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Different Role of cAMP Pathway on the Human Mast Cells HMC-1^{560} and HMC-1^{560,816} Activation

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ABSTRACT

HMC-1 are inflammatory cells that release vasoactive substances such as histamine. These cells have the c-kit receptor permanently activated in the membrane due to mutations in the proto-oncogene c-kit: Val-560 → Gly and Asp-816 → Val. Thus, there are two known cellular lines: HMC-1^{560} and HMC-1^{560,816}. These mutations are involved in a disease called mastocitosys. In the present paper both lines were used to study the influence of cAMP/PKA/PDEs pathway on the histamine release and Ca^{2+} signaling since this pathway is often involved in these process. For this, the cells were preincubated with cAMP/PKA/PDEs modulators such as dibutyryl cAMP (dbcAMP), forskolin, H89, rolipram, IBMX, or imidazole and then stimulated with ionomycin. When cells were stimulated with agents that increase cAMP levels, the histamine release was not modified in HMC-1^{560} but decreased in HMC-1^{560,816} cells. The same happened when PKA was blocked. Furthermore, PDEs role on histamine release was independent of cAMP in HMC-1^{560} cells and possibly also in HMC-1^{560,816} cells. By contrast, the modulation of PKA and PDEs together changed the response in both cellular lines, therefore a relationship between them was suggested. All these modulatory effects on histamine release are Ca^{2+}-independent. On the other hand, the effect of c-kit modulation on the cAMP/PKA/PDEs pathway was also checked. This receptor was blocked with STI571 (imatinib) and BMS-354825 (dasatinib), but only the last one caused a decrease in the cellular response to ionomycin. This article demonstrates for the first time than the cAMP/PKA/PDEs pathway is involved in the activation of HMC-1^{560} and HMC-1^{560,816} cells. J. Cell. Biochem. 115: 896–909, 2014.

KEY WORDS: HMC-1; MAST CELLS; cAMP/PKA/PATHWAY; c-KIT RECEPTOR; IMATINIB; DASATINIB

Mast cells are inflammatory cells located in peripheral tissues like skin, lung, intestinal sub-mucosa layer, and thymus. These cells are originated from the bone marrow and circulate in the blood as CD34+ cells precursor. Subsequently mast cells pass through the endothelial cells to be deposited on peripheral tissues, where they differentiate and mature. These cells are part of the immune system and act by releasing vasoactive substances such as histamine or serotonin, contained within the secretory granules.

Stem cell factor (SCF) is a fundamental protein which induces the development of mast cells from their progenitors and promotes its maturation and terminal differentiation [Tsai et al., 1991]. The effects of SCF are regulated through a type transmembrane tyrosine kinase receptor, also known as c-kit and encoded by the proto-oncogene c-kit. Thus, SCF and c-kit are critical molecules in regulating development, proliferation, and survival of mast cells.

Mutations involving a gain of function in c-kit gene are associated with a higher growth of mast cells, higher release of vasoactive substances and excessive accumulation of these cells at one or more tissues. These alterations describe a disease called mastocytosis [Valent et al., 2001]. The HMC-1 (Human Mast Cell) line express activated c-kit receptor permanently in its membrane [Butterfield et al., 1988]. There are two known mutations in proto-oncogene c-kit: HMC-1^{560} and HMC-1 ^{560,816}, that cause the auto-phosphorylation and activation, thereby causing cell proliferation independently of the binding of ligand [Furitsu et al., 1993; Kitayama et al., 1995; Ma et al., 1999; Longley et al., 2001]. These two cellular lines show a different behavior regarding the response to drugs, phenotype and

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growth. Both lines present a glycine amino acid change at position 560 for valine (Val-560 → Gly) and the second line also has the aspartate amino acid at position 816 instead of valine (Asp-816 mutation → Val).

