Calcium-pH Crosstalks in the Human Mast Cell Line HMC-1: Intracellular Alkalinization Activates Calcium Extrusion Through the Plasma Membrane Ca^{2+}-ATPase

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Abstract   The human mast cell line (HMC-1) has been used to study the relationship between intracellular pH and cytosolic calcium (Ca^{2+}) in mast cells. Thapsigargin (TG) caused store-operated Ca^{2+} entry, that is enhanced by the PKC activator PMA. NH_4Cl-induced alkalinization showed an inhibitory effect on TG-sensitive stores depletion (not on TG-insensitive stores), and also on final cytosolic Ca^{2+} levels reached in response to both TG and the ionophore ionomycin. Loperamide, a positive modulator of store-operated channels, induced a slight Ca^{2+} entry by itself, and also increased TG-induced Ca^{2+} entry. This enhancement was not enough to reverse the inhibitory effect of NH_4Cl-induced alkalinization. When comparing the effect of NH_4Cl-induced alkalinization on Ca^{2+} levels, with those observed using Ca^{2+} channel blockers (namely Ni^{2+} and SKF-96365), cytosolic profiles for this ion are different, either in modified saline solution or in HCO_3^--free medium. Thus, it seems unlikely that the inhibitory effect of NH_4Cl-induced alkalinization on Ca^{2+} is taking place by blockage of Ca^{2+} entry. Furthermore, inhibition of the plasma membrane Ca^{2+}-ATPase (an important mechanism for Ca^{2+} efflux) with sodium orthovanadate (SO) matches with the inhibition of the negative effect on Ca^{2+} levels elicited by NH_4Cl. Data indicate that NH_4Cl-induced alkalinization might be activating Ca^{2+} efflux from the cell, by stimulation of the plasma membrane Ca^{2+}-ATPase, and also confirm our previous finding that Ca^{2+} is a secondary signal to activate HMC-1 cells. J. Cell. Biochem. 99: 1397–1408, 2006.   © 2006 Wiley-Liss, Inc.

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Mast cells are a cell type often used in signal transduction studies because they secrete their granules through a fast exocytosis process, which can be used as a functional model. Mast cells are mainly involved in pathophysiological episodes such as asthma, allergies, inflammation or intestinal helminth infestations. They also produce cytokines that stimulate the pro-liferation and function of leukocytes [McNeil, 1996], take part in angiogenesis [Norrby, 1995], tissue reparation, coagulation [Okayama et al., 1995], and in some rare disorders such as mastocytosis [Sperr et al., 2001]. Therefore, mast cell lines are frequently used in studies of mast cell biology.

Up to now, only two mast cell lines obtained from humans are available: the LAD 1/2 line (Laboratory of Allergic Diseases) [Kirshenbaum et al., 2003], and the human mast cell line HMC-1 [Butterfield et al., 1988]. LAD cells present a duplication time of about 2–3 weeks, a fact that makes difficult its continuous use for investigations needing a large number of cells. The only mast cell line with human origin that has been widely studied so far is the line HMC-1. These

cells show a variable expression of the FcΣRI receptor [Nilsson et al., 1994], thus responding inconsistently to IgE-dependent signals. HMC-1 cells contain 0.9 pg histamine per cell, and show a high expression of the kit receptor for stem cell factor (SCF), which is constitutively phosphorylated owing to different activating mutations present in the c-kit proto-oncogene [Sundstrom et al., 2003]. Because of this fact, HMC-1 cells are growth-factor independent, with a doubling time of about 80 h.

We have chosen the human mast cell line HMC-1 for our studies because its growth and behavior are very regular, and also because there were many still unknown aspects concerning to activation process of these kind of cells. The possible influence of important transduction pathways, such as Ca^{2+} signaling or intracellular pH, in the exocytosis process remain also to be defined. These process are often studied in mast cells from different species but remained unknown in the HMC-1 line, that has particular characteristics. It is very important to know the secretory reponse in this cell line since it is involved in mastocytosis. In this disease mast cells degranulation induce different symptoms like pruritus, flushing, syncope, gastric distress, nausea and vomiting, diarrhea, bone pain and neuropsychiatric disturbances. These symptoms are variably controlled by adequate medications but it is basic to know mast cells activation to predict their behavior.

In a previous report [Pernas-Sueiras et al., 2005], we have started the characterization of the activation process (measured as histamine release) in the human mast cell line HMC-1, and also described its regulation by Ca^{2+} signaling and intracellular pH. In that work, it is a very remarkable observation that an intracellular alkalinization, even in the complete absence of Ca^{2+} changes, is enough signal to elicit histamine release in HMC-1 cells. This fact described in a human cell line is in good agreement with similar results obtained in rat mast cells [Alfonso et al., 2000, 2005]. Interestingly, Ca^{2+} entry in HMC-1 cells was found to be reduced in previously alkalinized versus non-alkalinized cells. This finding suggest a clear influence of intracellular pH on Ca^{2+} entry in this cellular model, that the present paper tries to further explore.

Although pH is generally considered as a parameter that has to be under control by the cell, in order to integrate all their regulatory functions, in the last years it is becoming more evident that (at least in mast cells) pH could be a modulatory signal itself, with different mechanisms of control [Vilariño et al., 1998; Alfonso et al., 2000, 2005]. Thus, the aim of the present work is to further characterize how Ca^{2+} signaling and intracellular pH are linked in the human mast cell line HMC-1.

METHODS

Chemicals

Ammonium chloride (NH_4Cl) was from Panreac (Barcelona, Spain); thapsigargin (TG), ionomycin and [1-[®-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole.HCl] (SKF-96365) 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), loperamide, nickel chloride (Ni^{2+}) and sodium orthovanadate (SO) were from Sigma-Aldrich (Madrid, Spain).

Cell Cultures

HMC-1 cell line 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 Ca^{2+} and pH measurements cells were centrifuged (1,500 rpm, 5 min, 4°C) and washed twice with modified 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 plus 0.1% bovine serum albumin (BSA).

For experiments carried out in bicarbonate-free medium, cells were prepared as described above, but bicarbonate-free medium was used to perform the assays, being the composition of this solution (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; SO_4^{2-} 1.2; HEPES 10; glucose 1 g/L.

