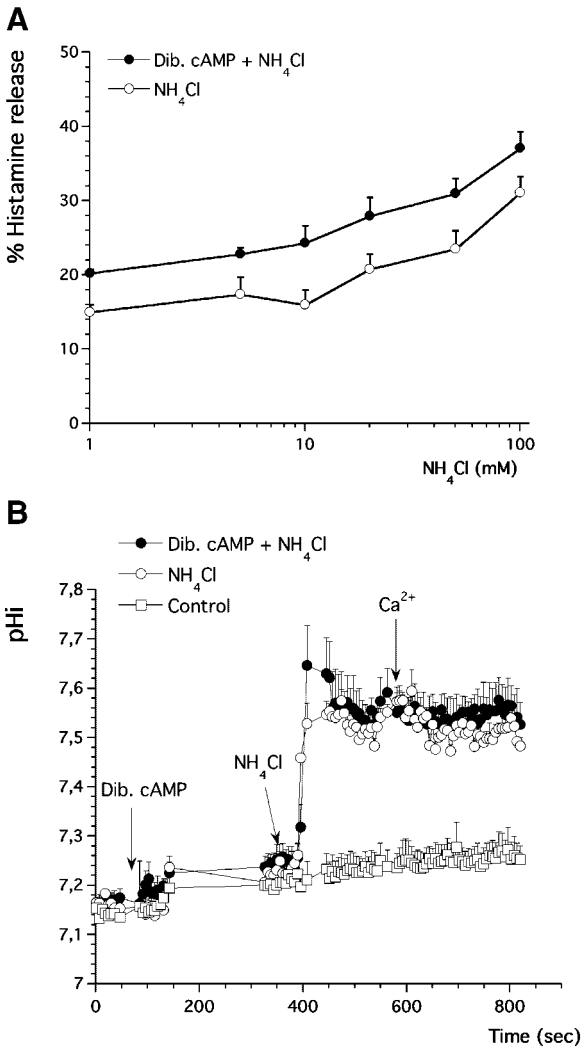


Fig. 3. Effect of dibutyryl cAMP plus NH_4Cl on histamine release and intracellular pH in HMC-1 cells. **A**: Dose-response of histamine released in the presence of NH_4Cl in saline solution. Cells were preincubated (closed circles) or not (open circles) with 200 μM dibutyryl cAMP. **B**: Intracellular pH profile in cells stimulated with 200 μM dibutyryl cAMP plus 50 mM NH_4Cl. First arrow indicates the addition of dibutyryl cAMP, second indicates the addition of NH_4Cl, and third indicates that extracellular Ca^{2+} is restored. Mean ± SEM of three experiments (approximately 40 cells/single experiment).

and the adenylate cyclase inhibitor SQ22,536 (1 μM) failed to affect NH_4Cl-induced histamine release (data not shown).

So far, data presented in this study show that NH_4Cl-induced alkalinization, and subsequent histamine release, can be modulated by different intracellular kinase pathways. To check if these effects could be also observed in the case of other stimuli that use different mechanism of action, we carried out experiments with ionomycin, which affects Ca^{2+} signaling.

It is a first remarkable finding that none of the cAMP active drugs employed with ionomycin (H89, SQ22,536, forskolin and dibutyryl cAMP) showed any effect on ionomycin-induced histamine release (Table II). However, other kinase pathways studied did show significant effects on the degranulation process elicited by the ionophore. Figure 4 shows the results obtained in experiments with the PKC activator PMA (100 ng/ml) and ionomycin. After a 10 min preincubation with PMA, histamine release elicited by ionomycin is clearly enhanced, until that values reach a plateau at a maximum response of approximately 20% (Fig. 4A). Figure 4B presents the Ca^{2+} profile obtained with PMA and ionomycin. In cells only treated with ionomycin, there is a first increase on cytosolic Ca^{2+} that can be attributed to the emptying of intracellular Ca^{2+} pools, since in that moment cells are placed in a Ca^{2+}-free medium. After extracellular Ca^{2+} is restored to the medium, there is another increase of this ion in the cytosol, which can be attributed to extracellular Ca^{2+} influx. In cells that have been preincubated with PMA, the ionomycin-induced Ca^{2+} stores emptying takes place normally, and no difference can be observed when compared to cells only treated with ionomycin. However, preincubation with PMA significantly increases extracellular Ca^{2+} entry elicited by ionomycin, when this ion is restored to the medium. PMA-treated cells reach values that are about 100 nM Ca^{2+} higher than those observed in cells only treated with ionomycin.