The World Health Organization's classification of mastocytosis (2001) distinguishes between processes that are limited to the skin (cutaneous mastocytosis) and cumulative processes at the level of other tissues: bone marrow and/or other extracutaneous organs (systemic mastocytosis). The 560 mutation is common in patients with cutaneous mastocytosis, while the 816 mutation can be found in 80% of patients with systemic mastocytosis. At the current time, there is no cure for mastocytosis, but a symptomatic therapy is used. Control of mastocytosis symptoms is primarily based on regulating the release of mast cell chemical mediators. In this sense, drugs such as Imatinib (STI571) are used, which acts as a specific inhibitor of the c-kit receptor and has proven to be useful in cases where there are certain mutations, but not useful in the mutation Asp-816 → Val [Valent et al., 2001; Ma et al., 2002]. In systemic mastocytosis, a symptomatic treatment after the chemotherapy is used, but it is noteworthy that in the case of mast cell leukemia, even chemotherapy does not produce a long lasting effect.

The adenosine 3′,5′-cyclic monophosphate (cAMP) is a nucleotide that acts as a second messenger in intracellular processes, participating in signal transduction involved in the cellular response. It is synthesized from adenosine triphosphate (ATP) by the enzyme adenylate cyclase (AC) in response to stimulation of G-protein-coupled receptor (GPCR). It can carry out its function through activation of cAMP-dependent protein kinase A (PKA), activating ion channels dependent of cyclic nucleotides or acting as guanine nucleotide exchange factors for the small G protein Rap (Epac) [Bos, 2003; Kopperud et al., 2003; Gloerich and Bos, 2010]. In addition, cAMP is also able to activate the expression of genes whose promoter contains one or several CREs sequences (cyclic AMP response elements) [Borrelli et al., 1992] such as CREB (cAMP-responsive element-binding protein), CREM (cyclic AMP response element modulator), and ATF-1 (activating transcription factor). cAMP intracellular concentration is regulated by a balance between the activity of AC and phosphodiesterases (PDEs) (Fig. 1) [Houslay, 1998]. PDEs are enzymes that catalyze the hydrolysis of phosphodiester bonds and convert the cyclic nucleotides in non-cyclic forms. The superfamily of PDEs is divided into 11 families (PDE1-PDE11), with different amino acid sequence, sensitivity to inhibitors, regulatory mechanisms, and affinity for cAMP or cGMP [Landells et al., 2000; Corbin and Francis, 2002].

cAMP is involved in the regulation of exocytosis in HMC-1^{560} cells [Pernas-Sueiras et al., 2006], but its effect is unknown in HMC-$1^{560,816}$ cells. The response of both cellular lines to the c-kit inhibitor STI571 is the same, after 24 h of incubation the spontaneous release of histamine is reduced [Lober et al., 2008]. This finding is interesting because, apparently, STI571 is not useful in the treatment of all mastocytosis. With regard to the PDEs, its inhibition is associated with a lower histamine release in rat mast cells [Alfonso et al., 1995] and many asthma treatments are based on inhibiting these enzymes. Therefore, the cAMP/PKA/PDEs signaling pathway is key in the activation of mast cells [Alfonso et al., 2000], so the aim of this work was to study its influence on histamine release in HMC-1^{560} and HMC-$1^{560,816}$ cells and its role in the cellular activation.

MATERIALS AND METHODS

CHEMICALS

Ionomycin, forskolin and H89 were from Alexis Corporation (Laufelingen, Switzerland). Fura-2 acetoxymethyl ester (FURA-2 AM) was from Molecular Probes (Leiden, The Netherlands). Bovine serum albumin (BSA), poly-L-lysine, N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dibutyryl cAMP), 3-isobutyl-1-methylxanthine (IBMX), and 1,3-Diaza-2,4-cyclopentadiene (Imidazole) were from Sigma–Aldrich (Madrid, Spain). Rolipram was from Tocris (Bristol, UK). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti-$PKA_{RII\alpha}$ was from BD Biosciences (NJ). Anti-PDE4A was from abcam® (Cambridge, UK). Anti-mouse IgG and cAMP Biotrak Enzymeimmnunoassay (EIA) System were purchased from GE Healthcare (Barcelona, Spain). Polyvinylidene flouride (PVDF) membrane and anti-actin were from Millipore (Temecula). Polyacrylamide gels molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ and inmun-star WesternC kit were from BioRad® (Barcelona, Spain). Protease Inhibitor Complete Tablets and Phosphatase Inhibitor Cocktail Tablets were from Roche (Spain). Dasatinib was from Santa Cruz Biotechnology (Heidelberg, Germany). STI571 was provided by Dr. Luis Escribano Mora (Instituto de Estudios de Mastocitosis, Castilla la Mancha, Spain).

