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Modulatory Effect of HCO_3^- on Rat Mast Cell Exocytosis: Cross-Talks between Bicarbonate and Calcium

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HCO_3^- modulation of histamine release and its relationship with the Ca^{2+} signal were studied in serosal rat mast cells. Histamine release was induced by Ca^{2+} mobilizing stimuli, namely compound 48/80, thapsigargin, Ca^{2+} chelators, ionophore A23187, and PMA and ionophore A23187 in a HCO_3^--buffered medium or a HCO_3^--free medium. The presence of HCO_3^- reduced histamine release by 48/80, Ca^{2+} chelators, A23187, and PMA/A23187, but increased histamine release induced by thapsigargin. Histamine release by PMA was significantly higher in a HCO_3^--free medium than in a HCO_3^--free medium, as it was the PMA potentiation of histamine release by A23187. $[Ca^{2+}]_i$ changes induced by these drugs were measured in fura-2-loaded mast cells. In thapsigargin and EGTA or BAPTA preincubated mast cells $[Ca^{2+}]_i$ increase was higher in a HCO_3^--buffered medium than in a HCO_3^--free medium in the presence of Ca^{2+}. On the contrary, in compound 48/80 and PMA/A23187 activated mast cells the $[Ca^{2+}]_i$ increase is the same both in the presence and in the absence of HCO_3^-. The effect of HCO_3^- on histamine release in serosal rat mast cells depends on the stimulus, but it is not related to the presence of Cl^-. In thapsigargin-stimulated mast cells the effect of HCO_3^- on histamine release may be related to the Ca^{2+} signal, but in compound 48/80, EGTA, and PMA/A23187-activated mast cells there is no relationship between intracellular Ca^{2+} and the inhibitory effect of HCO_3^- on histamine release. Additionally, the PKC pathway is implicated in the inhibitory effect of HCO_3^- on histamine release, the higher the chelation of calcium rendering the higher the enhancement of the response after adding calcium in the absence of HCO_3^-. © 1999 Academic Press

Key Words: **mast cell; intracellular calcium; histamine release; bicarbonate; compound 48/80; thapsigargin; PMA; A23187.**

The rat mast cell, a model used to study exocytosis and for its role in the allergic response, provides a suitable biological tool to understand the mechanisms that relate exocytosis to cytosolic pH. HCO_3^- ions are involved in different cellular functions, generally through their role in pH_i regulation (1, 2). Out of the different mechanisms that regulate cytosolic pH, the effect of HCO_3^- on mast cells has received very little attention, bearing in mind that most of the published works reporting research with these or similar cells do not use this ion on the medium (3). The HCO_3^- ions play also a role in the modulation of serosal rat mast cell function; indeed the presence of HCO_3^- in the medium reduces compound 48/80-induced histamine release (4). These authors demonstrate that the effect of HCO_3^- is not directly related to the regulation of pH_i by this ion, since the reduction of histamine release by HCO_3^- versus control without HCO_3^- occurs even when pH_i values are unchanged in HCO_3^--containing or HCO_3^--free medium. Compound 48/80 is a widely used univalent stimulus to activate the rat mast cell response (5).

The mechanism of action of compound 48/80 is by now not fully understood. It seems to act through a direct activation of G proteins, although 48/80 does activate more than one G protein which elicit different signaling pathways (6). Another generally admitted feature of compound 48/80 mechanism of action is a $[Ca^{2+}]_i$ increase (7). Compound 48/80 increases PLC activity and hence the cytosolic levels of inositol phosphates (8, 9); since cytosolic IP_3 depletes intracellular Ca^{2+} stores and induce a Ca^{2+} entry in rat mast cells (10), this is probably the mechanism of the Ca^{2+} signal induced by compound 48/80 in mast cells (7).

Thus far, no group has directly studied the role of cytosolic pH regulation on calcium levels in rat mast cells, much less the role of HCO_3^- (11). The Ca^{2+} signal

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Abbreviations used: BAPTA, 1,2-bis-(-2-aminophenoxy)ethane-*N*,*N*,*N*′-*N*′-tetraacetic acid acetoxymethylester; $BAPTA_i$, intracellular BAPTA; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; EGTA, ethylene glycol bis-*N*,*N*,*N*′,*N*′-tetraacetic acid); $EGTA_o$, extracellular EGTA; PMA, phorbol 12-myristate 13-acetate; pH_i, cytosolic pH; pH_o, external pH.

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can be elicited in mast cells by other Ca^{2+} mobilizing stimuli with different mechanisms of action. Thapsigargin, an intracellular Ca^{2+}-ATPase inhibitor (12), or Ca^{2+} chelators (10) are able to elicit the Ca^{2+} signal without an increase of IP_3 levels. Both mechanisms induce a passive depletion of intracellular Ca^{2+} stores and a Ca^{2+} entry in mast cells (10, 13). Thapsigargin causes histamine release when the Ca^{2+} influx takes place, but not in the absence of external Ca^{2+} (14). Another Ca^{2+} dependent stimulus with a completely different mechanism of action is the calcium ionophore A23187 (15). Moreover the synergistic action of the phorbol ester PMA and ionophore A23187 to induce histamine release revealed the implication of both PKC and calcium signaling pathways on mast cell degranulation (16, 17). We studied the role of both signaling pathways in HCO_3^- modulation of histamine release in rat mast cells.

METHODS

Chemicals and solutions. Fura-2/AM was purchased from Molecular Probes (Eugene, OR). Compound 48/80, ionophore A23187, ionophore Br-A23187, EGTA (ethylene glycol bis-*N,N,N′,N′*-tetraacetic acid), PMA (phorbol 12-myristate 13-acetate) and BAPTA/AM (1,2-bis-(-2-aminophenoxy)ethane-*N,N,N′-N′*-tetraacetic acid acetoxymethylester) were from Sigma Chemical (St. Louis, MO). Thapsigargin was from Alexis (Läufelfingen, Switzerland) and Percoll was from Pharmacia (Uppsala, Sweden). The HCO_3^--buffered medium contains (in mM): Na^+, 142.3; K^+, 5.94; Ca^{2+}, 1; Mg^{2+}, 1.2; Cl^-, 126.1; HCO_3^-, 22.85; $PO_4H_2^-$, 1.2 and SO_4^{2-}, 1.2. The composition of the HCO_3^--free solution was (in mM): Na^+, 146.89; K^+, 5.94; Ca^{2+}, 1; Mg^{2+}, 1.2; Cl^-, 125.94; $PO_4H_2^-$, 7.70; PO_4H^{2-}, 12.49 and SO_4^{2-},1.2. Glucose (1 mg/ml) was added to the solutions and pH was adjusted to 7.20. Mast cell purification was performed with a HCO_3^--buffered solution containing BSA 1 mg/ml adjusted to pH 7.20.