The incubation medium was equilibrated with CO_2 prior to use. In all the experiments, pH (7.40) was maintained constant by bubbling

CO_2 during the experiment. All experiments were carried out at least three times, by duplicate.

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) in a bath at 37°C, for 10 min. After this time, loaded cells were washed with modified saline solution BSA free (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 vs. intracellular Ca^{2+} was made according to the method of Grynkiewicz [Grynkiewicz et al., 1985]. The calibration of the fluorescence values vs. 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

TG is a tumor-promoting sesquiterpene lactone that inhibits the Ca^{2+}-ATPase from intracellular Ca^{2+} pools, thus promoting its depletion and, according to the store-operated model, an external Ca^{2+} influx inducer [Thastrup et al., 1987; Hoth and Penner, 1992]. In HMC-1 cells, this drug induces a store-operated Ca^{2+} (SOC) entry that reaches values of 500 nM, but this is not enough to induce histamine release [Pernas-Sueiras et al., 2005]. To further understand the effect of TG, we have studied this drug in combination with the protein kinase C (PKC) activator PMA, that has been reported to promote histamine release in rat serosal mast cells, also affecting Ca^{2+} signaling [Vilariño et al., 1999, 2001]. Figure 1A shows that preincubation with PMA (100 ng/ml) does not modify depletion of TG-sensitive intracellular
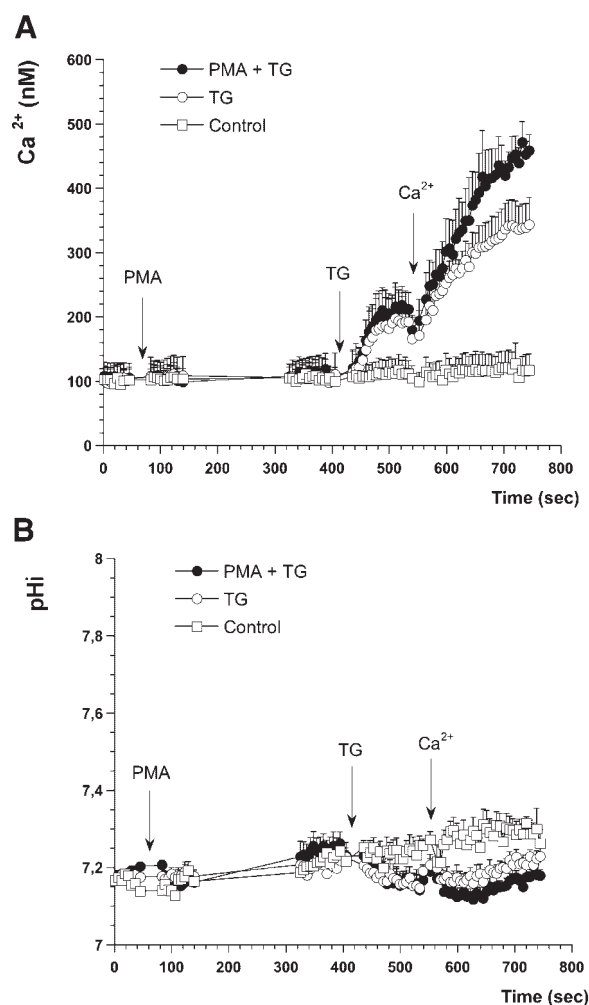

Fig. 1. Effect of PMA plus TG on cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells. **A**: Cytosolic Ca^{2+} profile in cells stimulated with 100 ng/ml PMA plus 2 µM TG. First arrow indicates the addition of PMA, second indicates the addition of TG, and third indicates that extracellular Ca^{2+} is restored. **B**: Intracellular pH profile in cells subject to the protocol described in (A). Mean ± SEM of three experiments (approximately 50 cells/single experiment).

Ca^{2+} pools (in a Ca^{2+}-free medium), but increases TG-induced Ca^{2+} entry when this ion is restored to the extracellular medium. The concentration of TG used was 2 μM. TG does not modify intracellular pH in HMC-1 cells [Pernas-Sueiras et al., 2005], and as Figure 1B shows, preincubation with PMA does not change this fact.

In a previous work with HMC-1 cells, we have demonstrated that NH_4Cl evokes a dose-dependent histamine release, that matches a dose-dependent intracellular pH increase [Pernas-Sueiras et al., 2005]. Figure 2 shows
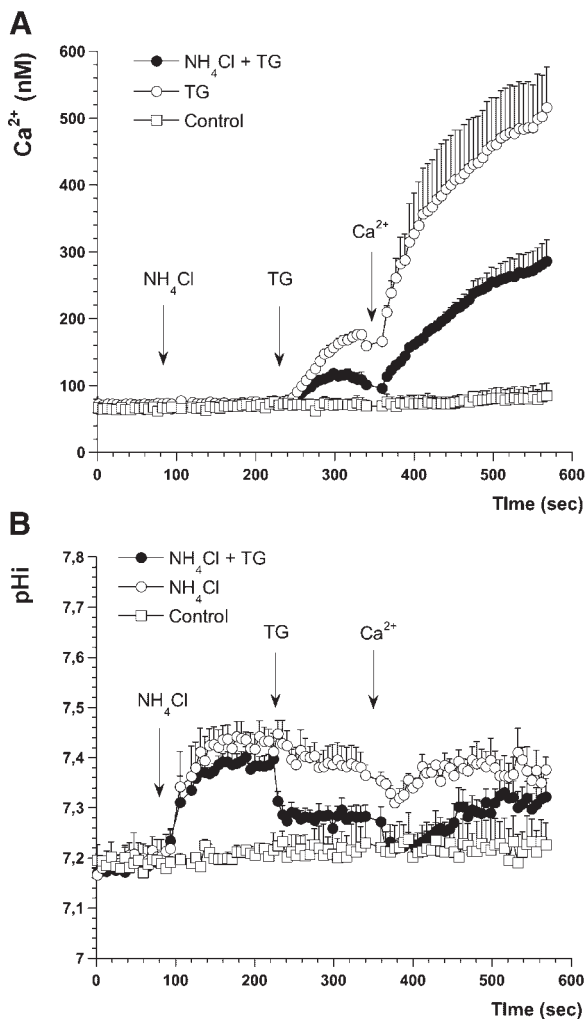