CELL CULTURES

HMC-1^{560}cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and HMC-$1^{560,816}$ cells were kindly provided by Dr. Luis Escribano Mora with permission from Dr. J. Butterfield. They were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen) and 100 IU/ml penicillin + 100 μg/ml streptomycin (Gibco, Invitrogen) in an atmosphere containing 5% CO_2. The medium was renewed once a week.

CELL PREPARATION

For histamine release assays, cells were centrifuged (1,500 rpm, 5 min, 4°C) and then washed twice with saline solution (umbreit). 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^{2-}_4 1.2; SO^{2-}_4 1.2; and glucose 1 g/L. For Ca^{2+} measurement cells were treated in the same conditions. In all assays the incubation medium was equilibrated with CO_2 prior to use, to adjust the final pH to 7.20. Experiments were carried out at least three times, by duplicate.

HISTAMINE RELEASE ASSAYS

When the medium reached 37°C, 100 μl of a cell suspension with an approximate density of 1.5–2 × 10^6 cells/ml were added to each tube. HMC-1 cells were preincubated with modulating drugs of cAMP/PKA pathway and then incubated with ionomycin. In the experiment with c-kit inhibitors, HMC-1 cells were first incubated with STI571 (25 nM) and dasatinib (8 μM for HMC-1^{560} and 10 μM for HMC-$1^{560,816}$) for 24 h. Then, cells were stimulated with PDE inhibitors/activators and/or cAMP active drugs and after incubated with ionomycin (25 μM). Incubations were carried out into a bath at 37°C for 10 min. The incubations were stopped by immersing tubes in a cold bath. After