Mast cell isolation and purification. Mast cells were obtained by lavage of pleural and peritoneal cavities of Sprague–Dawley rats (400–800 g), as described earlier (3). Briefly, rats were blooded and 10 ml of the purification solution were introduced in the peritoneal cavity through a small hole. After a smooth massage, the solution was recovered with mast cells suspended in it. The pleural cavity lavage was performed with 5 ml of the solution in the same way. Total cells collected from one rat were centrifuged at 900 rpm for 4 min and suspended in a final volume of 1 ml. Purification was carried through 4 ml of 95% isotonic Percoll at 1500 rpm for 10 min. Then mast cells were washed twice with the purification solution. The purity of mast cells was higher than 95% and viability was higher than 97% as judged by trypan blue staining.

$[Ca^{2+}]_i$ measurements. Purified mast cells were incubated in the purification solution with 0.3 μM fura-2/AM for 10 min at 37°C and then washed twice with a BSA-free purification solution. Mast cells were resuspended at 400.000 cells/ml in a quartz cuvette with continuous stirring. Experiments were carried out at 37°C. The experiments were performed in a Perkin–Elmer LS-50 B spectrofluorometer with automatic calculation of the ratio values for excitation at 340 nm/380 nm and emission at 500 nm. The calibration and calculation of $[Ca^{2+}]_i$ was performed as described (18). R_{max} was obtained with 0.1% Triton-X 100 in the presence of 1 mM external Ca^{2+} and R_{min} with 60 mM EGTA. The drugs stock solutions were 100 fold concentrated in freshly made DMSO.

Measurement of histamine release. The experiments of histamine release were performed with the total cellular population. The cells from one rat were centrifuged at 900 rpm 4 min and suspended in a small volume. Then mast cells were distributed in a small volume in incubation tubes with the required medium, yielding a final incubation volume of 500 μL and an approximate concentration of 1×10^5 mast cells/tube. The incubations were performed at 37°C. Once the cellular suspension reached the incubation temperature the stimulus was added 40 fold concentrated. Histamine release was stopped at the required time in an ice-cooled bath. The cellular suspension was centrifuged at 2000 rpm 3 min. The pellets were resuspended in 500 μL of 0.1 N HCl and sonnicated for 60 s. Histamine was assayed fluorometrically both in the pellet (non released histamine) and in the supernatants (released histamine) by the Shore's method (19), that was adapted to a microplate. Fluorescence was measured in a microplate Labsystems Fluoroskan II fluorometer with a 355 nm excitation filter and a 460 nm emission filter. The results are shown as a percentage of histamine release with respect to total histamine content, and corrected for spontaneous histamine release measured without stimulation and in the same conditions. The equation used was $HR = (S - ER)/(S + P) \times 100$, being HR the percentage of histamine release; S, supernatant fluorescence, ER, media of fluorescence of spontaneous release supernatants; P, pellet fluorescence.

Statistical analysis. All the experiments were carried out at least three times in duplicate. 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 means ± SEM.

RESULTS

The presence or absence of HCO_3^- ions in the external medium sets a different response of mast cells to compound 48/80 (4). To check if the effect of HCO_3^- is related to the Ca^{2+} signal, we studied the Ca^{2+} signal and the histamine release induced by different Ca^{2+}-mobilizing stimuli, namely compound 48/80, thapsigargin, Ca^{2+} chelators, ionophore A23187 and the synergic combination of PMA and ionophore A23187.

We first observed that, in our experimental conditions, mast cells stimulated with compound 48/80 release more histamine in a HCO_3^--free medium than in a HCO_3^--buffered one. Mast cells were incubated for 10 min with increasing concentrations of compound 48/80 in a HCO_3^--buffered or a HCO_3^--free medium in the presence of 1 mM external Ca^{2+}. The dose–response curve of compound 48/80 was shifted to the left in a HCO_3^--free medium versus a HCO_3^--buffered one (Fig. 1). As we said above similar results were obtained in a HCO_3^--buffered medium (4). In the same experiment another group of mast cells were suspended in a Cl^--free, HCO_3^--free medium and yielded a dose–response curve that matches exactly with that obtained in the HCO_3^--free medium.

We next recorded the $[Ca^{2+}]_i$ changes elicited by compound 48/80 in fura-2-loaded mast cells. Mast cells were stimulated with 1 μg/ml compound 48/80 since at this concentration the biggest difference was observed between the mast cell response in both media (Fig. 1). Mast cells were preincubated for 5 min in Ca^{2+}-free conditions in a HCO_3^--buffered or a HCO_3^--free medium, and then 1 μg/ml compound 48/80 and 1 mM $CaCl_2$ were added simultaneously to the medium. After

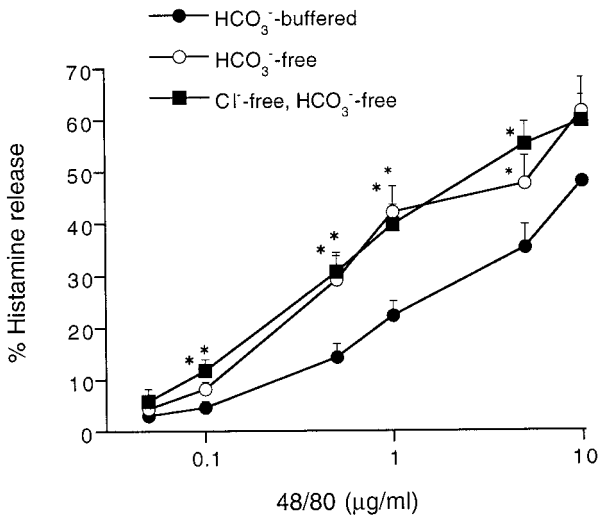

FIG. 1. Dose–response curve of histamine release by compound 48/80 in a HCO_3^--buffered, a HCO_3^--free and a Cl^--free, HCO_3^--free medium. Mast cells were suspended in a HCO_3^--buffered, a HCO_3^--free or a Cl^--free, HCO_3^--free medium and stimulated with increasing concentrations of compound 48/80 for 10 min. Then histamine release was measured and expressed as a percentage (media ± SEM, $n = 8$) (* significant differences).