Fig. 2. Effect of NH_4Cl plus TG on cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells. **A:** Cytosolic Ca^{2+} profile in cells stimulated with 50 mM NH_4Cl plus 2 μM TG. First arrow indicates the addition of NH_4Cl, second indicates the addition of TG, and third indicates that extracellular Ca^{2+} is restored. **B:** Intracellular pH profile in cells subject to the protocol described in (A). Mean ± SEM of four experiments (approximately 40 cells/single experiment).

the effect of NH_4Cl addition on TG-induced Ca^{2+} changes. Cytosolic Ca^{2+} profile is represented in Figure 2A. After addition of TG, there is a first Ca^{2+} increase due to internal stores depletion and, when this ion is restored to the extracellular medium, cytosolic Ca^{2+} increases until values close to 500 nM. If NH_4Cl is added prior to TG, there is a significant inhibition of TG-induced Ca^{2+} pools depletion and also cytosolic Ca^{2+} levels reach lower values when this ion is restored to the medium (only 300 nM). Under these conditions, intracellular pH changes are shown in Figure 2B. NH_4Cl induces an intracellular alkalinization: pH quickly reaches approximate values of 7.45 and remains elevated over control cells during all the experiment. In cells where TG is added after NH_4Cl-induced alkalinization, pH sharply decreases after this point.

In order to compare data obtained with TG and NH_4Cl, we carried out the same experiments using the Ca^{2+} ionophore ionomycin, that has been reported as a stimulus to induce histamine release in mast cells, including the human mast cell line HMC-1 [Hsu and MacGlashan, 1996; Teofoli et al., 1999; Pernas-Sueiras et al., 2005]. Figure 3A shows that ionomycin promotes important Ca^{2+} stores depletion, in the absence of external Ca^{2+}, and subsequent Ca^{2+} entry (values over 500 nM) when this ion is restored to the medium. Addition of NH_4Cl prior to ionomycin does not modify depletion of internal Ca^{2+} pools but, as found in experiments carried out with TG, cytosolic Ca^{2+} levels reach lower values when this ion is restored to the medium. pH profile under these conditions is shown in Figure 3B. NH_4Cl addition induces an intracellular alkalinization, as it was described in Figure 2B. This pH increase shows a slight decrease after addition of ionomycin to the cells, but differences are not significant.

For the next experiments, we have used TG as stimulus for Ca^{2+} signaling, and NH_4Cl to modulate intracellular pH. We have described that each one of these drugs only modify one parameter, without any effect on the other, a fact that makes them a very useful tool for our studies.

To check if the inhibitory effect of NH_4Cl-induced alkalinization on Ca^{2+} levels could be reversed by a higher Ca^{2+} entry, we carried out experiments using loperamide. This drug has been reported as a positive modulator of SOC channels in several cellular models, including
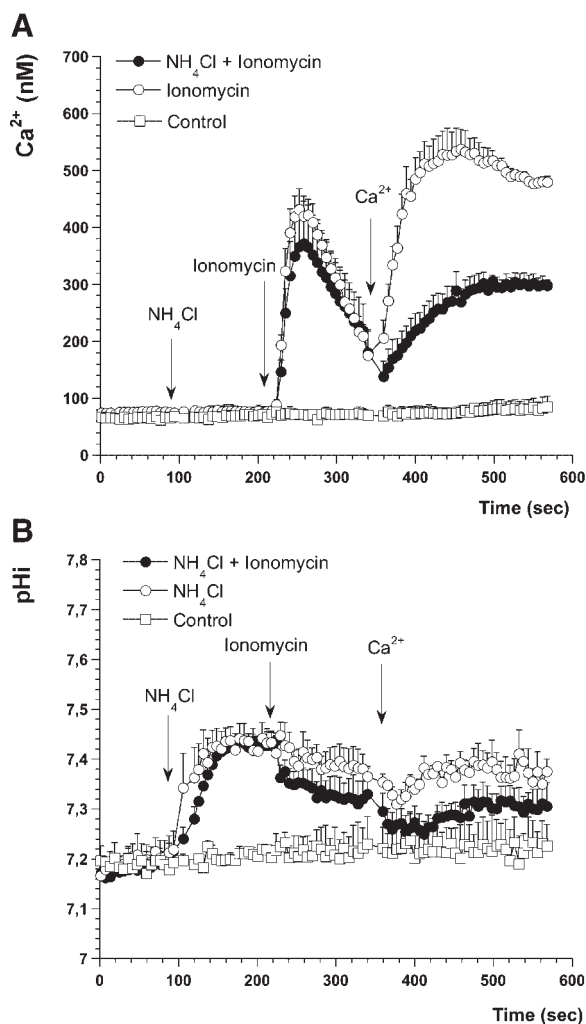
Fig. 3. Effect of NH_4Cl plus ionomycin on cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells. **A**: Cytosolic Ca^{2+} profile in cells stimulated with 50 mM NH_4Cl plus 0.1 µM ionomycin. First arrow indicates the addition of NH_4Cl, second indicates the addition of ionomycin, and third indicates that extracellular Ca^{2+} is restored. **B**: Intracellular pH profile in cells subject to the protocol described in (A). Mean ± SEM of four experiments (approximately 40 cells/single experiment).

RBL-2H3 mast cells and human T lymphocytes [Harper et al., 1997; Chen et al., 2000; Daly and Harper, 2000]. Results of these experiments are shown in Figure 4. When cells are incubated with loperamide in a Ca^{2+}-free medium, there is no effect on intracellular Ca^{2+} levels. When this ion is restored, loperamide-treated cells show a dose-dependent increase in intracellular Ca^{2+} levels over control cells, between 75 and 100 nM Ca^{2+} (Fig. 4A). If cells are preincubated with 15 µM loperamide prior to addition of TG, in a Ca^{2+}-free medium, it is observed (Fig. 4B) that TG-induced stores depletion takes place nor-

mally. But when this ion is restored to the extracellular medium, Ca^{2+} entry is significantly enhanced in loperamide-treated cells (values close to 700 nM) versus those only incubated with TG (values reaching 500 nM Ca^{2+}).