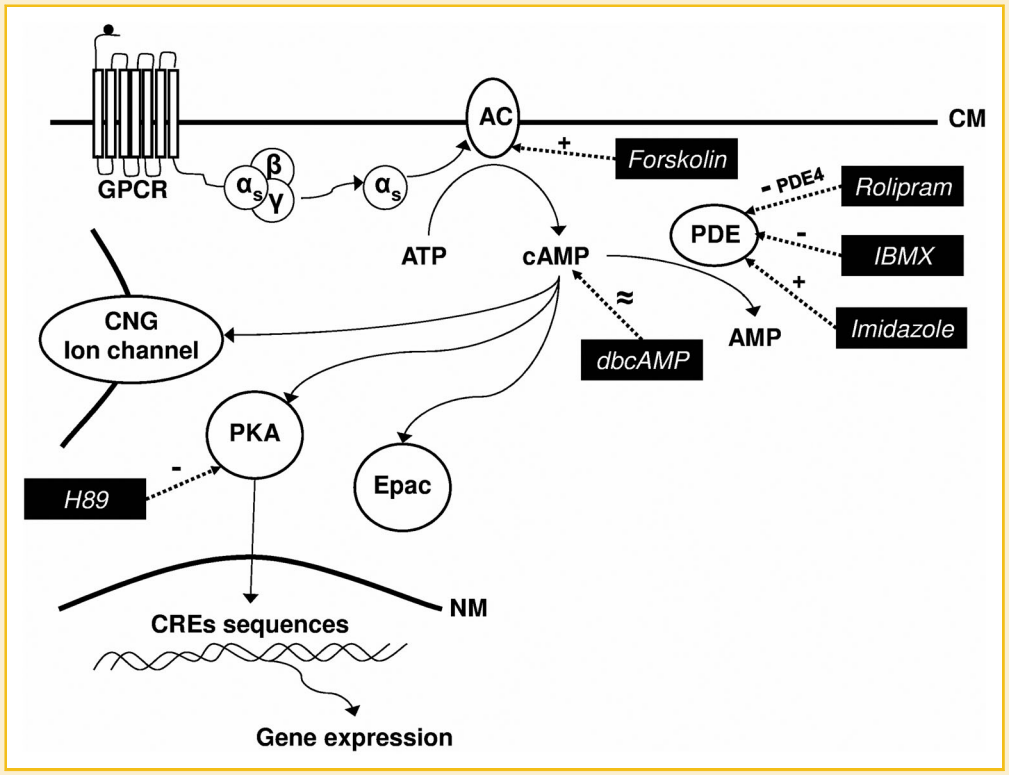

Fig. 1. cAMP/PKA/PDEs signaling pathway and effects of modulating drugs. AC, adenylate cyclase; PDE, phosphodiesterase; PKA, protein kinase A; CREB, cAMP-responsive element-binding protein; cAMP, 3′,5′-cyclic monophosphate; GPCR, G -protein-coupled receptor; dbcAMP, dibutyryl cAMP; IBMX, 3-isobutyl-1-methylxanthine.

centrifugation at 2,300 rpm for 10 min (4°C), the supernatants were collected and decanted into other tubes for histamine determination. Appropriate controls to determine spontaneous histamine release in the absence of stimuli were used in each experiment. Controls of vehicles (DMSO) and sodium butyrate were checked and no cellular activation was observed.

Histamine release was tested with a multi-mode plate reader (Synergy^{TM} 4, BioTek Instruments, Vermont) both in pellets and supernatants according to Shore′s method [Shore, 1971]. A solution of trichloroacetic acid (14%) was added to avoid protein interferences in the histamine determination. To form the fluorescent complex 0.04% orthophthaldialdehyde was used. To ensure total histamine, pellets were sonicated for 60 s in 0.2 ml of 0.1 N HCl. Results shown are expressed as the percentage of histamine released from the total histamine content.

CAMP DETERMINATION

Hundred microliter of HMC-1^{560} and of HMC-1^{560,816} cells with an density of 4×10^6 cells/ml were added to its corresponding tubes and then were incubated to 37°C in a bath for 10 min. Nine hundred microlitres of 86% ethanol and 1N HCl (99:1) were added and the tubes were immediately submerged in liquid nitrogen. Samples were stored at −80°C until cAMP determination. cAMP was measured using the protocol described by Amersham for measurement of cAMP by acetylation EIA procedure. Previously samples were thawed and dried by centrifugal evaporation.

MEASUREMENT OF CYTOSOLIC FREE CA^{2+}

For Ca^{2+} measurement cells were centrifuged (1,500 rpm, 5 min, 4°C) and then washed twice with umbreit containing 0.1% BSA (1500 rpm, 5 min, 4°C). HMC-1 cells were loaded with FURA-2AM (0.2 μM) in a bath at 37°C for 10 min. After this time, loaded cells were washed with saline solution (1,500 rpm, 10 min, 4°C). Cells were attached to glass coverslips treated with poly-L-lysine, and these 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. Fluorescence values were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175 W xenon lamp, and the used wavelengths were selected with filters. The excitation wavelengths were 340 and 380 nm, with emission at 505 nm. The calibration of the fluorescence values versus intracellular Ca^{2+} was made according to the method of Grynkiewicz et al. [1985].

CELL VIABILITY

After exposure to differents dasatinib concentrations (1 nM–40) μM for 24 h in culture medium, cells were centrifuged (1,500 rpm, 5 min, 4°C). The pellets were resuspended in saline solution with MTT (250 μg/ml) and then incubated at 37°C during 30 min in darkness. After washing twice with saline solution cells were sonicated for 1 min. The coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader.