the addition of compound 48/80 mast cells undergo an immediate $[Ca^{2+}]_i$ rise, with an initial spike followed by an elevated $[Ca^{2+}]_i$ plateau (Fig. 2A). This profile of $[Ca^{2+}]_i$ rise is similar to that elicited by antigen in RBL-2H3 cells (13). There was no difference between the $[Ca^{2+}]_i$ rise in a HCO_3^--buffered medium and in a HCO_3^--free medium (Fig. 2A). We observed that basal $[Ca^{2+}]_i$ is a bit high due to dye bleaching during the 5 min preincubation. Histamine release was measured in the same conditions. 1 μg/ml compound 48/80 and 1 mM $CaCl_2$ were added simultaneously to mast cells suspended in a HCO_3^--buffered or a HCO_3^--free medium and histamine release was measured at increasing times. The kinetic of histamine release by compound 48/80 is very fast ((20) and Fig. 2B) and the presence or absence of HCO_3^- make no difference in this kinetic (Fig. 2B). At 90 s after compound 48/80 stimulation histamine release was significantly higher in a HCO_3^--free medium than in a HCO_3^--buffered one (Fig. 2B), meanwhile at this time $[Ca^{2+}]_i$ is the same in both media (Fig. 2A). Since compound 48/80 is an external Ca^{2+} independent stimulus, which means that it does not need the presence of external Ca^{2+} to induce histamine release (21), the same experiment was repeated but in the absence of external Ca^{2+}. Mast cells were suspended in HCO_3^--buffered or HCO_3^--free medium without Ca^{2+} and added with 1 μg/ml compound 48/80. $[Ca^{2+}]_i$ rises upon addition of compound 48/80 in the absence of external Ca^{2+} showing a similar profile to that shown in the presence of Ca^{2+}, but significantly higher in a HCO_3^--buffered medium than in a HCO_3^--free medium (Fig. 3A). The $[Ca^{2+}]_i$ profile induced by compound 48/80 is similar in the presence or absence of external Ca^{2+} (Figs. 2A and 3A), which might suggest that there is no Ca^{2+} entry in compound 48/80-stimulated mast cells in a HCO_3^--buffered medium. However, we can not compare both plots because these experiments were performed in different days and with different mast cell populations, and mast cells show a high heterogeneity in their Ca^{2+} response (unpublished results in a fluorescence microscope and (22)). Regardless the major difference on calcium levels, in the absence of external Ca^{2+} histamine release by 1 μg/ml compound 48/80 was the same in a HCO_3^--buffered and in a HCO_3^--free medium (Fig. 3B).

Thapsigargin is a Ca^{2+} dependent stimulus in rat serosal mast cells, that only releases histamine in the presence of external Ca^{2+} (14, 23, 24). This drug is an

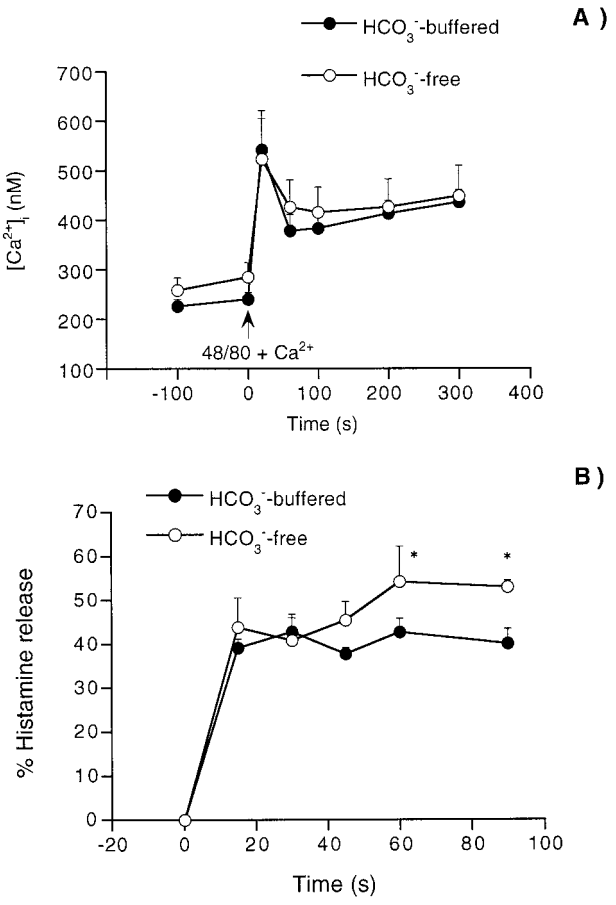

FIG. 2. Kinetics of cytosolic Ca^{2+} changes (A) and histamine release (B) induced by compound 48/80 in the presence of Ca^{2+} in mast cells suspended in a HCO_3^--buffered or a HCO_3^--free medium. (A) Fura-2 loaded mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium in the absence of Ca^{2+}. After 5 min of preincubation 1 μg/ml compound 48/80 and 1 mM Ca^{2+} were added simultaneously to the medium. (B) Mast cells were preincubated for 5 min in the absence of Ca^{2+} and then 1 μg/ml compound 48/80 and 1 mM Ca^{2+} were added to the external medium. Histamine release was measured at sequential times (media ± SEM, $n = 3$) (* significant differences).