Different PKC inhibitors were also checked with ionomycin: Gö6976, and both low and high GF concentrations, failed to modify ionomycin-induced histamine release and also did not affect to Ca^{2+} changes induced by the ionophore (Table II). Results obtained with the PKCδ inhibitor rottlerin are presented in Figure 5. Histamine released in response to ionomycin is not affected by preincubation of the cells with rottlerin (Fig. 5A). However, Ca^{2+} profile elicited by the ionophore is dramatically modified by rottlerin, as Figure 5B addresses. After preincubation of the cells with rottlerin, ionomycin-induced emptying of Ca^{2+} stores takes places normally. However, when this ion is restored to the medium, rottlerin-treated cells show a nearly complete abolition of extracellular Ca^{2+} entry, that contrast with values of cytosolic Ca^{2+} of about 500 nM in cells only treated with ionomycin. Values of

TABLE II. Summary of Effects of Different Kinase Pathways on Ionomycin-Induced Histamine Release

Drug	Kinase family: Effect on kinase	Effect on histamine release
cAMP Pathway		
Forskolin 30 μM	Activation of adenylate cyclase	No effect
Dibutyryl cAMP 200 μM	cAMP analog	No effect
SQ22,536 1 μM	Inhibition of adenylate cyclase	No effect
H89 1 μM	Blockage of PKA	No effect
PKC		
PMA 100 ng/ml	Unspecific activation	Increases release
Gö6976 100 nM	Inhibition of Ca^{2+}-dependent isozymes	No effect
GF102903X 50 nM	Inhibition of Ca^{2+}-dependent isozymes	No effect
GF102903X 500 nM	Inhibition of Ca^{2+}-independent isozymes	No effect
Rottlerin 10 μM	Inhibition of PKCδ	No effect

rottlerin-treated cells are close to 100 nM and are not significantly different from those obtained for control cells (no drugs added). Interestingly, Figure 5C shows that rottlerin does not modify the slight alkalinization elicited by ionomycin.

DISCUSSION

Data shown in this study confirm our previous findings about exocytosis in HMC-1 cells: an intracellular alkalinization is a sufficient signal to promote histamine release (even in the complete absence of Ca^{2+} changes) while an increase in cytosolic Ca^{2+} is not [Pernas-Sueiras et al., 2005b]. Results presented in this work indicate that alkalinization-induced exocytosis in HMC-1 cells is modulated by several intracellular pathways.

In this work we have tried to study how the modification of different kinase pathways would affect Ca^{2+} concentration, pH profile and histamine release elicited by two stimuli with different mechanism of action: ionomycin and NH_4Cl. We have chosen NH_4Cl because it is a useful tool to promote an intracellular pH increase, without affecting cytosolic Ca^{2+} levels [Pernas-Sueiras et al., 2005b]. Thus, this drug allows us to directly study the relationship between intracellular pH and histamine release. On the other hand, ionomycin causes