Since 15 µM loperamide was found to enhance TG-induced Ca^{2+} entry, we checked if this synergistic effect might be enough to revert the inhibitory effect of alkalinization on Ca^{2+} levels. Results are shown in Figure 4C. When NH_4Cl and TG are sequentially added to cells that have been preincubated with loperamide, it is still observed that alkalinization is impairing TG-induced Ca^{2+} pools depletion. When extracellular Ca^{2+} is restored, there is a remarkable difference on the cytosolic Ca^{2+} levels reached by the cells, depending on the treatment received. Cells incubated with loperamide plus TG reach values of about 700 nM (as seen in Fig. 4B); cells preincubated with NH_4Cl prior to addition of loperamide plus TG show cytosolic Ca^{2+} values close to 450 nM, similar to those obtained for cells that only have been treated with TG. Thus, it seems that alkalinization is still showing an inhibitory effect on cytosolic Ca^{2+} levels, since in cells preincubated with NH_4Cl the loperamide-induced enhancement of Ca^{2+} entry is not observed.

So far, results presented in this study show that NH_4Cl-induced alkalinization has an inhibitory effect on TG-sensitive Ca^{2+} stores depletion, and also on cytosolic Ca^{2+} increase elicited by TG in HMC-1 cells. We have observed this inhibitory effect on cytosolic Ca^{2+} levels either if alkalinization is induced prior to Ca^{2+} stores depletion or after that, but before Ca^{2+} restoration to the medium [Pernas-Sueiras et al., 2005]. In order to check if this inhibition might be due to a blockage of Ca^{2+} entry produced by the intracellular alkalinization, we carried out experiments where NH_4Cl was added after the Ca^{2+} entry was fully established. We have compared the response to NH_4Cl with the response obtained for two Ca^{2+} channel blockers widely used: nickel (Ni^{2+}) and SKF-96365. Both compounds have been used to block Ca^{2+} influx in many different cellular models, including mast cells [Fischer et al., 1998; Matsubara et al., 2004; Roman et al., 2004; Pernas-Sueiras et al., 2005]. Data obtained from experiments using NH_4Cl, Ni^{2+}, and SKF-96365 are represented in Figure 5. TG induces SOC entry, until approximate values of 500 nM Ca^{2+}. After

addition of NH_4Cl there is a significant decrease on cytosolic Ca^{2+} concentration: values reach a plateau at about 250 nM (Fig. 5A, open circles). Later addition of Ni^{2+} to these cells produces a

new decrease on cytosolic Ca^{2+} levels, approximately until the initial basal values. If Ni^{2+} is added after Ca^{2+} entry is established (Fig. 5A, closed circles), cytosolic concentration of this ion significantly decreases and quickly reaches basal values, that are not already modified by the later addition of NH_4Cl. Figure 5B shows pH profile under these conditions. After addition of NH_4Cl, both prior or after Ni^{2+} addition, an intracellular alkalinization is immediately observed (pH increases 0.3 units). Figure 5C shows the Ca^{2+} profile obtained in experiments where SKF-96365 was used instead of Ni^{2+}. This profile is very similar to the one presented in Figure 5A: first, a decrease in cytosolic Ca^{2+} concentration after NH_4Cl-induced alkalinization, and then, a new decrease (until basal values) after the addition of the Ca^{2+} entry blocker SKF-96365. pH results using this drug are also very similar to those obtained with Ni^{2+} (data not shown). Therefore, results shown in Figure 5 suggest that NH_4Cl and the Ca^{2+} entry blockers might be acting in a different way, since Ca^{2+} profiles obtained for these compounds are different.

Since it seems unlikely that NH_4Cl-induced alkalinization was blocking Ca^{2+} entry, we checked if it might be activating Ca^{2+} efflux from the cytosol to the extracellular medium. To test this hypothesis, we first carried out the same experiments shown in Figure 5, but in a bicarbonate-free (HCO_3^--free) medium, in order to determine if any HCO_3^- exchanger present in the membrane might account for Ca^{2+} extrusion. Results are presented in Figure 6. In Figure 6A and B it is observed that, in a HCO_3^--free medium, Ca^{2+} profiles after addition of NH_4Cl, or the Ca^{2+} entry blockers (Ni^{2+} and SKF-96365) are very similar to those obtained in modified saline solution, containing HCO_3^- ions. Figure 6C shows the pH results
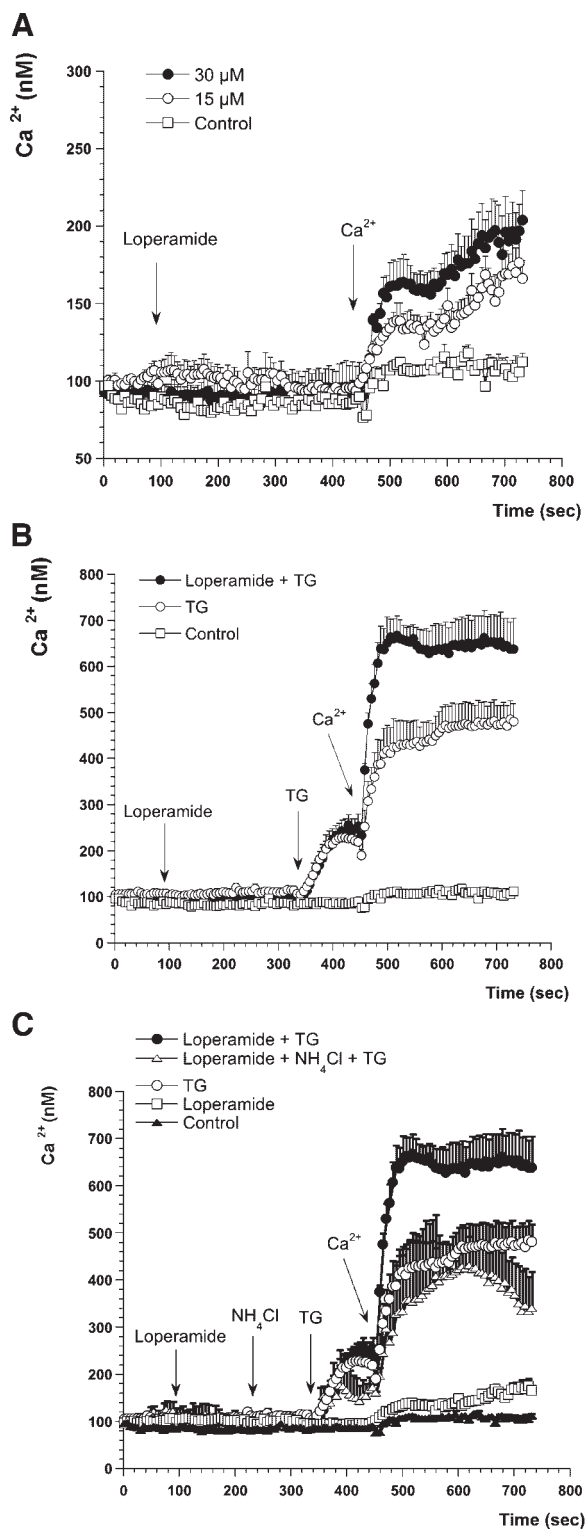