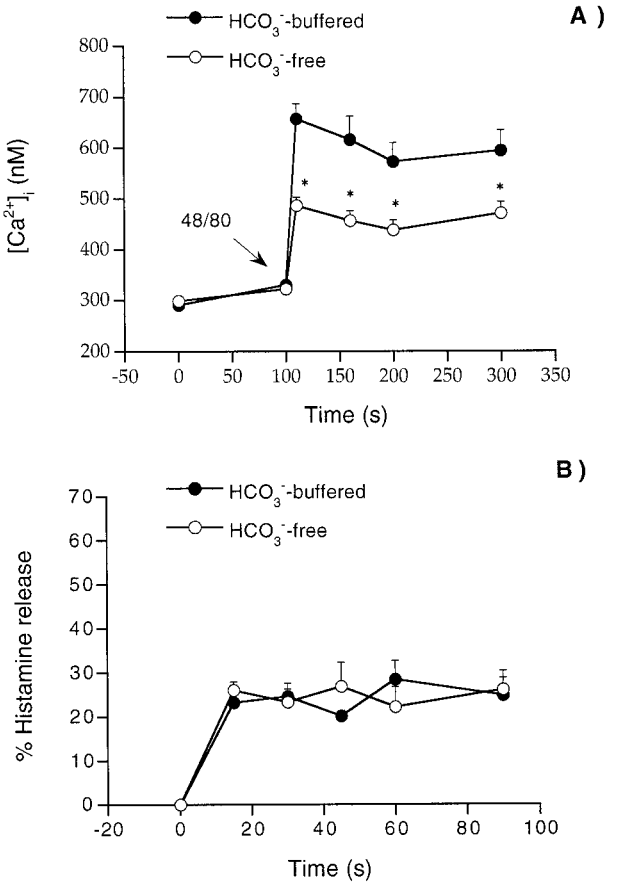

FIG. 3. Kinetics of cytosolic Ca^{2+} changes (A) and histamine release (B) induced by compound 48/80 in the absence of external Ca^{2+} in mast cells suspended in a HCO_3^--buffered or a HCO_3^--free medium. (A) Fura-2 loaded mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium in the absence of Ca^{2+}. After 5 min of preincubation 1 μg/ml compound 48/80 was added to the medium. (B) Mast cells were preincubated for 5 min in the absence of Ca^{2+} and then 1 μg/ml compound 48/80 was added to the external medium. Histamine release was measured at sequential times (media ± SEM, $n = 3$) (* significant differences).

intracellular Ca^{2+}-ATPase inhibitor (12) commonly used to elicit depletion of intracellular Ca^{2+} stores and Ca^{2+} entry without an increase of cytosolic IP_3 levels in a variety of cellular models, included mast cells (13, 25). Mast cells were stimulated with 0.05 μM thapsigargin in the presence of external Ca^{2+}, and $[Ca^{2+}]_i$ started to rise slowly immediately after the addition of thapsigargin. During the first 100 s the $[Ca^{2+}]_i$ rise was similar either in the presence or absence of HCO_3^-, but after that time the $[Ca^{2+}]_i$ became higher in a HCO_3^--buffered medium compared to a HCO_3^--free one (Fig. 4A). Histamine release was measured in these cells, which were recovered from the quartz cuvette 300 s after thapsigargin addition and cooled in an ice bath. We used the very same cells used to study calcium because the response to thapsigargin is highly variable among populations. Histamine release was higher in a HCO_3^--buffered medium than in a HCO_3^--free one (Fig. 4B). In thapsigargin-stimulated mast cells, $[Ca^{2+}]_i$ rise and histamine release are higher in the presence of HCO_3^- than in its absence, hence in this case the effect of HCO_3^- on the Ca^{2+} entry could be related to HCO_3^- modulation of histamine release.

Ca^{2+} chelators, as well as thapsigargin, induce Ca^{2+} entry by passive depletion of intracellular Ca^{2+} stores (10). $[Ca^{2+}]_i$ was recorded in mast cells preincubated in the presence of 1 mM external EGTA ($EGTA_o$) for 10 min and then added with 1 mM free Ca^{2+}. In the presence of $EGTA_o$ the basal $[Ca^{2+}]_i$ in resting cells was lower than in its absence. The addition of Ca^{2+} induced a fast $[Ca^{2+}]_i$ increase both in the presence and absence of HCO_3^-, but $[Ca^{2+}]_i$ reached higher values in a HCO_3^--buffered medium than in a HCO_3^--free medium (Fig. 5A). Additionally histamine release was measured in mast cells preincubated in the presence of external or

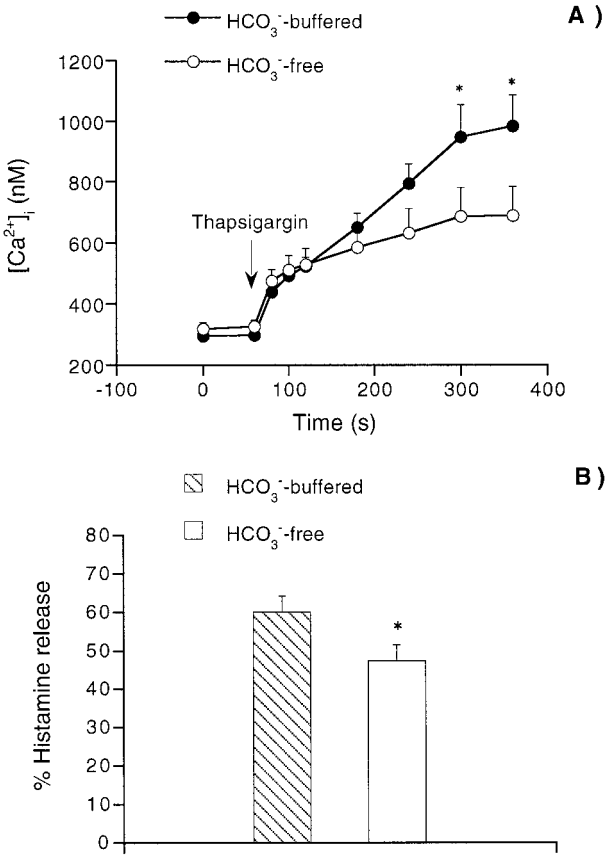

FIG. 4. Thapsigargin-induced $[Ca^{2+}]_i$ changes (A) and histamine release (B) in a HCO_3^--buffered or a HCO_3^--free medium in the presence of 1 mM external Ca^{2+} in mast cells. (A) Fura-2 loaded mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium with 1 mM external Ca^{2+}. After a steady-state $[Ca^{2+}]_i$ was reached, we added 0.05 μM thapsigargin. (B) In the same experiment, the mast cells were recovered from the quartz cuvette 300 s after the addition of thapsigargin and degranulation was stopped in an ice-cooled bath. Histamine release was measured and expressed as a percentage (media ± SEM, $n = 3$) (* significant differences).