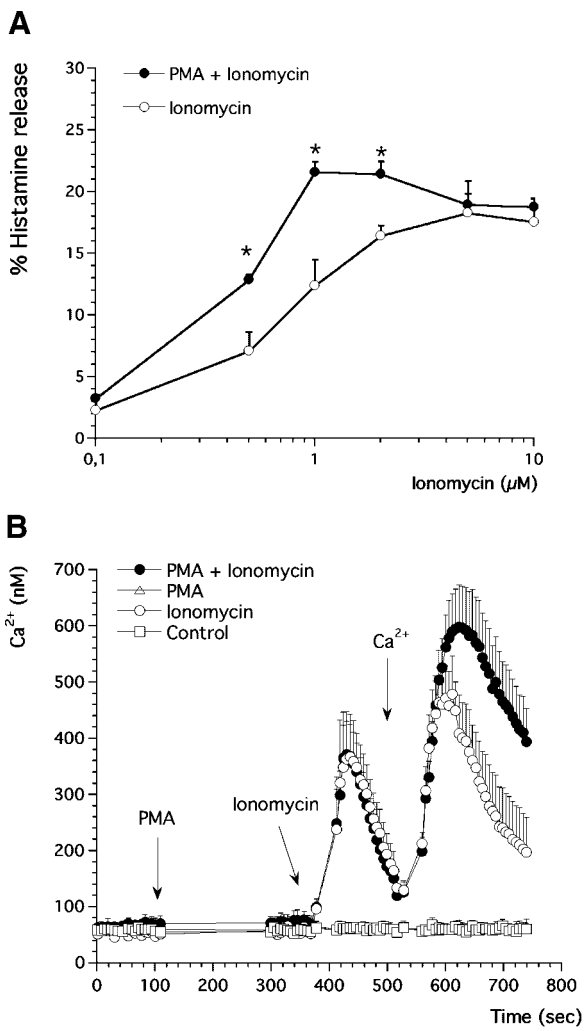


Fig. 4. Effect of PMA plus ionomycin on histamine release and cytosolic Ca^{2+} concentration in HMC-1 cells. **A**: Dose-response of histamine released in the presence of ionomycin in saline solution. Cells were preincubated (closed circles) or not (open circles) with 100 ng/ml PMA. Significant differences are marked with an asterisk. **B**: Cytosolic Ca^{2+} profile in cells stimulated with 100 ng/ml PMA plus 0.1 μM ionomycin. First arrow indicates the addition of PMA, second indicates the addition of ionomycin, and third indicates that extracellular Ca^{2+} is restored. Mean ± SEM of four experiments (approximately 45 cells/single experiment).

a slight intracellular alkalinization, but also a very important cytosolic Ca^{2+} increase [Pernas-Sueiras et al., 2005b].

In this study we have presented data pointing to an important influence of PKC on histamine release, Ca^{2+} signaling and pH regulation in HMC-1 cells. The role of PKC on the modulation of Ca^{2+} entry is still controversial. While some authors describe an inhibition of Ca^{2+} entry by PKC [Baranska et al., 1995; Shibata et al., 1996; Haverstick et al., 1997; Lee et al., 1997], other works describe a dual regulation [Petersen and Berridge, 1994; Petersen and Berridge, 1995], or even no effect of PKC on Ca^{2+} entry [Wolfe et al., 1996; Scott et al., 1998]. In RBL-2H3 cells, contradictory results have also been published about this issue. In some works, PMA has been described to inhibit Ca^{2+} entry [Parekh and Penner, 1995; Kuchtey and Fewtrell, 1999] while, in others, it has been proposed that PKC had no influence on Ca^{2+} entry [Wolfe et al., 1996; Scott et al., 1998]. In a previous work, we have observed that PKC stimulation with PMA increased TG-induced store-operated Ca^{2+} entry in HMC-1 cells, without affecting internal stores depletion. PMA alone did not induce changes on internal Ca^{2+} [Pernas-Sueiras et al., 2005a]. Data presented in this study, in experiments using ionomycin, revealed that PMA also enhances ionomycin-induced Ca^{2+} entry. We can rule out a direct activation of Ca^{2+} entry by PMA, since this drug was not found to have any effect on Ca^{2+} levels when acting alone, and also because in the present study we have found that NH_4Cl still does not induce Ca^{2+} entry after preincubation of the cells with PMA. It seems clear that, in HMC-1 cells, the positive modulation of PKC promotes an enhancement of TG- and ionomycin-induced Ca^{2+} entry, although more studies should be carried out to determine how this effect is taking place.

It is also a very remarkable finding that inhibition of PKCδ with rottlerin resulted in a dramatic reduction (or even abolition) of ionomycin-induced Ca^{2+} entry, but no effect was observed on ionomycin-induced alkalinization. A similar puzzling result has already been

