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EFFECT OF SIGNAL TRANSDUCTION PATHWAYS ON THE ACTION OF THAPSIGARGIN ON RAT MAST CELLS

CROSSTALKS BETWEEN CELLULAR SIGNALLING AND CYTOSOLIC pH

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Abstract—Thapsigargin elicits histamine release on rat mast cells, and this effect is increased if cells are pretreated with thapsigargin before the addition of external calcium. Okadaic acid does not modify the response of mast cells to thapsigargin, while sodium fluoride or the phorbol esther 12-*O*-tetradecanoylphorbol-13-acetate (TPA) increases several fold the sensitivity of cells to thapsigargin. On the other hand, pertussis and cholera toxins inhibit the response to thapsigargin. Thapsigargin increases the activity of the Na^+–H^+ exchanger, this effect being blocked by fluoride and not modified by TPA. The metals cadmium and lanthanum completely block the effect of TPA or thapsigargin on the Na^+–H^+ exchanger. The influx of ^{45}Ca in rat mast cells is not modified by thapsigargin, but if cells are treated with thapsigargin before the addition of calcium, the influx is markedly increased in the first 2 min before returning to normal. Our results indicate that exocytosis is modulated by crosstalks between intracellular calcium, cytosolic pH and external calcium.

Key words: mast cells; Na^+–H^+ exchanger; propionate; BCECF; thapsigargin; fluoride

Rat mast cells are an exocytotic cellular model used to study the biology of the secretory process. There are many papers devoted to the study of the role of calcium and other intracellular signals such as cAMP, DAG§ or G-proteins, [1], but very little is known about the modulatory role of monovalent cations on these cells. It is known that in the absence of extracellular sodium, mast cells are more sensitive to low calcium concentrations [2] and that the blockade of potassium efflux triggers the release of histamine and the uptake of Ca^{2+} [3]. Thus far, there is no integrated theory to explain the links between calcium and monovalent cations.

The role of sodium on the cellular physiology of inflammatory cells is not known. It has been described in the activity of the Na^+–H^+ transporter [4], the relationship of the Na^+–K^+ pump with calcium [5] and some functional regulation of the Na^+–H^+ transporter on A23187-stimulated arachidonic acid and histamine release [6]. We have recently observed that in sodium-free conditions, mast cells can desensitize to A23187 at very low extracellular calcium concentrations (manuscript submitted for publication). This observation implies that there is a functional relationship between calcium and intracellular pH, since in sodium-free conditions the Na^+–H^+ transporter is inhibited.

The cellular physiology of calcium in mast cells is not yet understood, either pertaining calcium influx through the plasma membrane or the mechanisms that regulate calcium release from internal reservoirs. According to the capacitative model of Putney [7], calcium influx is related to the amount of internal calcium in the reservoirs, but this has never been studied in rat mast cells. Thapsigargin is a tumor-promoter sesquiterpene lactone that potently and selectively inhibits the Ca^{2+}-ATPase of intracellular stores [8], thus increasing cytosolic calcium. Therefore, this drug is a suitable tool to understand how mast cells regulate calcium levels. The approach that we make in this work is to establish a link between calcium influx and the modification of the signal transduction pathways with different drugs. Also, since cytosolic alkalinization has been reported to be related to the release of calcium from the internal reservoirs [9, 10], we study the modifications induced by thapsigargin on cytosolic pH, focusing our study on the Na^+–H^+ exchanger, which is relevant to elevate cytosolic pH.

MATERIALS AND METHODS

Chemicals. Thapsigargin and the phorbol ester TPA were from the Sigma Chemical Co. (St Louis, MO, U.S.A.); lanthanum chloride, cadmium chloride, sodium fluoride and orthophthalaldehyde were from Merck (Darmstadt, Germany); okadaic acid was from the LC Services Co. (Boston, MA, U.S.A.). ^{45}Ca (sp. act. 14.4 mCi/mg) was purchased from Du Pont (Germany), Percoll® was from Pharmacia (Uppsala, Sweden), and 2,7-bis(carboxyethyl)-5(6)-carboxyluorescein acetoxymethyl ester (BCECF/AM) was obtained from Molecular

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§ Abbreviations: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; DAG, diacylglycerol.