Fig. 4. Effect of loperamide on cytosolic Ca^{2+} concentration in HMC-1 cells. **A**: Variation of cytosolic Ca^{2+} levels in the presence of 15 μM (open circles) or 30 μM (closed circles) loperamide. First arrow indicates the addition of loperamide and second indicates that extracellular Ca^{2+} is restored. **B**: Cytosolic Ca^{2+} profile in cells stimulated with 15 μM loperamide plus 2 μM TG. First arrow indicates the addition of loperamide, second indicates the addition of TG, and third means that extracellular Ca^{2+} is restored. **C**: Cytosolic Ca^{2+} profile in cells stimulated with 15 μM loperamide, 50 mM NH_4Cl and 2 μM TG. First arrow indicates the addition of loperamide, second indicates the addition of NH_4Cl, third indicates the addition of TG, and fourth means that extracellular Ca^{2+} is restored. Mean ± SEM of three experiments (approximately 45 cells/single experiment).

obtained for NH_4Cl and Ni^{2+} under these conditions. By comparing this pH profile with the one shown in Figure 5B (that represents the same experiments, but in modified saline solution), there is a major difference in the response of control and TG-treated cells: in modified saline solution (Fig. 5B), intracellular pH of
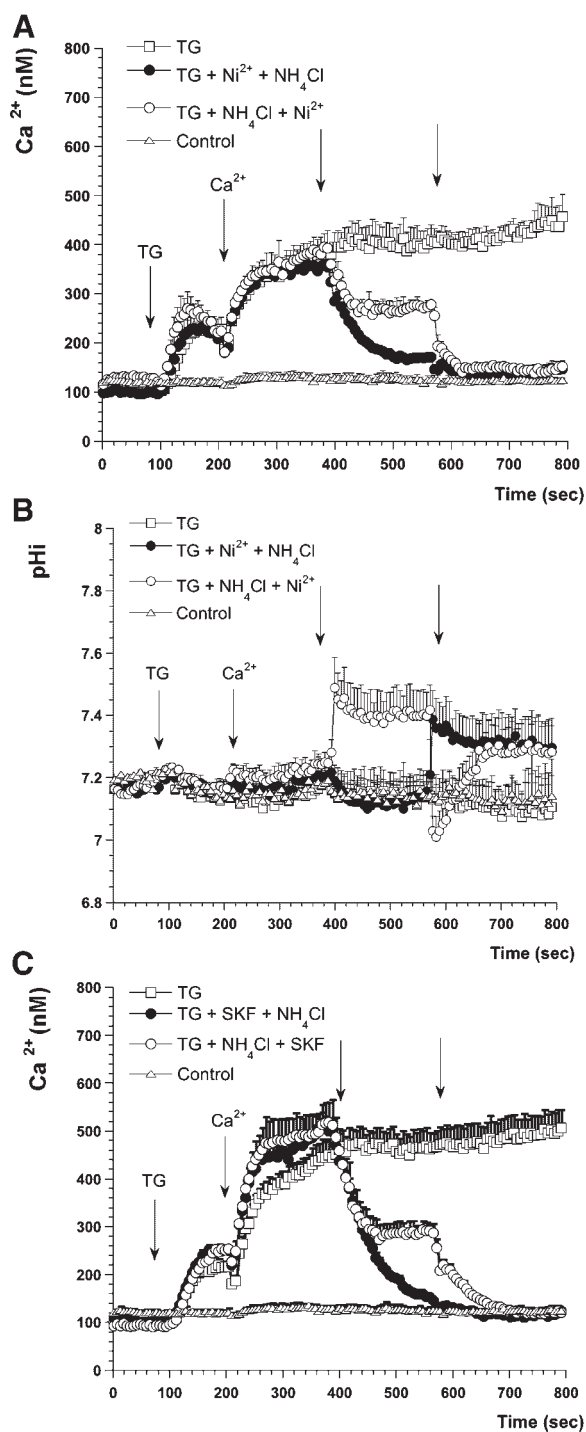
these cells remains in basal values (7.2) during all the experiment (about 15 min); however, in a HCO_3^--free medium (Fig. 6C), cells show an important decrease on intracellular pH, which takes place gradually during the time course of the experiment (final pH falls about 0.4 units). It is also noteworthy that NH_4Cl still induces, in a HCO_3^--free medium, an intracellular alkalinization with a similar magnitude to that found in modified saline solution. This alkalinization also matches with a significant decrease in cytosolic Ca^{2+} concentration (Fig. 6A and C, open circles).

In second place, we have studied the possible influence of an important mechanism for Ca^{2+} extrusion, namely the plasma membrane Ca^{2+}-ATPase. We checked the effect of SO, which has been reported to inhibit the plasma membrane Ca^{2+}-ATPase [Chiesi et al., 1991; Romero and Romero, 2003]. Data from these experiments are shown in Figure 7. When cells are preincubated with SO prior to addition of TG, in a Ca^{2+}-free medium, internal Ca^{2+} stores depletion takes place normally. After this ion is restored to the medium, TG-induced Ca^{2+} entry is strongly augmented: values over 700 nM Ca^{2+} are found in orthovanadate-pretreated cells, while cells only incubated with TG reach values of 400 nM (Fig. 7A and B). When Ca^{2+} entry is later blocked by addition of Ni^{2+} (Fig. 7A), cytosolic Ca^{2+} concentration immediately falls until values close to 250 nM Ca^{2+}. However, when NH_4Cl is added to cells instead of Ni^{2+} (Fig. 7B), cytosolic Ca^{2+} concentration does not decrease: it remains elevated at about 700 nM, after an initial fast and transient decreasing peak.