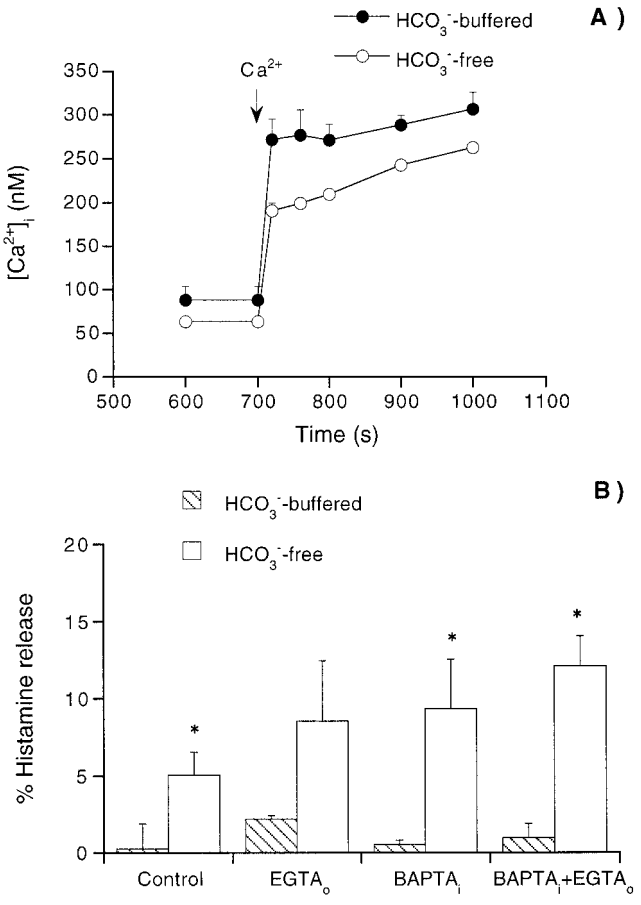

FIG. 5. $[Ca^{2+}]_i$ changes (A) and histamine release (B) in mast cells preincubated with Ca^{2+} chelators and added with Ca^{2+} in a HCO_3^--buffered or a HCO_3^--free medium. (A) Fura-2 loaded mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium with 1 mM external EGTA. After 10 min 2 mM $CaCl_2$ was added to the medium yielding a free Ca^{2+} concentration of 1 mM. (B) Mast cells and BAPTA-loaded mast cells were preincubated for 10 min in the nominal absence of Ca^{2+} or in the presence of 1 mM EGTA. Then 1 mM free Ca^{2+} was added to normal mast cells and 5 mM free Ca was added to BAPTA-loaded mast cells. After 10 min of incubation histamine release was stopped in an ice-cooled bath and the percentage of histamine release was measured (media ± SEM, $n = 3$) (* significant differences).

internal Ca^{2+} chelators. Mast cells were incubated for 10 min in the presence of 1 mM $EGTA_o$ in a HCO_3^--buffered or a HCO_3^--free medium. Control mast cells were incubated in the nominal absence of Ca^{2+}. After this preincubation 1 mM free Ca^{2+} (1 mM $CaCl_2$ in control mast cells and 2 mM $CaCl_2$ in $EGTA_o$-preincubated mast cells) was added to the medium. Histamine release was measured 10 min later. As shown, results clearly indicate that Ca^{2+}-depleted mast cells release histamine in response to calcium, therefore, calcium itself can be a stimulus for histamine release. The results show a higher release of histamine in a HCO_3^--free medium than in a HCO_3^--buffered medium when the stimulus is the addition of Ca^{2+} after depletion of intracellular stores by the absence of external Ca^{2+} or by external Ca^{2+} chelation (Fig. 5B). Simultaneously mast cells were loaded with BAPTA in order to induce the depletion of intracellular Ca^{2+} stores and to eliminate any $[Ca^{2+}]_i$ rise. Mast cells were incubated in the presence of 16 μM BAPTA/AM for 20 min and then washed. BAPTA-loaded mast cells were preincubated for 10 min in a Ca^{2+}-free medium or in the presence of 1 mM $EGTA_o$ and then 5 mM free Ca^{2+} was added. There is a significantly higher histamine release upon addition of Ca^{2+} in a HCO_3^--free medium than in a HCO_3^--buffered medium (Fig. 5B).

Next, we combined the action on two signaling pathways present in mast cells: the Ca^{2+} signal and the PKC activation, due to the well known potentiation by PMA of the ionophore A23187-induced histamine release (16, 17). Mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium and preincubated for 10 min with 100 ng/ml PMA in the nominal absence of Ca^{2+}, and then 2 μM ionophore A23187 and 1 mM $CaCl_2$ were added to the medium. Control cells followed the same time course in the absence of PMA. $[Ca^{2+}]_i$ changes and histamine release were measured under these conditions. The addition of PMA induced no change in $[Ca^{2+}]_i$. Ionophore A23187 and Ca^{2+} added together after a 10 min preincubation with PMA, induced a $[Ca^{2+}]_i$ rise that showed no difference in the presence or absence of HCO_3^- (Fig. 6A), similar results were obtained for control cells (data not shown). On the other hand histamine release was measured in the same conditions at sequential times after the addition of ionophore A23187 and Ca^{2+}. Mast cells response to PMA plus A23187 was much higher in a HCO_3^--free medium than in a HCO_3^--buffered one (Fig. 6B). In fact, the increase of histamine release induced by the absence of HCO_3^- is smaller in control cells stimulated only with ionophore A23187 (Fig. 6C) than in PMA preincubated mast cells (Fig. 6B). The potentiating effect of PMA over the ionophore-elicited histamine release is also higher in a HCO_3^--free than in a HCO_3^--buffered medium (Figs. 6B and 6C). Moreover PMA alone induced a 20% histamine release after 10 min of incubation in a HCO_3^--free medium without a $[Ca^{2+}]_i$ increase, but in a HCO_3^--buffered medium PMA did not induce histamine release (Fig. 6D).

Since PMA effect and Ca^{2+} chelation seem to be related to the effect of HCO_3^- on mast cells, the experiments with Ca^{2+} chelators were repeated in the presence of PMA. Firstly, histamine release was measured in mast cells activated with PMA in a Ca^{2+}-free medium and an EGTA-containing medium (Fig. 7A). In a Ca^{2+}-free medium, PMA induced a higher histamine release in the presence of HCO_3^- than in its absence, as it was previously described (Fig. 6D). On the contrary, in the presence of 1 mM $EGTA_o$, a 10 min incubation with 100 ng/ml PMA induced the same mast cell response in the either presence or absence of HCO_3^-