Probes (Eugene, OR, U.S.A.). Okadaic acid was dissolved in a 1:10 DMSO:water stock solution, and the final concentration of DMSO was less than 0.1%. Thapsigargin and TPA were dissolved in a stock solution of DMSO, final concentration being always less than 0.1%. All stock solutions were stored at −70°.

Mast cell preparation. Mast cells were obtained by lavage of pleural and peritoneal cavities of Sprague–Dawley rats (200–400 g) as described previously [11]. The composition of the physiological saline solution was (mM): Na^+, 142.3; K^+, 5.94; Ca^{2+}, 1; Mg^{2+}, 1.2; Cl^-, 126.1; CO_3^-, 22.85; $PO_4H_2^-$, 1.2; SO_4^{2-}, 1.2, giving a final osmotic pressure of 300 ± 5 mOsm/Kg H_2O. In all experiments the incubation medium was equilibrated with CO_2 and the final pH was adjusted to 7.0 prior to use. During the short time of each experiment (maximum 10 min), the pH in the solution remained constant.

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

Cell purification. Cells pooled from three rats were purified by centrifugation through 3 mL of isotonic Percoll at 400 *g* for 10 min. Percoll was eliminated by washing three times with the medium described above [12] 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% [12].

Cell incubation. Twenty-five microlitres of a freshly prepared concentrated solution of each drug were added to the incubation medium to attain a final volume of 0.925 mL and preincubated. When the medium reached 37°, 25 μL of a cell suspension containing 1–1.5 × 10^5 mast cells, were added to each tube. Incubations were carried out in a bath at 37° for 10 min, and for a further 10 min after addition of the stimulus.

Incubations were stopped by immersing the tubes in a cold bath. After centrifugation at 1000 g_{max} for 5 min, the supernatants were collected and decanted into other tubes for histamine determination. Incubations in sodium-free conditions were carried out in sucrose isotonized with Tris, pH 7.0. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were executed in every experiment.

Histamine release assay. Histamine was assayed spectrofluorometrically both in the pellet (residual histamine) and supernatants—released histamine—by Shore's method [13] in a spectrofluorometer Kontron SFM 25. However, 0.1% orthophthalaldehyde was employed. Trichloroacetic acid was added (7%, final concentration) to prevent reaction because protein interferes with histamine assay. To ensure total histamine, pellets were sonnicated for 60 sec in 0.8 mL of 0.1 N HCl. Results are expressed as a percentage of histamine released with respect to total histamine content.

Cell labeling and pH_i *measurement.* Approximately 1 × 10^6 mast cells were incubated with 1 μM BCECF/AM for 20 min at 37° and washed three times (400 *g*, 2 min) after the incubation. All the fluorescence measurements were carried out in a final volume of 2.5 mL in a thermostated cuvette with continuous stirring. A spectrofluorometer (Shimadzu RF-5000) was used for the experiments, with automatic calculation of the ratio values for excitation at 500–440 nm and emission at 530 nm. We checked for BCECF leakage and we did not observe any loss of the dye for the first 15 min. The calibration of fluorescence vs pH_i was made using nigericin in K^+ solution as per Thomas *et al.* [14]. 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. In order to plot the average of several experiments, and to avoid loosing the information about the variability of the assay, we do not show the original plots, but the average ± SEM of several plots. To do this, we averaged the values obtained from each plot at 15 sec intervals.

Analysis of pH_i *recovery after cytoplasmic acid loading.* The activity of the Na^+–H^+ exchanger was measured as the rate of pH_i recovery in acid-loaded cells, in this case propionic acid, as per Livne *et al.* [15]. The free propionate anion is in equilibrium with the protonated acid, which is membrane-permeable and able to enter the cell. Once inside the cell, it dissociates to form an equilibrium between the free propionate anion and the proton, which lowers the pH_i. In the presence of extracellular Na^+, the Na^+–H^+ exchanger acts to elevate the pH_i value to its original level by exchanging intracellular H^+ with extracellular Na^+. The osmotic pressure after the addition of propionate was slightly changed (up to 40 mOsm in addition to the medium). This increase over the normal osmotic pressure did not change the values of intracellular pH, as checked with equivalent osmotic concentrations of NaCl or KCl.