WESTERN BLOTTING

Cells were incubated first with dasatinib (8 μM for HMC-1^{560} and 10 μM for HMC-$1^{560,816}$) during 24 h. Afterwards, cells were centrifuged and washed twice with saline solution and incubated with ionomycin (25 μM) during 10 min. Then, pellets were resuspended in lysis buffer with the following composition: 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X Complete Protease Inhibitor and 1X Phosphatase Inhibitor Cocktail. The determination of protein concentration was carried out using Bradford assay and BSA as protein standard. Samples were blotted to PVDF membrane by reduced SDS–PAGE. To determine the protein size and also to monitor the progress of an efectrophoretic run Precision Plus Protein™ Standards Kaleidoscope™ molecular weight marker was used. After, the membrane was blocked with 0.5% BSA in washing buffer (PBS + 0.1% Tween), then it was incubated 10 min with primary antibody (anti-AKAP149 (2:1,000), anti-$PKA_{RII\alpha}$ (1.5:1,000) or anti-PDE4 (1:1,000)) and then the membrane was washed three times with washing buffer and incubated for 10 min with secondary peroxidase-labelled antibody. The incubations were made in the equipment SNAP i.d™ Protein Detection System (Millipore). A chemiluminescence detection kit (inmun-star WesternC; BioRad®) was used to determine the levels of protein expression. Relative protein expression was calculated in relation to actin expression for each experiment. To chemiluminescence measures Diversity GeneSnap software (Syngene) was used. The experiments were carried out three times by duplicate.

STATISTICAL ANALYSIS

Results were analyzed using the Student's *t*-test for unpaired data and two-way analysis of variance (ANOVA). A probability level of 0.05 or smaller was used for statistical significance. Results were expressed as the mean ± SEM of three experiments.

RESULTS

EFFECTS OF CAMP/PKA/PDES ON CELLULAR ACTIVATION: HISTAMINE RELEASE AND CA^{2+} INFLUX

The cAMP/PKA/PDEs signaling pathway was checked to determine its influence on the histamine release activated by ionomycin both in HMC-1^{560} and HMC-$1^{560,816}$ cells. Ionomycin has been widely used to induce Ca^{2+} influx from the extracellular medium and thus cause histamine release in mast cells. In the present paper, the cAMP/PKA/PDEs pathway was modulated by using different drugs: dbcAMP as cAMP analogue, forskolin as AC activator, H89 as PKA blocker, IBMX as nonspecific PDE inhibitor, and rolipram as Type IV PDE (PDE4) inhibitor, Figure 1. In previous studies carried out with HMC-1^{560} cells, we reported that neither 200 μM dbcAMP nor 30 μM forskolin or 1 μM H89 cause any effect on ionomycin-induced exocytosis [Pernas-Sueiras et al., 2006]. Therefore the effect of these drugs in addition to IBMX and rolipram was studied in HMC-$1^{560,816}$ cells, Figure 2. Although the inhibitory effect of some of these drugs can be observed with low ionomycin concentrations, the histamine release induced by 25 μM ionomycin was siginificantly decreased when these cells were incubated with any of the drugs used (16%, 9%, 11%, 30%, and 27% inhibition with dbcAMP, forskolin, H89, IBMX, and rolipram, respectively). Thus, the concentration of 25 μM ionomycin was selected to perform the following assays. Next, to evaluate if the effect of these drugs is only related with variations in cAMP levels or to another effect, forskolin, H89, and IBMX were combined with dbcAMP in both lines. As it is shown in Figure 3A and B, in HMC-1^{560}, the combined effect of forskolin and H89 with dbcAMP did not change the ionomycin-induced histamine release. However, IBMX treatment caused a 46% inhibition of the exocytosis. When IBMX was combined with dbcAMP a similar inhibition was observed (Fig. 3C). When HMC-$1^{560,816}$ cells were incubated with dbcAMP plus forskolin, Figure 3D–F, the decrease of the histamine release was higher, showing an additive effect (19% inhibition). The treatment with H89 combined with dbcAMP caused a similar inhibition than control of each drug. Instead, the treatment of HMC-$1^{560,816}$ cells with dbcAMP plus IBMX caused a summatory effect (51% inhibition). All these effects are summarized in Table I.

Since the results show that the two cellular lines have different behavior when cAMP levels are modified, the basal levels of this cyclic nucleotide were measured. In HMC-1^{560} cells, the basal amount of cAMP is 42 fmol ± $1.6/4 \times 10^5$ cells, while in HMC-$1^{560,816}$ cells is 1.6 fmol ± $0.3/4 \times 10^5$ cells. Therefore HMC-$1^{560,816}$ cells have 97% less basal cAMP than HMC-1^{560} cells.