DISCUSSION

In a previous report [Pernas-Sueiras et al., 2005], we have already presented data pointing

Fig. 5. Effect of Ca^{2+} channel blockers (Ni^{2+} and SKF-96365) and NH_4Cl on cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells. **A**: Cytosolic Ca^{2+} profile in cells incubated with 2 µM TG, 50 mM NH_4Cl and 1 mM Ni^{2+}. First arrow indicates the addition of TG and second indicates that extracellular Ca^{2+} is restored. In the case of open circles, third arrow indicates the addition of NH_4Cl, and fourth indicates the addition of Ni^{2+}. In the case of closed circles, third arrow indicates the addition of Ni^{2+}, and fourth indicates the addition of NH_4Cl. **B**: Intracellular pH profile in cells subject to the protocol described in (A). **C**: Cytosolic Ca^{2+} profile in cells incubated with 2 µM TG, 50 mM NH_4Cl and 30 µM SKF-96365, following the same protocol described in (A). Mean ± SEM of three experiments (approximately 50 cells/single experiment).

out the importance of intracellular pH regulation in the human mast cell line HMC-1: we have demonstrated that an intracellular alkalinization is a sufficient signal to evoke exocytosis in this cellular model, while an increase in cytosolic Ca^{2+} is not. These observations
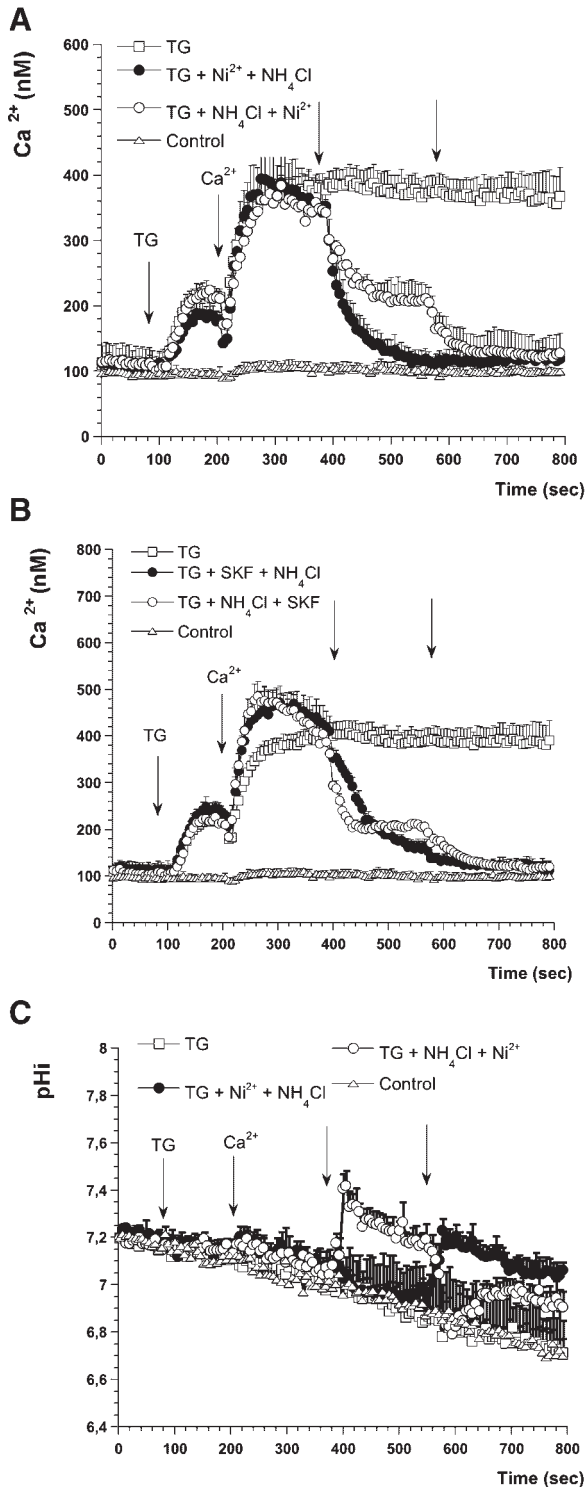
and data obtained in several cellular models [Alfonso et al., 2000; Roman et al., 2004; Alfonso et al., 2005] strongly suggest that pH could be not only an important condition for enzymes or receptors to work, but also a modulatory signal itself. In this paper we have tried to further characterize the relationship between cytosolic Ca^{2+} and pH regulation.

Loperamide is an opioid agonist commonly used as antidiarrheal agent [Awouters et al., 1983; Ooms et al., 1984; Burleigh, 1988]. It has been reported that this drug is able to enhance SOC entry elicited by receptor activation, TG or ionomycin, but not if Ca^{2+} levels are increased by a mechanism different from SOC channels [Harper et al., 1997; Chen et al., 2000; Daly and Harper, 2000; Roman et al., 2004]. HL-60 cells, RBL-2H3 mast cells and human T lymphocytes are cellular models where this drug increases SOC entry, but does not induce Ca^{2+} entry by itself. On the other hand, loperamide alone was found not only to increase SOC entry, but also to promote Ca^{2+} entry in astrocytoma 1321N cells. Our results show that this drug does not have an effect on internal Ca^{2+} stores, since it shows no effect when it is added to the cells in Ca^{2+}-free conditions. However, when this ion is restored to the medium, loperamide alone is able to induce a dose-dependent Ca^{2+} entry, probably due to activation of SOC channels. Also, if SOC entry is induced in the presence of TG after the cells are preincubated with loperamide, a remarkable enhancement of TG-induced Ca^{2+} entry is observed. Thus, our results indicate that HMC-1 cells present the SOC mechanism, and also that loperamide enhances SOC entry in this cellular model.