The activity of the Na^+–H^+ exchanger is estimated by the rate of pH_i recovery after the addition of Na-propionate, which changes the pH_i immediately after its addition. As reported by Ozaki *et al.* [16], the pH_i change is blunted by the time required for mixing the solution and by the effect of the exchanger activated immediately after acid loading. Therefore, the method of Guggenheim [17], which does not require a knowledge of the magnitude of the initial reaction, was employed to determine the rate constants for Na^+–H^+ exchanger activity. The principle of Guggenheim's plot has been described previously [4]. With this transformation, we obtain the plot $\log_{10}(v_i' - v_i)$ against t; the straight line so obtained will have a slope $-k\log_{10}e$, v_i being the reading at time i, and k the velocity constant (pH units/min) of the transporter.

^{45}Ca *experiments.* Mast cells were incubated with the isotope in a volume of 300 μL at 37° in a calcium-buffered medium and pH 7.2. The amount of isotope used was 0.25 μCi/250,000 mast cells. The uptake of calcium was determined measuring the activity on the pellet obtained after centrifugation (13,000 rpm, 30 sec) of 300 μL of cellular suspension on top of 100 μL of dibutylphthalate in a 400 μL conic tube. The samples were measured in a scintillation beta counter (Kontron Instruments, Switzerland).

Statistical analysis. Results were analysed 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

Figure 1 shows the pattern of histamine release elicited by thapsigargin. The working range is 0.001–0.5 μM (Fig. 1A), and the effect is clearly more potent if calcium is added after the cells were treated with thapsigargin. In this case, the potency of calcium is up to 4-fold higher when added 10 min after thapsigargin (Fig. 1B). Also, the response elicited by thapsigargin is notably less potent when added simultaneously, maximum response being 30%, while the maximum response obtained with preincubation with thapsigargin is 60%. The dose–response profile is shifted to the left if calcium is added after thapsigargin (Fig. 1C), although the same plateau is obtained in both cases, with a maximum response slightly higher than 80%. The shift to the left is, expressed as ED_{50}, 0.0067 ± 0.001 and 0.028 ± 0.001, both preincubating and without preincubation with thapsigargin before the addition of calcium, respectively.

In order to study the effect of the functional modification of the different transduction pathways on the cell response, we used several drugs which modify different pathways, namely okadaic acid (phosphatase inhibitor), TPA (PKC activator), sodium fluoride (unspecific G-protein activator) and cholera and pertussis toxin (which modify the function of G_s- and G_i-proteins). The response of rat mast cells to thapsigargin is not altered by 1 μM okadaic acid (Fig. 2A). This concentration of okadaic acid is sufficient to modify the response of these cells [18]. On the other hand, both the unspecific G-protein activator fluoride (Fig. 2B) and the phorbol ester TPA (Fig. 2C) do modify the response to thapsigargin. Both drugs displace the dose–response curve of thapsigargin to the left, 10 mM fluoride being less potent than TPA, with ED_{50} decreases of 2.5- and 4.4-fold for sodium fluoride and TPA, respectively. In control experiments, neither TPA nor NaF elicit histamine release at these concentrations.

Figure 3 shows the effect of 10 ng/mL cholera toxin (Fig. 3A) and 50 ng/mL pertussis toxin (Fig. 3B) on histamine release elicited by thapsigargin. Both toxins inhibit the effect of thapsigargin, cholera toxin progressively and pertussis toxin very sharply between 0.005 and 0.01 μM thapsigargin. The effect of both toxins is very slow, maximal inhibition being observed after 180 min preincubation with pertussis and after 90 min preincubation with cholera toxin. Therefore, Fig. 3 shows the effect of 3 and 1.5 hr preincubation with pertussis and cholera toxins, respectively. Under these conditions, we obtained mediator release with concentrations of thapsigargin which did not elicit any response in previous experiments (Figs 1 and 2), although we do not have an explanation for this phenomenon.