The following set of assays was designed to study if there is any direct relationship between PKA and PDE. Surprisingly any separated effect of PKA or PDE inhibition over histamine release was abolished when the inhibition was simultaneously done. As Figure 4A shows, rolipram treatment of HMC-1^{560} cells caused a 21% inhibition of the ionomycin-induced histamine release but this effect was suppressed in the presence of H89. Furthermore, H89 treatment reverses the inhibitory effect of IBMX and induces a 17% higher response, Figure 4B. In HMC-$1^{560,816}$ cells, the inhibitory effects of H89, rolipram, or IBMX were reversed when H89 was co-incubated with rolipram or IBMX. In both cases the ionomycin-induced exocytosis was 8% stimulated, Figure 4C and D.

In order to know if the changes observed in histamine release were linked to changes in citosolyc Ca^{2+} concentration, the influence of cAMP and PDEs on Ca^{2+} signaling was studied. As Figure 5 shows, the stimulation of HMC-1^{560} and HMC-$1^{560,816}$ cells with 2.5 μM ionomycin in a Ca^{2+} -free medium, induced an increase of 350 nM in cytosolic Ca^{2+} levels due to Ca^{2+} -pools depletion. Immediately thereafter, this ion returned to basal levels and when 1mM Ca^{2+} was restored to the medium and a large Ca^{2+} influx is measured. When HMC-1^{560} cells were preincubated with dbcAMP and then stimulated with ionomycin, Figure 5A, a significantly increase of Ca^{2+} influx took place when 1 mM Ca^{2+} was restored to the medium (100 nM Ca^{2+} influx-increase). As Figure 5B, the preincubation of HMC-1^{560} cells with the nonspecific PDE activator imidazole caused a significant increase in the Ca^{2+} -pools emptying induced by ionomycin. In these conditions, when Ca^{2+} was restored to the extracellular medium a faster and larger Ca^{2+} influx was also observed (cytosolic Ca^{2+} concentration reached 750 nM). As Figure 5C shows, the preincubation of HMC-$1^{560,816}$ cells with dbcAMP shows a delay in Ca^{2+} influx, this is evidenced when Ca^{2+} was restored to the medium. Furthermore, the preincubation with imidazole, Figure 5D, caused an increase in Ca^{2+} influx activated by ionomycin (Ca^{2+} concentration reached 650 nM), while no effect in pools depletion was observed. All other drugs used to modulate the