Data obtained in our experiments clearly indicate that Ca^{2+} is a secondary signal to activate HMC-1 cells, since an important Ca^{2+}

Fig. 6. Effect of Ca^{2+} channel blockers (Ni^{2+} and SKF-96365) and NH_4Cl on cytosolic Ca^{2+} concentration and intracellular pH in HMC-1 cells incubated in a HCO_3^--free medium. **A:** Cytosolic Ca^{2+} profile in cells incubated with 2 μM TG, 50 mM NH_4Cl and 1 mM Ni^{2+}. First arrow indicates the addition of TG and second indicates that extracellular Ca^{2+} is restored. In the case of open circles, third arrow indicates the addition of NH_4Cl, and fourth indicates the addition of Ni^{2+}. In the case of closed circles, third arrow indicates the addition of Ni^{2+}, and fourth indicates the addition of NH_4Cl. **B:** Cytosolic Ca^{2+} profile in cells incubated with 2 μM TG, 50 mM NH_4Cl and 30 μM SKF-96365, following the same protocol described in (A). **C:** Intracellular pH profile in cells subject to the protocol described in (A). Mean ± SEM of four experiments (approximately 40 cells/single experiment).
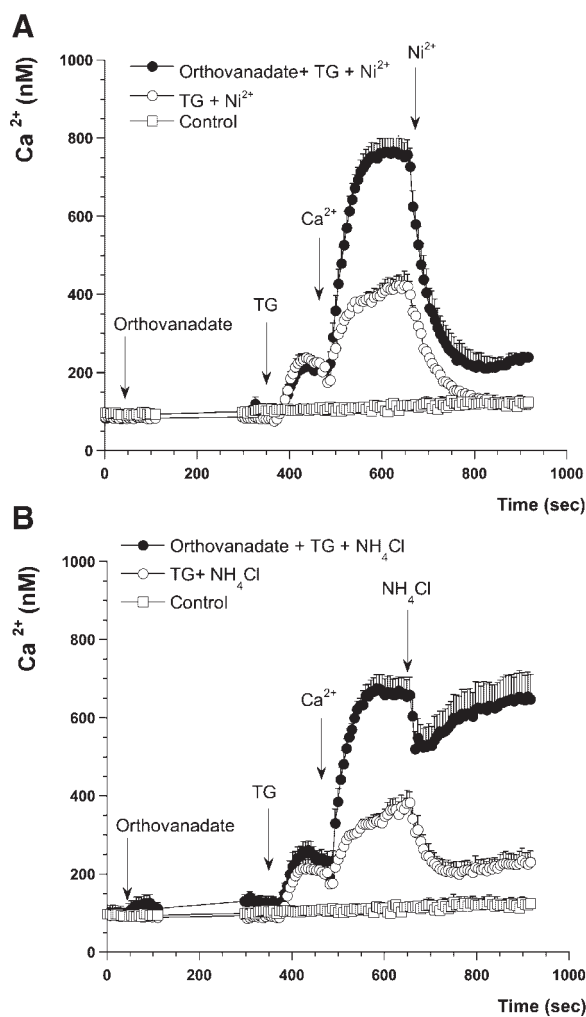
Fig. 7. Effect of sodium orthovanadate (SO) on cytosolic Ca^{2+} concentration in HMC-1 cells stimulated with TG. **A:** Cytosolic Ca^{2+} levels in cells incubated with 10 mM SO, 2 µM TG and 1 mM Ni^{2+}. First arrow indicates the addition of SO, second indicates the addition of TG, third means that extracellular Ca^{2+} is restored and fourth indicates the addition of Ni^{2+}. **B:** Cytosolic Ca^{2+} profile in cells incubated with 10 mM SO, 2 µM TG and 50 mM NH_4Cl, following the same protocol described in (A). Mean ± SEM of four experiments (approximately 45 cells/single experiment).

entry elicited by TG, without affecting intracellular pH, was not enough to evoke histamine release [Pernas-Sueiras et al., 2005]. The PKC activator PMA, that has been reported to promote histamine release in rat mast cells, also affecting to Ca^{2+} signaling [Vilariño et al., 1999, 2001], failed to trigger histamine release in HMC-1 cells. PMA increases TG-induced Ca^{2+} entry, but even with these higher ion levels, no histamine release was observed (data not shown). Thus, PKC activation or cytosolic Ca^{2+} increase are not sufficient signals to

induce histamine release in HMC-1 cells, an this is a clear difference with respect to rat mast cells.

In several cellular models, an intracellular alkalinization has been reported to induce Ca^{2+} release from internal stores, and subsequent SOC influx [Guse et al., 1994; Nitschke et al., 1996; Alfonso et al., 2000]. Furthermore, NH_4Cl-induced alkalinization was found to enhance SOC entry elicited by TG [Marumo and Wakabayashi, 2003; Wakabayashi et al., 2003]. Relationship between cytosolic Ca^{2+} and intracellular pH is different in HMC-1 cells, since we have already demonstrated that an intracellular alkalinization or acidification does not change cytosolic Ca^{2+} concentration [Pernas-Sueiras et al., 2005], and in this study we have described how NH_4Cl-induced alkalinization negatively modulates cytosolic Ca^{2+} levels.

It is a remarkable finding that, in HMC-1 cells, an intracellular alkalinization was found to impair TG-sensitive Ca^{2+} stores depletion, but had no effect on ionomycin-induced Ca^{2+} release from internal stores. In both cases, a negative modulation of Ca^{2+} levels was observed after this ion was restored to the medium. These data are different to those obtained with rat mast cells, where TG and NH_4Cl were found to release Ca^{2+} from the same intracellular pools [Alfonso et al., 2000]. However, several reports have shown that changes in intracellular pH with different compounds, including NH_4Cl, do modify depletion of Ca^{2+} pools, increasing or decreasing ion sequestration by affecting intracellular buffering mechanisms [Bode et al., 1994; OuYang et al., 1994; Hoyt and Reynolds, 1998; Potapenko et al., 2004]. Also, variation of lysosomal pH with NH_4Cl is able to induce lysosomal Ca^{2+} efflux to the cytosol, probably trough channels or transporters which are pH-dependent [Christensen et al., 2002]. Our data indicate that NH_4Cl-induced alkalinization is impairing only the action of TG, but not the action of the ionophore ionomycin on Ca^{2+} stores. So, it is likely that the event responsible for this inhibition is related to TG or the ATPase. Also, it is taking place in a fast way, since TG is added to the cells about 100 s after NH_4Cl. We can hypothesize that NH_4^+ ions might affect the union of TG to its binding place. It is also a possible explanation that NH_4Cl-induced alkalinization is affecting the Ca^{2+}-ATPase isoform present in HMC-1 cells,

causing a conformational change and, thus, affecting the union of TG to its binding place. But it is also reasonable that alkalinization might be activating, at least partially, the Ca^{2+}-ATPase present in the internal pools, thus counteracting, or avoiding, the inhibitory action of TG on the ATPase.