The activation of the Na^+–H^+ exchange can increase cytoplasmic pH, and the optimal pH for Ins(1, 4, 5)P_3 to induce Ca^{2+} mobilization is 8 [10]. Hence, the use of the PKC activator TPA (100 ng/mL) may facilitate the release of internal calcium. The effect of thapsigargin under several conditions on the activity of cytosolic pH recovery is shown in Fig. 4C. The recovery of cytosolic pH on rat mast cells is mostly due to the Na^+–H^+ antiporter, this effect being inhibitable with amiloride. As previously reported, TPA is a potent activator of Na^+–H^+ antiporter activity [4], this effect not being modified by 0.05 μM thapsigargin (Fig. 4A). Thapsigargin

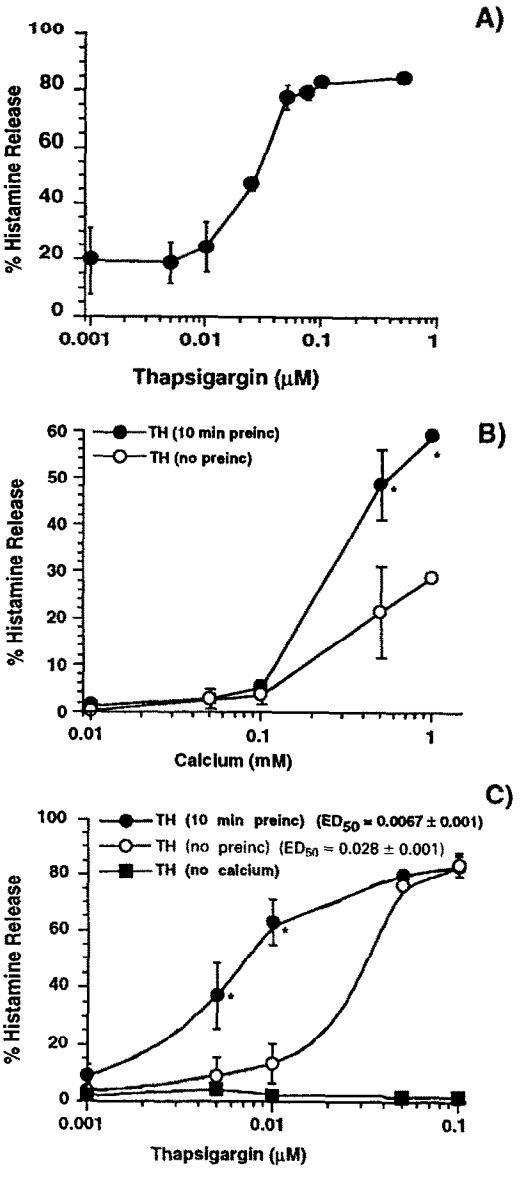

Fig. 1. (A) Histamine release elicited by thapsigargin. Mean ± SEM of nine experiments. (B) Effect of 10 min preincubation with thapsigargin on the sensitivity of the cells to calcium in a calcium-free medium before the addition of external calcium. Mean ± SEM of four experiments. (C) Effect of 10 min preincubation with thapsigargin on the sensitivity to thapsigargin in a calcium-free medium before the addition of external calcium. Significant differences with respect to control are indicated with an asterisk. Mean ± SEM of four experiments.