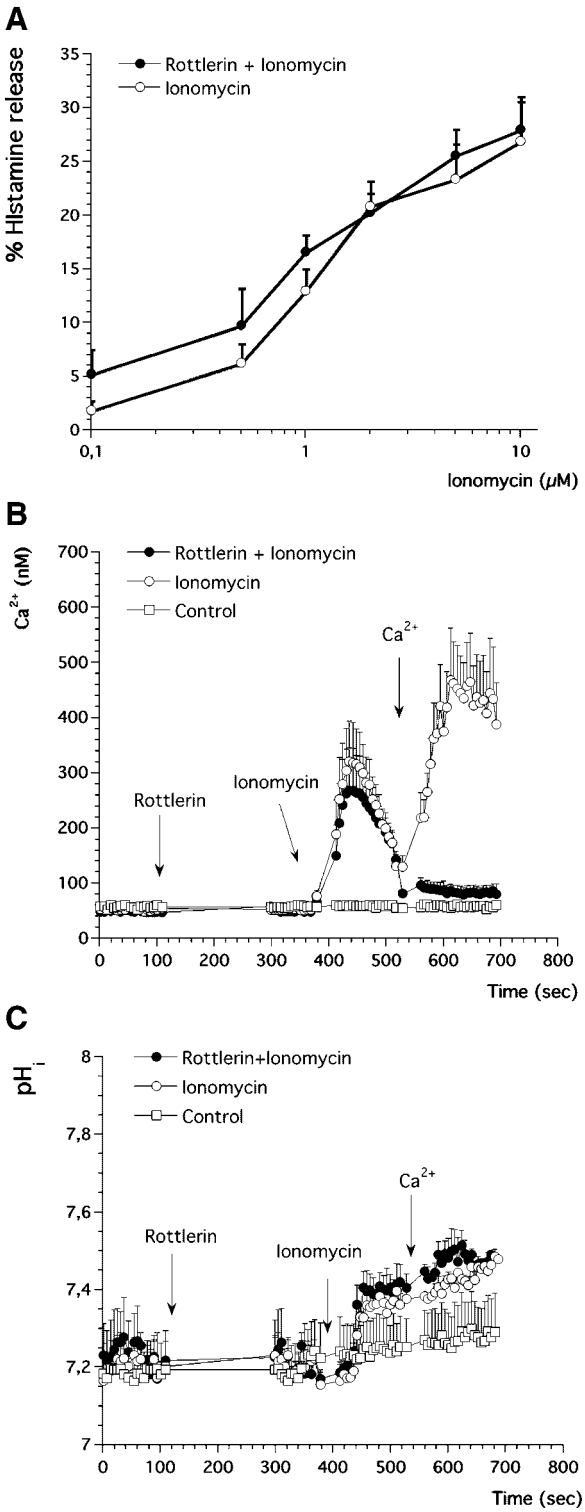


Fig. 5. Effect of rottlerin plus ionomycin on histamine release, cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells. **A**: Dose-response of histamine released in the presence of ionomycin in saline solution. Cells were preincubated (closed circles) or not (open circles) with 10 μM rottlerin. **B**: Cytosolic Ca^{2+} profile in cells stimulated with 10 μM rottlerin plus 0.1 μM ionomycin. First arrow indicates the addition of rottlerin, second indicates the addition of ionomycin, and third indicates that extracellular Ca^{2+} is restored. **C**: Intracellular pH profile in cells subject to the protocol described in (B). Mean ± SEM of four experiments (approximately 45 cells/single experiment).

described in rat mast cells, where rottlerin was found to inhibit Ca^{2+} entry in a bicarbonate-free- but, interestingly, not in a bicarbonate-buffered medium [Vilarino et al., 2001]. Previous findings in HMC-1 cells, where Ca^{2+} profiles obtained for certain experiments were the same in solutions with or without bicarbonate [Pernas-Sueiras et al., 2005a], and also the fact that experiments described above were carried out in physiological solution containing this ion, seem to indicate that this might be another difference between rat mast cells and HMC-1 cells. It is very important to bear in mind that, even when ionomycin-induced Ca^{2+} entry has been nearly abolished by rottlerin, histamine release evoked by the ionophore takes place normally, since rottlerin does not modify the effect of ionomycin on intracellular pH. This observation contrast with previous findings in RBL-2H3 cells, where a primordial role on degranulation has been proposed for PKCδ, because rottlerin was able to inhibit ionophore-induced exoytosis [Cho et al., 2004]. Again, it is observed that there are differences between HMC-1 cells and other mast cells types. Also, the fact that ionomycin triggers histamine release with nearly no extracellular Ca^{2+} entry is another evidence that supports our hypothesis that Ca^{2+} signaling is a secondary signal to activate HMC-1 cells. Further experiments should be performed to deeper understand how rottlerin inhibits in such a dramatic way Ca^{2+} entry.