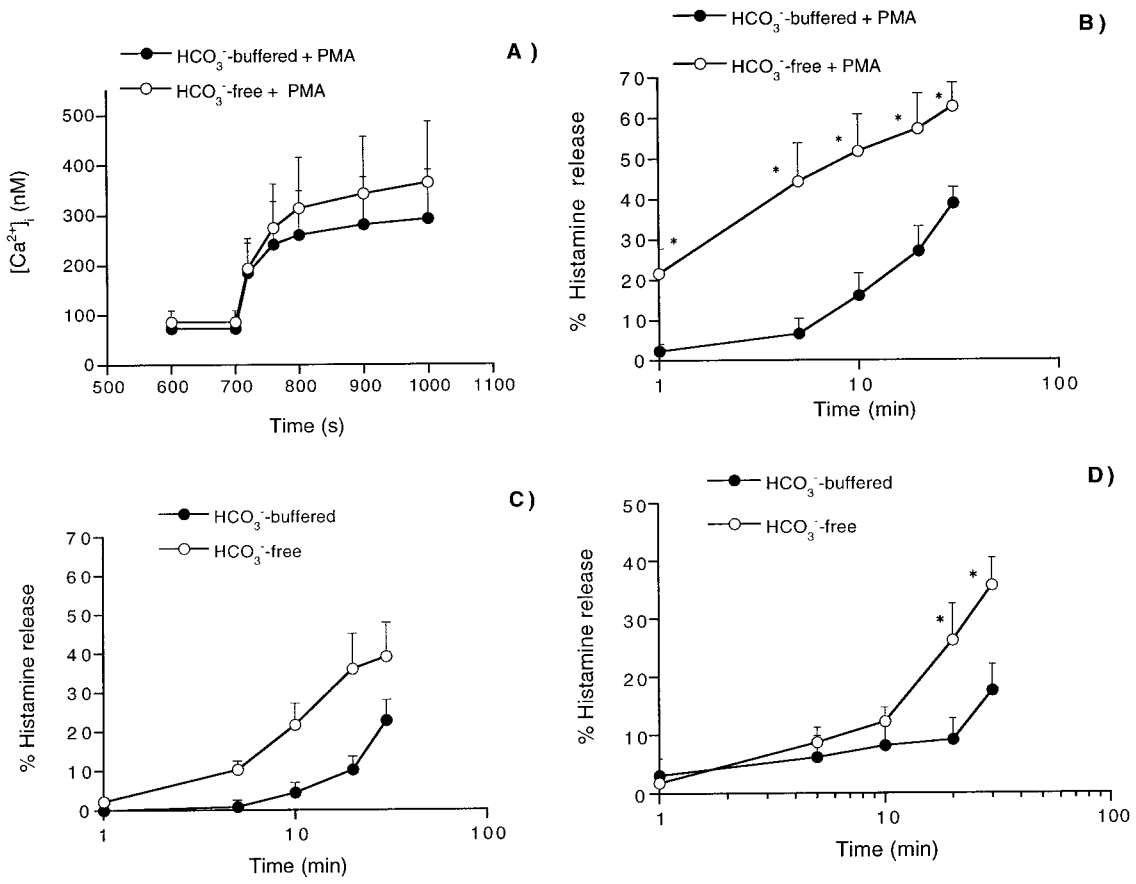

FIG. 6. Kinetics of $[Ca^{2+}]_i$ changes (A) and histamine release (B and C) induced by PMA plus ionophore A23187 in fura-2-loaded rat mast cells. (A) Fura-2-loaded rat mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium both of them without Ca^{2+}. After a 10 min preincubation with 100 ng/ml PMA, we added 2 μM ionophore Br-A23187 and 1 mM Ca^{2+} simultaneously (media ± SEM, $n = 3$). (B) Rat mast cells were suspended in a HCO_3^--buffered or a HCO_3^--free medium without 1 mM external Ca^{2+} and preincubated for 10 min with 100 ng/ml PMA. Then 2 μM ionophore A23187 and 1 mM Ca^{2+} were added to the medium and histamine release was measured at sequential times. (media ± SEM, $n = 5$). (C) The control for histamine release which followed the same time course without PMA (media ± SEM, $n = 5$) (* significant differences). (D) Histamine release kinetics in cells stimulated with PMA in a HCO_3^--buffered or a HCO_3^--free medium in the presence of 1 mM Ca^{2+} (media ± SEM, $n = 5$) (* significant differences).

(Fig. 7A). Secondly, the stimulation of mast cells with Ca^{2+} chelators and the posterior addition of Ca^{2+} were performed in the presence of 100 ng/ml PMA (Fig. 7B). In this experiment, mast cells were preincubated in the presence of 100 ng/ml PMA and 1 mM $EGTA_o$ and/or $BAPTA_i$ for 10 min, and then $CaCl_2$ was added to the medium. In the case of BAPTA-loaded mast cells, the concentration of external free Ca^{2+} was 5 mM; in the absence of BAPTA the external free Ca^{2+} concentration was 1 mM. Histamine release was measured 10 min after the addition of Ca^{2+}. Mast cells preincubated with PMA in a Ca^{2+} free medium elicited a 30% histamine release 10 min after the addition of Ca^{2+} in a HCO_3^--free medium, and less than 5% in the presence of HCO_3^- (Fig. 7B). In the presence of Ca^{2+} chelators, histamine release upon addition of Ca^{2+} in PMA-preincubated mast cells is higher in a HCO_3^--free than in a HCO_3^--buffered medium (Fig. 7C). Additionally, PMA induced a potentiation of the response of mast cells preincubated with Ca^{2+} chelators (compare Figs. 5B and 7C), which demonstrates a synergistic interaction between PKC and calcium signaling pathways.

DISCUSSION

Effect of HCO_3^- on histamine release. In this paper, we study the modulatory effect of bicarbonate on rat mast cell secretion. This study is important because bicarbonate is usually omitted on the composition medium by all researchers, though it is a fundamental part of the composition of the external medium in physiological conditions. All previous work of our group has been carried out in the presence of bicarbonate, and recently, a paper by another group has included this ion on the composition medium (4). Our results demonstrate that HCO_3^- ions do modulate mast cell degranulation and that its effect depends on the degranulating stimulus. Compound 48/80, addition of