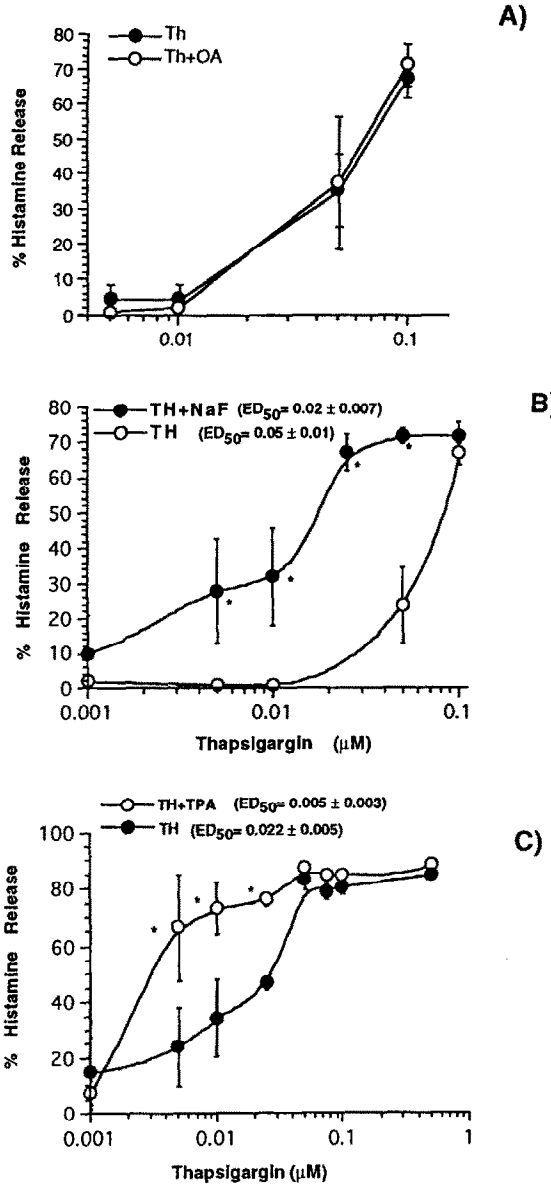

Fig. 2. (A) Effect of 1 μM okadaic acid on histamine release elicited by thapsigargin. Mean ± SEM of five experiments. (B) Effect of 10 mM sodium fluoride on histamine release elicited by 0.01 μM thapsigargin. Significant differences with respect to the control are indicated with an asterisk. Mean ± SEM of four experiments. (C) Effect of 100 ng/mL TPA on histamine release elicited by thapsigargin. Significant differences with respect to control are indicated with an asterisk. Mean ± SEM of five experiments.

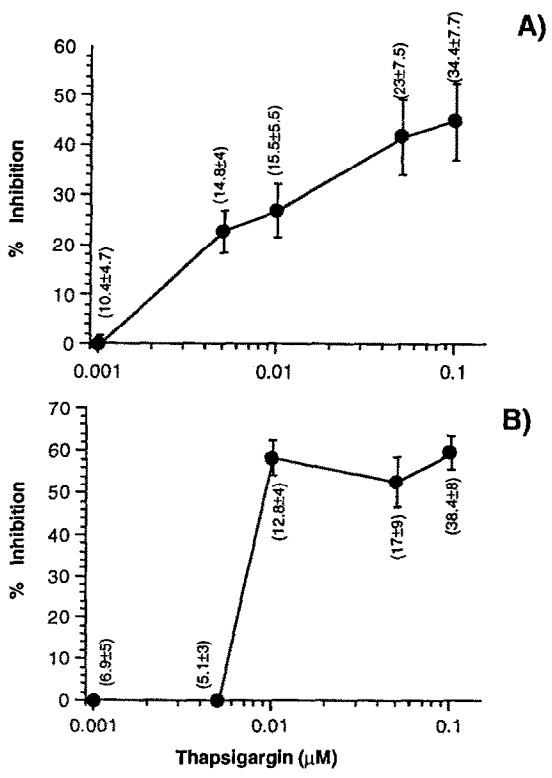

Fig. 3. (A) Effect of 10 ng/mL cholera toxin on histamine release elicited by thapsigargin. Cells were preincubated for 90 min with the toxin before adding thapsigargin. Histamine release values are indicated in parenthesis. Mean ± SEM of four experiments. (B) Effect of 50 ng/mL pertussis toxin on histamine release elicited by thapsigargin. Cells were preincubated for 180 min with the toxin before adding thapsigargin. Histamine release values are indicated in parenthesis. Mean ± SEM of four experiments.

itself stimulates the activity of the antiporter, but not as much as 100 ng/mL TPA, although these results do not differ statistically. Sodium fluoride (10 mM) does not modify the activity of the antiporter, but it is able to inhibit the action of thapsigargin (Fig. 4B). These effects are clearly calcium dependent, since in the absence of external calcium there is no significant change on the activity of the exchanger (Fig. 4C and D).

Since thapsigargin activates calcium influx, we studied the effect of blocking this influx. Figure 5 shows the effect of the calcium blockers cadmium (Fig. 5A) and lanthanum (Fig. 5B) on the activation of the Na^+–H^+ antiporter triggered by TPA and thapsigargin. The effect is essentially the same with both metals. They block the activation of the antiporter produced by TPA or thapsigargin.

The results obtained in Fig. 5 point to an important role in calcium influx, and therefore, we studied the kinetics of ^{45}Ca entry in the cells. The results are shown in Fig. 6. The most striking observation is the clear increase in calcium uptake when cells were preincubated with thapsigargin in a calcium-free medium. Under these conditions, the cells become activated with thapsigargin and within the first minutes after the addition of calcium, the influx is 71% higher than that of the control.