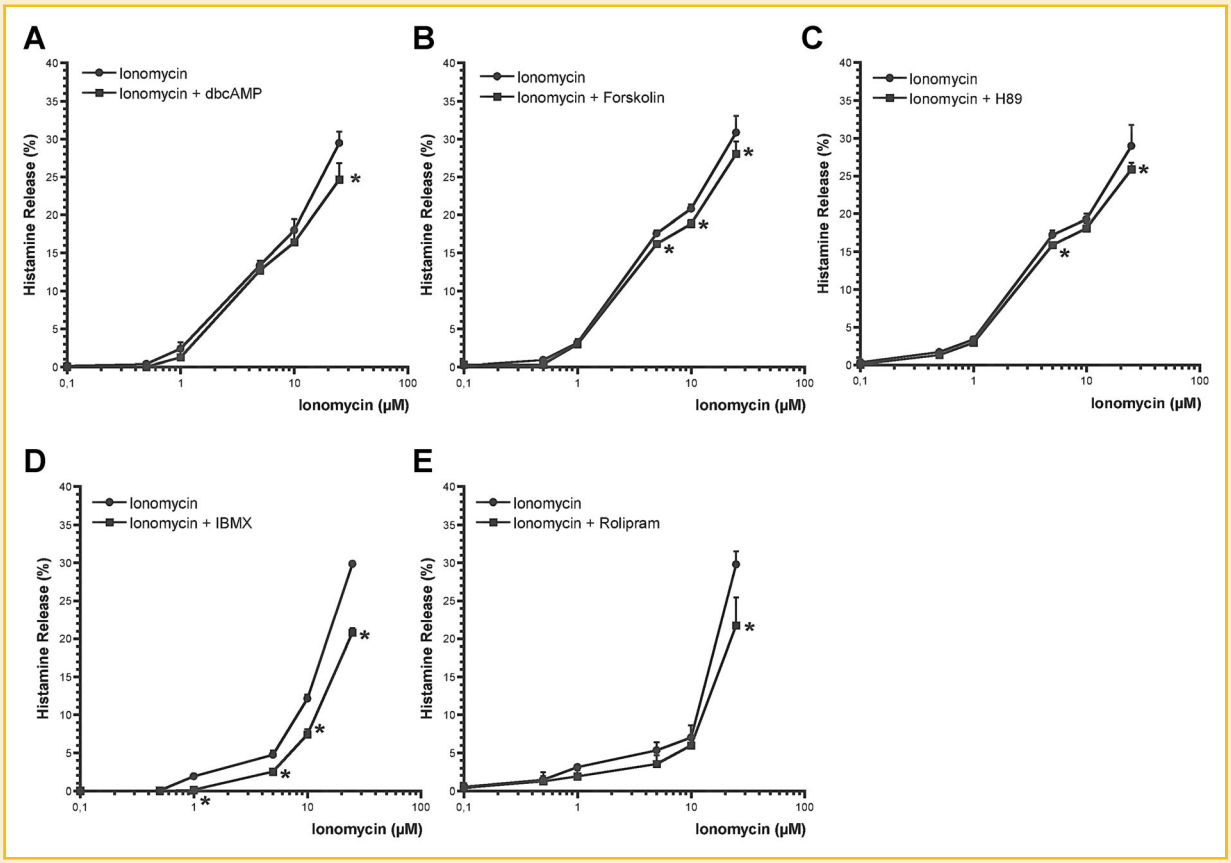

Fig. 2. Effect of modulating drugs of cAMP/PKA/PDE pathway on histamine release induced by ionomycin in HMC-1560,816 cells. Cells were incubated with 200 μM dbcAMP (A), 30 μM forskolin (B), 1 μM H89 (C), 500 μM IBMX (D), and 5 μM rolipram (E), and then stimulated with different concentrations of ionomycin for 10 min at 37°C. Mean ± SEM of three experiments. (*) Significant differences with respect to ionomycin control.

cAMP/PKA pathway did not modify the cytosolic Ca^{2+} concentration (results not showed). The effects of all these drugs on Ca^{2+} signaling in HMC-1^{560} and HMC-$1^{560,816}$ cells are summarized in Table II.

CAMP/PKA/PDES PATHWAY—C-KIT RECEPTOR RELATIONSHIP

Since HMC-1^{560} and HMC-$1^{560,816}$ cells have different response to the modulation of cAMP/PKA/PDEs pathway and the c-kit receptor is different in both cellular lines, the link between the receptor and the pathway was studied. Two TyrK inhibitors were used: STI571 (imatinib), that inhibits proliferation and induces apoptosis in cells that carry the 560 mutation [Valent et al., 2001; Ma et al., 2002], and BMS-354825 (dasatinib), that decreases cellular survival and proliferation in malignant or normal human cells [Ustun et al., 2011]. In previous studies 25 nM STI571 was used both in HMC-1^{560} and HMC-$1^{560,816}$ cells but no modifications in ionomycin-activated histamine release were observed [Lober et al., 2008]. In the present paper, HMC-1^{560} and HMC-$1^{560,816}$ cells were preincubated for 24 h with STI571 and then treated with dbcAMP and the inhibitor rolipram. As Figure 6A shows, STI571 did not change the effect of dbcAMP over the histamine release induced by ionomycin in HMC-1^{560} cells. However, the inhibitory effect of rolipram disappeared when HMC-1^{560} cells were preincubated with STI571 (Fig. 6B and C). In HMC-$1^{560,816}$ cells, STI571 treatment blocked the inhibitory effect of dbcAMP (Fig. 6D), rolipram (Fig. 6E), and both (Fig. 6F) over the ionomycin-induced histamine release.