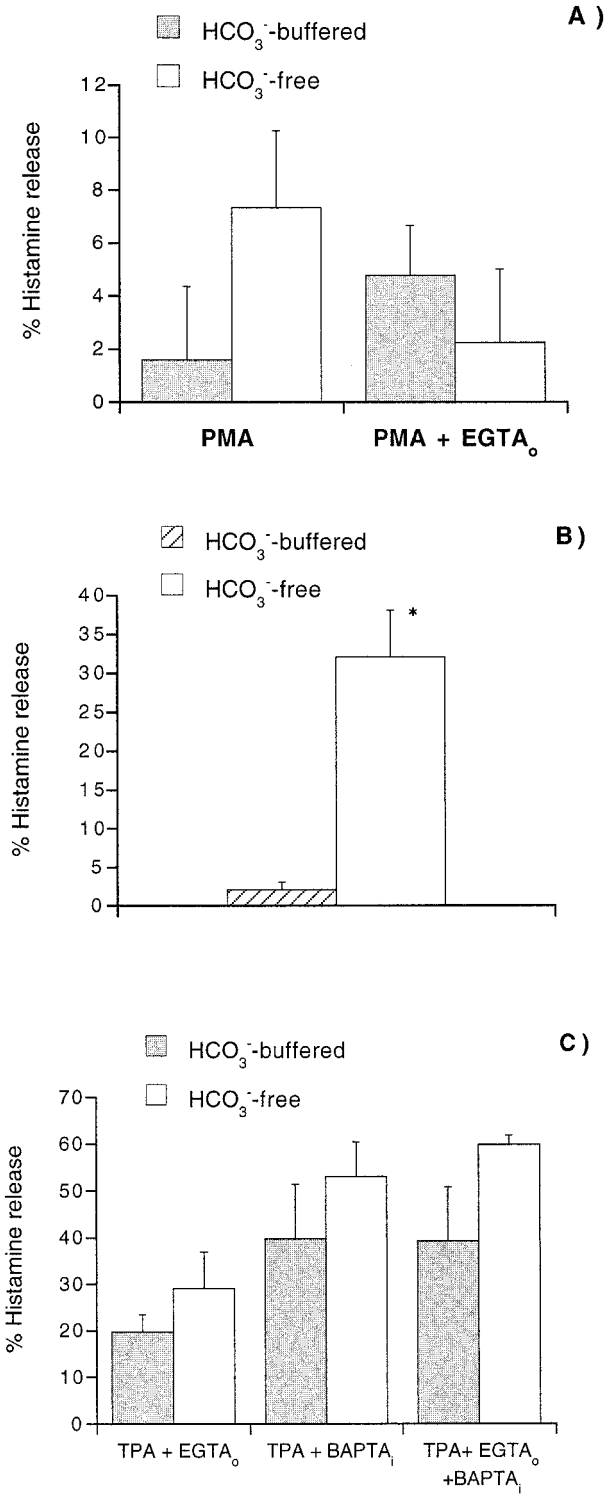

FIG. 7. Histamine release in mast cells preincubated with Ca^{2+} chelators and PMA in a HCO_3^--buffered or a HCO_3^--free medium. (A) Mast cells were incubated with 100 ng/ml PMA or 100 ng/ml PMA and 1 mM EGTA for 10 min and histamine release was measured. (B) Mast cells were preincubated for 10 min with 100 ng/ml PMA, in the nominal absence of Ca^{2+}. Then 1 mM free Ca^{2+} was added (media ± SEM, $n = 3$). (C) Mast cells and BAPTA-loaded mast cells were preincubated for 10 min with 100 ng/ml PMA, in the nominal absence of Ca^{2+} or in the presence of 1 mM $EGTA_o$. Then 1 mM free Ca^{2+} was added to normal mast cells and 5 mM free Ca^{2+} was added to BAPTA-loaded mast cells (media ± SEM, $n = 3$).

Ca^{2+} to Ca^{2+} depleted cells, the ionophore A23187 and the combinations of the phorbol ester PMA and ionophore A23187 or PMA and Ca^{2+} chelators induced higher histamine release in a HCO_3^--free medium than in a HCO_3^--buffered medium. Similar results have been described before for compound 48/80 in serosal rat mast cells (4). On the contrary, thapsigargin-induced histamine release was higher in a HCO_3^--buffered medium than in a HCO_3^--free one. An inhibition of antigen and ionomycin-induced secretion by the absence of HCO_3^- was described in RBL-2H3 cells, a mast cell line (26). In the absence of external Ca^{2+} compound 48/80-induced histamine release is the same in the presence or absence of HCO_3^-, thus the effect of HCO_3^- on histamine release seems to be related to Ca^{2+} entry and not to depletion of intracellular Ca^{2+} stores. On the other hand, the different modulatory effect of HCO_3^- on histamine release by these stimuli must be due to the different mechanism of action of each drug, and suggest that HCO_3^- may modulate more than one signaling pathway.

Is the effect of HCO_3^- on histamine release related to intracellular Ca^{2+}? We hypothesized that the effect of HCO_3^- on histamine release could be mediated by the intracellular Ca^{2+} signal. The Ca^{2+} signal is modulated by other ion fluxes such as a Cl^- influx (7, 27), which may provide the driving force for the Ca^{2+} entry. The modification of the HCO_3^- gradient may alter Cl^- or other ion fluxes and thus Ca^{2+} influx. Although we have recently reported that bicarbonate influx is independent of Cl^- channels (11), the presence of HCO_3^- in the medium increases intracellular Cl^- concentration (4). This observation might be linked to the levels of HCO_3^- and Ca^{2+}. However, in our experimental conditions, the stimulation of mast cells with compound 48/80 in a Cl-free and HCO_3^--free medium yield a higher histamine release than in control conditions with Cl^- and HCO_3^-, and the same histamine release as in a Cl^--containing, HCO_3^--free medium. Thus the inhibitory effect of HCO_3^- is not mediated by a decrease of an inward Cl^- current, since in the absence of external Cl^-, which inhibits the Cl^- current, histamine release is the same as in Cl^--containing, HCO_3^--free conditions. Our results demonstrate that there is no relationship between the inhibitory effect of HCO_3^- on histamine release and the Ca^{2+} signal, or at least, that the nature of the effect is stimulus-dependent, thus relating the effect to a transducing signal. In fact, compound 48/80 induced a higher histamine release in a HCO_3^--free medium than in a HCO_3^--buffered medium in the presence of 1 mM Ca^{2+}, but the $[Ca^{2+}]_i$ increase

was equal in mast cells suspended in both media. Moreover, in the nominal absence of external Ca^{2+} the histamine release elicited by compound 48/80 is the same when HCO_3^- is absent or present, but the intracellular Ca^{2+} rise is significantly lower in a HCO_3^--free medium than in a HCO_3^--buffered one. At this point, it seems that the intracellular Ca^{2+} rise is not quantitatively related to histamine release, and that the HCO_3^- effect on compound 48/80-induced degranulation is not mediated by Ca^{2+} but by some other signaling pathway.