DISCUSSION

Thapsigargin is a tumor-promoter sesquiterpene lactone that potently and selectively inhibits the Ca^{2+}-ATPase of intracellular stores, hence emptying these reservoirs by allowing the accumulated Ca^{2+}

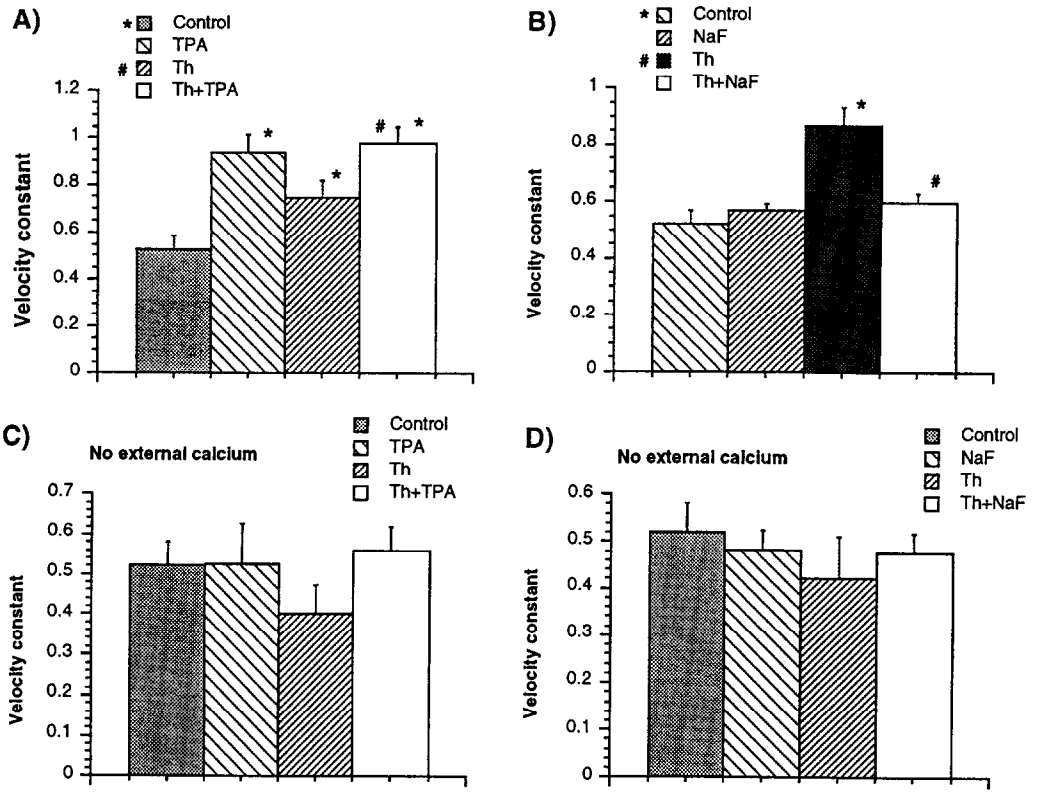

Fig. 4. (A) Effect of 100 ng/mL TPA and 0.05 μM thapsigargin on the rate of constant of the Na^+–H^+ antiport activity. Velocity constant units: pH units/min. Significant differences with respect to each control are indicated with a symbol. Mean ± SEM of six experiments. (B) Effect of 10 mM NaF and 0.05 μM thapsigargin on the rate constant of the Na^+–H^+ antiport activity. Velocity constant units: pH units/min. Significant differences with respect to each control are indicated with a symbol. Mean ± SEM of three experiments. (C) Effect of 100 ng/mL TPA and 0.05 μM thapsigargin on the rate constant of the Na^+–H^+ antiport activity in a calcium-free medium. Velocity constant units: pH units/min. Mean ± SEM of four experiments. (D) Effect of 10 mM NaF and 0.05 μM thapsigargin on the rate constant of the Na^+–H^+ antiport activity in a calcium-free medium. Velocity constant units: pH units/min. Mean ± SEM of four experiments.

to leak [8]. According to the capacitative model proposed by Putney [19], the depletion of Ca^{2+} from intracellular reservoirs signals the entry of calcium through the plasma membrane by an unknown mechanism. Therefore, thapsigargin increases cytosolic calcium by allowing calcium to leak from internal reservoirs, and by increasing the plasma permeability to calcium. The intracellular action of thapsigargin takes place mainly on the inositol triphosphate-dependent reservoirs [20].