Both for TG- or ionomycin-induced Ca^{2+} entry, alkalinization caused by NH_4Cl decreases final cytosolic Ca^{2+} levels reached by the cells. Thus, it might be possible that Ca^{2+} balance mechanisms are being affected, maybe by blockage of Ca^{2+} entry or maybe inducing Ca^{2+} efflux from the cell.

Significant differences were found for cytosolic Ca^{2+} profiles when comparing the effect of NH_4Cl with two well-known Ca^{2+} channel blockers, Ni^{2+} and SKF-96365. When Ca^{2+} entry was blocked, cytosolic levels of this ion quickly decreases until basal values, and no changes on intracellular pH were measured; while when NH_4Cl was employed to promote an intracellular alkalinization, cytosolic Ca^{2+} levels still showed an important decrease, but approximately half of the one seen with the blockers. Later addition of the blockers to the cells resulted in another decrease of cytosolic Ca^{2+} until basal values. NH_4Cl has already been reported to produce a slight decrease of cytosolic Ca^{2+} concentration due to flux of this ion to intracellular stores [Burns et al., 1991], and, interestingly, this drug was also found to evoke Ca^{2+} efflux from the cell in bovine aortic endothelial cells and in human lymphocytes [Danthuluri et al., 1990; Cabado et al., 2000]. Therefore, it might be possible that the negative modulation that we have observed in HMC-1 cells is caused by efflux of Ca^{2+} from the cells, and not by Ca^{2+} entry blockage. Different observations presented in this study account for this hypothesis. First, inhibition of TG-sensitive stores depletion when alkalinization was prior induced, and second, our idea mentioned above regarding the activation of the Ca^{2+}-ATPase from internal stores by the alkalinization. It would be possible that both Ca^{2+}-ATPase from intracellular pools and the one present in the plasma membrane are activated in a pH-dependent way in HMC-1 cells, thus producing Ca^{2+} efflux. Data obtained with Ca^{2+} channel blockers also support the hypothesis of Ca^{2+} efflux, since the profiles obtained for cytosolic levels of this ion are different when channels blockers or NH_4Cl are employed.

Mast cells are endowed with several mechanisms to tightly regulate intracellular pH, either directly, such as a Na^+/H^+ exchanger (which is the main responsible for H^+ extrusion) or a HCO_3^- influx mechanism, or indirectly with a Na^+/Ca^{2+} exchanger or a Na^+/K^+ ATPase [Bronner et al., 1989; Alfonso et al., 1994a,b; Friis et al., 1997; Praetorius et al., 1998; Vilariño et al., 1998; Alfonso et al., 1999]. However, little attention has been paid to these regulatory systems in HMC-1 cells and, thus, it remains elusive which mechanisms are present or not in this cellular model. Further studies should be done in the next future in order to describe and characterize these exchangers.

However, because HCO_3^- ions have been shown as an important contribution to pH regulation in rat mast cells [Vilariño et al., 2003], specially to maintain intracellular pH near neutrality [Tonnessen et al., 1990], we carried out experiments in HCO_3^--free medium, in order to check if the inhibitory effect of NH_4Cl-induced alkalinization on cytosolic Ca^{2+} levels was still observed. Under these conditions, a significant difference was observed: untreated (control) HMC-1 cells incubated in modified saline solution maintain intracellular pH constant on basal levels, during the time course of the experiment. However, control cells incubated in HCO_3^--free medium show a significant decrease (about 0.4 units) on intracellular pH, that takes places in a gradual way. This is a very remarkable finding, since it clearly indicates that HMC-1 cells might be endowed with a HCO_3^--dependent alkalinizing mechanism, which would have a primordial role on maintaining intracellular pH on basal levels (7.2), since in the absence of this ion, pH was found to significantly decrease. On the other hand, the same inhibitory effects of NH_4Cl-induced alkalinization on Ca^{2+} levels were found, both in modified saline solution or in HCO_3^--free medium, thus indicating that the possible mechanism of transport of HCO_3^- is not accounting for the impaired cytosolic Ca^{2+} levels. The last observation is another evidence that supports the hypothesis of a Ca^{2+} efflux activated by NH_4Cl as responsible of this inhibitory effect. To directly address this point, we have presented data obtained using SO, a drug that has been reported to inhibit the plasma membrane Ca^{2+}-ATPase [Chiesi et al., 1991; Romero and Romero, 2003]. Orthovanadate-preincubation was found to significantly

increase TG-induced Ca^{2+} entry. This is a logical finding, because this compound is blocking the plasma membrane Ca^{2+}-ATPase, one important mechanism of Ca^{2+} efflux. Thus, cytosolic Ca^{2+} increase is higher, since Ca^{2+} entry mechanisms are not affected, but the extrusion is impaired. After Ca^{2+} entry is blocked, cytosolic Ca^{2+} concentration immediately decreases. Since the plasma membrane Ca^{2+}-ATPase is still blocked by SO, several events might be accounting for this decay: first, blockage of Ca^{2+} entry; second, Ca^{2+} reuptake to intracellular pools not sensitive to TG, and finally, Ca^{2+} efflux to the extracellular medium through a possible Na^+/Ca^{2+} exchanger, described in other mast cells [Alfonso et al., 1999].

Clearly, further studies should be done in order to characterize Ca^{2+} exchange, and also to describe the transporters that take place on it. Nevertheless, it is noteworthy that very different results were obtained for NH_4Cl, since the inhibitory effect of NH_4Cl-induced alkalinization on Ca^{2+} levels is not observed in the presence of SO. In summary, results indicate that alkalinization might be activating Ca^{2+} efflux through the plasma membrane Ca^{2+}-ATPase, since inhibition of this pump matches the inhibition of the negative alkaline-induced effect on Ca^{2+} levels.

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