Relationship between PKC and intracellular pH was also studied in this study, using NH_4Cl as a good tool to induce intracellular pH increases without any changes on cytosolic Ca^{2+}. Since PKC has been described to enhance the activity of the Na^+–H^+ exchanger (an important mechanism for H^+ extrusion) [Grinstein et al., 1985; Alfonso et al., 1994a; Friis and Johansen, 1996; Alfonso et al., 1998], we checked if the stimulation or inhibition of PKC might affect the action of NH_4Cl on HMC-1 cells. PKC activation was found to enhance NH_4-induced alkalinization, an event that matched an increased release of histamine. In rat mast cells, it has been described that inhibition of PKC with the unspecific drug chelerythrine, or the calcium-dependent isoforms with 50 nM GF, resulted in a decrease of histamine release [Alfonso et al., 2005]. In HMC-1 cells, we have found no effect of different PKC inhibitors on NH_4Cl-induced histamine release. However, it is noteworthy that for both stimulus employed in our work, ionomycin and NH_4Cl, the activation of PKC resulted in an enhancement of exocytosis. In the case of NH_4Cl, the increased histamine release matched with a higher intracellular alkalinization, and this could be a possible explanation for that enhancement. This observation is in good agreement with reports that describe a PKC-mediated enhancement of the Na^+-H^+ exchanger activity [Grinstein et al., 1985; Alfonso et al., 1994a, 1998; Friis and Johansen, 1996], since an increased activity of this exchanger might account for a higher NH_4Cl-induced alkalinization and, thus, for an increased histamine release. However, it should be noted that activation of the Na^+–H^+ exchanger has usually been linked to the recovery of an intracellular acidification. Since some considerable differences between other types of mast cells and HMC-1 cells have already been found in our studies, we can speculate that, activation of PKC might be enough to activate the exchanger in this cellular model, but further experiments should be done to deeper understand this issue.

Ionomycin-induced histamine release is also enhanced by PMA. As described above, PMA was found to increase ionomycin-induced Ca^{2+} entry, and this higher cytosolic Ca^{2+} levels might be accounting for the increased histamine release. This fact would be in good agreement with previous findings in HMC-1 cells: we have already demonstrated that preincubation with TG strongly increases ionomycin-induced histamine release, by increasing final cytosolic Ca^{2+} levels, and without affecting the ionomycin-induced alkalinization [Pernas-Sueiras et al., 2005b]. So, it is possible to assume that once that alkalinization has triggered the process, an increased Ca^{2+} entry can enhance the release of histamine by these cells. In a similar way, the higher Ca^{2+} levels reached in the presence of PMA, might explain the enhancement of ionomycin-induced histamine release.

Another remarkable aspect to comment is the influence of cAMP-active drugs on NH_4Cl-induced histamine release. Previous works have proposed an inhibitory role of cAMP on exocytosis [Alm, 1984] and, in general, histamine release is correlated with increased intracellular Ca^{2+} and decreased cAMP levels [Izushi and Tasaka, 1989; Botana and MacGlashan, 1994; Takei and Endo, 1994]. A total lack

of effect of cAMP-active drugs on NH_4Cl-induced histamine release has also been described in rat mast cells, indicating that the cAMP-PKA system would not be important for exocytosis [Alfonso et al., 2005]. On the other hand, release of vascular endothelial growth factor in HMC-1 cells, induced by corticotropin-releasing hormone, is mediated through activation of adenylate cyclase and increased cAMP levels [Cao et al., 2005].