The action of thapsigargin on rat mast cells is known to activate histamine release synergistically with the phorbol ester TPA [21, 22]. Also, it has been reported that rat peritoneal mast cells release histamine to calcium if they were previously incubated with thapsigargin in a calcium-free medium [23]. Our results confirm these observations and show that thapsigargin activates histamine release by a mechanism very sensitive to the action of protein kinase C, as concluded from the synergism observed with the phorbol ester TPA. The general increase of kinase activity through the unspecific action of fluoride elicits an increase in the response, while TPA potentiates the effect of thapsigargin. Therefore, there may be some inhibitory effect on the effect of some transducing signal not dependent of PKC. This is further sustained with the effect observed with okadaic acid.

Okadaic acid is a polyether fatty acid which was first obtained from the marine sponges *Halichondria okadaii* and *Halichondria melanodocia* [24]. It potently and selectively inhibits protein-serine/threonine phosphatases 1 and 2A, two of the major cytosolic phosphatases [25–27]. Therefore, the effect of okadaic acid is to induce a general hyperphosphorylation in the cell. Our results clearly indicate that this effect does not affect the response to thapsigargin. In a previous study we have shown that okadaic acid enhances the response evoked by sodium fluoride [18]. Therefore, although the actions of fluoride and thapsigargin are similar and calcium dependent, the intracellular signals that modulate both stimuli are completely different. Similarly, we have also reported that cAMP-active drugs inhibit

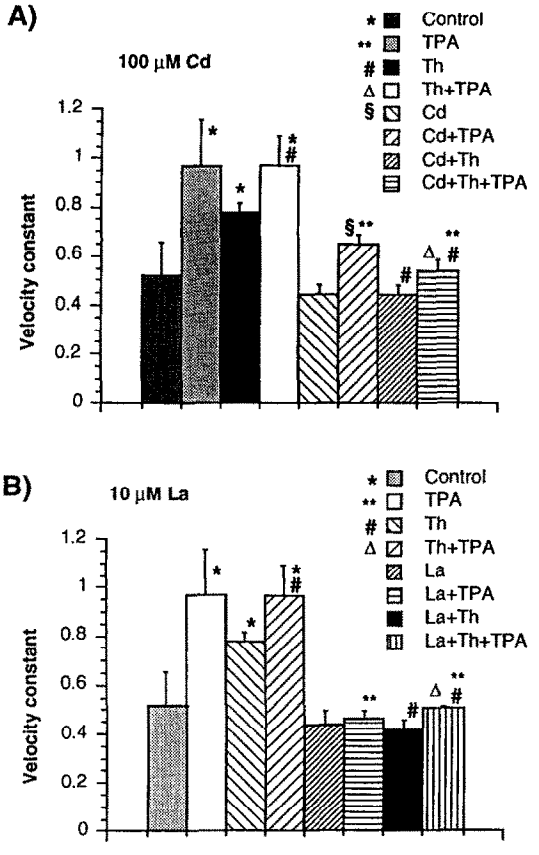

Fig. 5. (A) Effect of 100 ng/mL TPA and 0.05 μM thapsigargin on the rate constant of the Na^+–H^+ antiport activity both in the presence and the absence of 100 μM cadmium. Significant differences with respect to each control are indicated with a symbol. Mean ± SEM of three experiments. (B) Effect of 100 ng/mL TPA and 0.05 μM thapsigargin on the rate constant of the Na^+–H^+ antiport activity both in the presence and the absence of 10 μM lanthanum. Significant differences with respect to each control are indicated with a symbol. Mean ± SEM of three experiments, except Th and Th + TPA (five experiments).