The intracellular Ca^{2+} signal was also elicited by passive depletion of intracellular Ca^{2+} stores with thapsigargin and Ca^{2+} chelators in mast cells (10, 13). In both cases the $[Ca^{2+}]_i$ increase was higher in a HCO_3^--buffered medium than in a HCO_3^--free medium. Thapsigargin-stimulated mast cells showed a higher histamine release and a higher Ca^{2+} entry in a HCO_3^--buffered than in a HCO_3^--free medium. Similar results were obtained in RBL-2H3 cells, where the absence of HCO_3^- inhibits both degranulation and Ca^{2+} entry induced by antigen or ionomycin (26). Although RBL-2H3 cells show some differences with serosal rat mast cells, i.e., they are refractory to stimulation with compound 48/80 (28, 29), these authors also reported a parallel inhibition of degranulation and Ca^{2+} entry in the absence of HCO_3^-, as we found for thapsigargin in serosal rat mast cells. On the contrary, when the Ca^{2+} entry was induced by addition of external Ca^{2+} after extracellular or intracellular Ca^{2+} chelation, histamine release was higher in a HCO_3^--free medium than in a HCO_3^--buffered medium. These results demonstrate that only in the case of thapsigargin the effect of HCO_3^- on the Ca^{2+} entry could explain the negative modulation of HCO_3^- on histamine release; also, that the inhibitory effect of HCO_3^- on histamine release is not related to the Ca^{2+} signal.

The Ca^{2+} signal and the PKC pathway have a synergistic action in the induction of histamine release (14, 16, 17). The intracellular Ca^{2+} rise elicited by the combination of both drugs is the same in a HCO_3^--free and a HCO_3^--buffered medium in the presence of 1 mM external Ca^{2+}. However, the presence of HCO_3^- inhibits dramatically mast cell degranulation by PMA plus A23187. Moreover, in a HCO_3^--free medium PMA alone was able to cause histamine release, but not in a HCO_3^--buffered medium. The increase of histamine release caused by the absence of HCO_3^- is much higher in PMA preincubated mast cells than in control cells, and the potentiation by PMA of ionophore-induced histamine release was higher in a HCO_3^--free medium than in a HCO_3^--free one. As PMA is a specific activator of the PKC, all these results together confirm the implication of the PKC pathway on the inhibitory effect of HCO_3^- ions on histamine release in mast cells. Additionally, PMA is able to release histamine in the absence of external Ca^{2+} (our results and (30)), as PMA did not induce any $[Ca^{2+}]_i$ change indeed, histamine release took place in the absence of a $[Ca^{2+}]_i$ increase. This result support the theory that histamine release is not a completely Ca^{2+}-dependent process, as it was previously suggested (6, 16, 31) and further support previous work by other authors describing a stimulatory effect of PMA on histamine release in HCO_3^--free media (30, 32). Overall, the inhibitory effect of HCO_3^- over histamine release seems to be independent of the Ca^{2+} signal, but the striking results shown in Figs. 5B and 7C, where the tighter the chelation of cellular calcium the higher the release of histamine in the absence of HCO_3^-, suggests a link between bicarbonate and calcium on the degranulating process.

Is the effect of HCO_3^- on histamine release related to PKC? The activation of the PKC pathway by PMA induces a higher histamine release in a HCO_3-free medium than in a HCO_3^--buffered medium, either in the absence of Ca^{2+} or in combination with Ca^{2+} chelators or ionophore A23187 and Ca^{2+}. These results suggest that the inhibitory effect of HCO_3^- is mediated by the PKC pathway. Our results also confirm the well-known synergistic interaction between PKC and Ca^{2+}-signaling pathways, either when the Ca^{2+} signal is elicited by A23187 or Ca^{2+} chelators.

The inhibitory effect of HCO_3^- in PMA activated mast cells disappears when mast cells are preincubated with 1 mM $EGTA_o$, which demonstrates a dual interaction between PKC and calcium in the HCO_3^--induced effect. Moreover, the sensitivity of the PKC pathway to Ca^{2+} seems to be increased by the Ca^{2+} deprivation of mast cells. All together, these results suggest the implication of a Ca^{2+}-dependent isoform of PKC in mast cell degranulation.

HCO_3^- effect on the intracellular Ca^{2+} signal. Although it was not the aim of this work to study the role of HCO_3^- ions on the modulation of the intracellular Ca^{2+} signal on mast cells, it is worth discussing some features of the results related to Ca^{2+}, although a complete elucidation of the role of HCO_3^- on Ca^{2+} homeostasis requires further investigation.

Regarding the entry of Ca^{2+} ions from the external medium, passive depletion of intracellular Ca^{2+} stores by thapsigargin or Ca^{2+} chelators induced in the presence of Ca^{2+} a higher $[Ca^{2+}]_i$ increase in a HCO_3^--buffered medium than in a HCO_3^--free one. Therefore, the absence of HCO_3^- ions inhibits the Ca^{2+} entry. In rat mast cells IP_3 or passive depletion of intracellular stores activates a very specific Ca^{2+} entry called I_{CRAC} (calcium release activated calcium current) (10). In this case the effect of HCO_3^- on the Ca^{2+} entry could be mediated by regulation of pH_i by HCO_3^-. In our experimental conditions pH_i is more alkaline in mast cells suspended in a HCO_3^--buffered medium than in a HCO_3^--free medium (data not shown), and in other cellular types alkaline pH_i increases Ca^{2+} entry (33).

This effect of HCO_3^- is not shown in the $[Ca^{2+}]_i$ rise induced by compound 48/80 or PMA plus A23187. This is probably due to the activation of parallel signaling routes that may modulate the Ca^{2+} entry, such as the PKC (34, 35) or to activation of different Ca^{2+} entry pathways. In fact, compound 48/80 activates two Ca^{2+} entry pathways, a specific Ca^{2+} influx through CRAC channels and a Ca^{2+} influx through non-specific cationic channels (7).

On the other hand, depletion of intracellular Ca^{2+} stores is also modified by the presence of HCO_3^- ions. The compound 48/80-induced $[Ca^{2+}]_i$ increase in a Ca^{2+}-free medium is due to depletion of intracellular Ca^{2+} stores, and it is higher in a HCO_3^--buffered medium than in a HCO_3^--free one. These results could also be explained by the HCO_3^- pH_i regulation, since alkaline pH_i increases the affinity of IP_3R for IP_3, hence increasing the release of Ca^{2+} from intracellular stores (36). We have no explanation for the lack of effect of bicarbonate on calcium levels when the cells are activated with compound 48/80 in the presence of external calcium (Fig. 2A).

In summary, the results shown in Figs. 5B and 7C, where the tighter the chelation of cellular calcium the higher the release of histamine in the absence of bicarbonate, suggests a link between bicarbonate and calcium on the exocytosis machinery. This link is clearly related to PKC, as suggested by the highly negative modulatory effect of bicarbonate in cells treated with PMA. Nevertheless, no direct relationship could be demonstrated between the inhibitory effect of HCO_3^- on histamine release and the Ca^{2+} signal.

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