Our results also indicate that cAMP plays a modulatory role on exocytosis in HMC-1 cells. The adenylate cyclase inhibitor SQ22,536 and the PKA blocker H89 showed no effect on NH_4Cl-induced histamine release. But, interestingly, both forskolin and the cAMP analog dibutyryl cAMP were found to clearly enhance histamine release elicited by NH_4Cl. So, at least in the case of HMC-1 cells, cAMP might have a positive modulatory role on NH_4Cl-induced exocytosis, either by activation of PKA or by a direct interaction with a step of the signaling cascade that alkalinization might activate to evoke histamine release. In this sense, a direct interaction between cAMP and intracellular Ca^{2+} pools, without influence of PKA, has already been reported in human T lymphocytes [de la Rosa et al., 2001]. cAMP was also found to activate, independently of PKA, a Ras-like GTPase protein [de Rooij et al., 1998]. Studies investigating the cAMP-binding domains have recently proposed possible mechanisms to explain how cAMP might activate different effector proteins [Bos, 2003; Dodge-Kafka et al., 2005]. Thus, we can not rule out the possibility of any PKA-independent effect of cAMP on intracellular structures, maybe affecting the sensitivity or even modifying the role of an exchanger that could be accounting for alkaline-induced exocytosis. In this sense, influence of cAMP on Na^+, HCO_3^-, and Cl^- fluxes has been reported in different cellular models [Zegarra-Moran et al., 2001; Sheldon and Church, 2002]. cAMP has already been reported to be involved in the regulation of secretory granules pH, and also to enhance Ca^{2+}-induced granule exocytosis by rendering these granules more sensitive to Ca^{2+} [Tompkins et al., 2002; Lee et al., 2005]. Since in different cellular models, evidences showing a clear interaction between intracellular Ca^{2+} stores and cytosolic alkalinization have also been reported [Bankers-Fulbright et al., 2004; Rose et al., 2005], we can not exclude a complex link between these three elements, cAMP, cytosolic Ca^{2+}, and intracellular pH in HMC-1 cells, and further studies will help to understand how this relationship is established.

As described above with PMA, both forskolin and dibutyryl cAMP were found not only to increase histamine release, but also to enhance NH_4Cl-induced intracellular alkalinization. In all the cases, the effect seems to be more marked in the first seconds after NH_4Cl is added, since a higher initial pH peak was observed in cells preincubated with PMA, forskolin or dibutyryl cAMP. pH values also remain higher in these cells during the time course of the experiment, but less differences are seen between them and cells only treated with NH_4Cl. We have described in a previous work that a fast shift to a more alkaline pH, even without an increase over basal pH values, was enough signal to trigger an almost complete degranulation in HMC-1 cells. Furthermore, in experiments where the NH_4Cl-induced alkalinization was impaired, a considerable histamine release could be measured, in combination with only a fast, transient pH peak, after which pH values remain in basal values [Pernas-Sueiras et al., 2005b]. So, it is reasonable to propose that, in this cell line, histamine release is strongly dependent on fast pH changes, specially on the magnitude of the initial pH peak, that seems to be the key step to trigger the response. Furthermore, the positive modulatory role of PKC and cAMP on alkaline-induced exocytosis also seems to be taking place in that initial step.

Further studies should be carried out to determine how this pH increase is linked to exocytosis. The fact that TyrK inhibitors did not show any effect on NH_4Cl-induced histamine release might indicate that tyrosine phosphorylation is not relevant in the signal that alkalinization may initiate. Furthermore, since inhibition of the kinase activity of the Kit receptor by STI571 also showed a lack of effect on NH_4Cl-induced histamine release, it seems that the routes activated by this receptor, including the Ras-MAPK-ERK and the PI3K [Kempna et al., 2004] are not essential for exocytosis in this cellular model. Inhibition of PI3K with wortmannin also failed to affect NH_4Cl-induced histamine release, a fact that further confirms our hypothesis. However, it is necessary to mention that some drugs frequently used to treat patients suffering mast cell diseases (namely, STI571 in some types of

mastocytosis) act by inhibition of TyrK activity. Probably, several differences between doses and treatment times used, and also the fact that is difficult to correlate an in vivo response from a patient with the basic in vitro studies that we develop, might account to explain this apparent contradiction.

In summary, results shown in this work indicate that alkaline-induced exocytosis is modulated by two important signaling pathways, namely PKC and cAMP, a fact that accounts for the importance of pH as a rising signal transduction by itself.

ACKNOWLEDGMENTS

This work was mainly funded with grant FISS REMA-G03-007, and also partially with grants MCYT BMC2000-0441, SAF2003-08765-C03-02, REN2001-2959-C04-03, REN2003-06598-C02-01, INIA CAL01-068, Xunta PGID-T99INN26101, and PGDIDIT03AL26101PR.

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