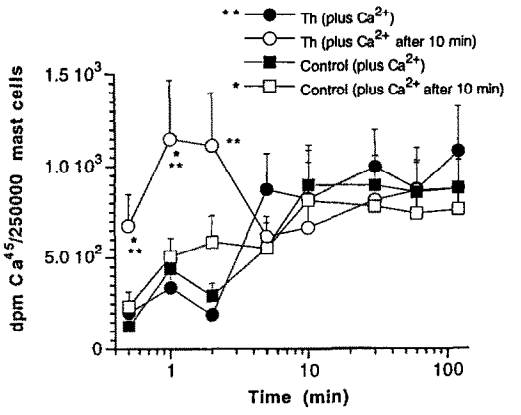

Fig. 6. ^{45}Ca uptake in cells treated with 0.5 μM thapsigargin. Thapsigargin was added in cells with external calcium (filled symbols) or 10 min before calcium (open symbols). Significant differences with respect to control (with 10 min preincubation) and thapsigargin (no preincubation before calcium) are indicated with one and two asterisks, respectively. Mean ± SEM of five experiments.

or slightly modify the response to fluoride, while cholera toxin strongly inhibits the response to thapsigargin. Since cholera toxin increases cAMP levels [28], this shows again that the intracellular mechanisms modulating the response to thapsigargin and fluoride are different.

The strong inhibition observed with pertussis toxin is very interesting. This drug has been reported to inhibit phospholipase C in mast cells [29], hence suggesting that the effect of thapsigargin is linked to the release of inositol triphosphate-dependent calcium. This result, together with the inhibition observed with cholera toxin, support a previous observation reporting an inhibitory effect of cAMP on calcium release from the endoplasmic reticulum [30]. The G-protein activation elicited by some cationic drugs such as compound 48/80 or mastoparan that leads to histamine release is also pertussis dependent [31]. Therefore, it is reasonable to think that these drugs have some mechanism in common with thapsigargin. In fact compound 48/80, mastoparan [32] or human GRF [33] activate histamine release by mobilizing calcium stored in intracellular reservoirs, similarly to thapsigargin [34]. Nevertheless, as far as we know, there is no description of direct G-protein activation by thapsigargin.

The mechanism by which both thapsigargin and fluoride elicit histamine release in an external calcium-dependent manner, suggest that both drugs increase calcium permeability. Putney's capacitative model suggest this phenomenon, and our results with ^{45}Ca further support this conclusion. The effect of thapsigargin added to cells in the presence of external calcium shows no increased calcium uptake, suggesting that the increased permeability is counteracted by an increased calcium extruding activity. This is supported for the fact that if thapsigargin is added in the absence of external calcium, the initial uptake of ^{45}Ca is 4-fold as compared to the control. But the mechanisms to eliminate calcium are activated rapidly and after 5 min the net uptake of calcium is again within control values. This elimination of internal calcium is ablated in the presence of lanthanum (results not shown), a known inhibitor of the calcium pump [35], which further supports our observation. Hoth and Penner have observed with different methodology that depletion of intracellular calcium stores activates a calcium current [36].

We have reported the stimulatory effect of the phorbol esther TPA on the Na^+–H^+ antiport in rat mast cells [4]. The effect of thapsigargin, although not as intense as TPA, is significant and calcium dependent, because in the absence of calcium this effect is not observed. Fluoride, on the other hand, does not modify the activity of the antiport by itself, and neutralizes the effect of thapsigargin on the antiport. The results obtained with lanthanum and cadmium, which are known to inhibit calcium influx in mast cells [37] and RBL-2H3 cells [38], suggest

two interesting conclusions. First, they confirm the calcium-dependent effect of thapsigargin on the antiport. Second, thapsigargin acts by increasing calcium turnover on the membrane, hence confirming the observation shown in Fig. 6. These results exclude the possibility that thapsigargin might act directly to activate the antiporter, because without a calcium influx, there is no effect.

We cannot elucidate the relationship between cytosolic pH, calcium and cellular exocytosis from our results. The actions of thapsigargin and TPA are synergistic on histamine release, but not on pH_i recovery. Some authors have pointed out opposite links between cytosolic pH and calcium, i.e. calcium mobilization is associated with intracellular acidification in vascular smooth muscle cells [39], while in platelets, protein kinase C enhances calcium mobilization coupled to alkaline cytoplasmic values [9]. This suggests that the relationships between calcium and pH are rather complex, in a similar way to the crosstalks between the different signal transduction pathways. Nevertheless, since the effect of TPA and thapsigargin are not additive, this suggests that the mechanisms by which both drugs activate the antiporter share several common steps, which supports previous observations in other cellular models suggesting that one of the roles of PKC is to alkalinize the cytosol to favor calcium release [40].

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