Next, dasatinib inhibition was studied. First, to select the drug concentration, the effect over cellular viability was tested. Cellular viability was checked after 24 h incubation in the presence of different concentrations of dasatinib. A decrease in cell viability was observed when dasatinib concentration was increased, in these conditions, 8 and 10 μM dasatinib were chosen as IC50 for HMC-1^{560} HMC-$1^{560,816}$ cells, respectively (data not shown). Following, the c-kit receptor was inhibited by treatment for 24 h with dasatinib, then the cells were incubated with dbcAMP, rolipram, H89, and combinations of these drugs and stimulated with iomomycin. Figure 7A and B show the effect of dasatinib in HMC-1^{560} cells. The histamine release activated by ionomycin significantly decreased (16% inhibition) when HMC-1^{560} cells were treated with dasatinib, while the increase of cAMP levels did not produce any change. In these conditions, the inhibitory effect of rolipram and IBMX disappeared and the histamine released within these treatments was similar to ionomycin control; additionally, the inhibitory effect of dasatinib disappeared. On the contrary, H89 treatment enhanced the inhibitory effect of dasatinib (Fig. 7B). When HMC-1^{560} cells were treated with dasatinib and then

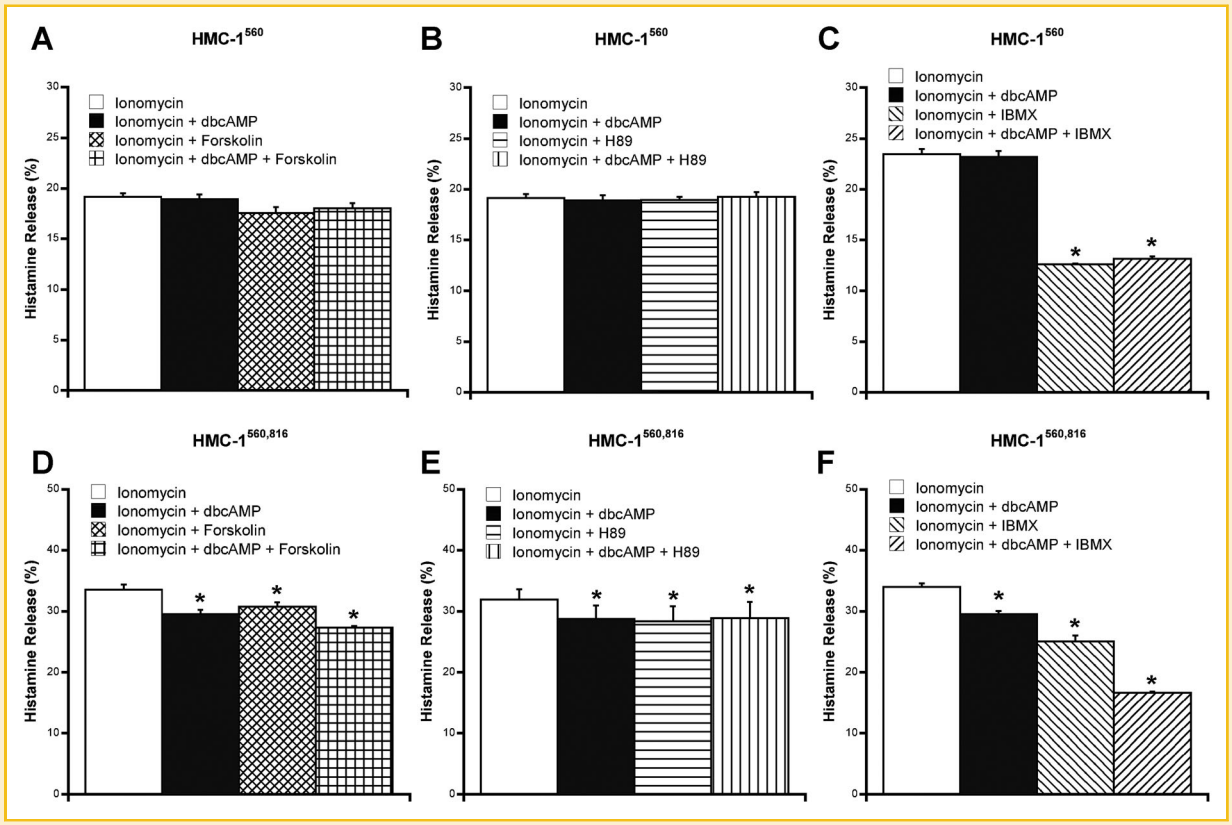

Fig. 3. Effect of cAMP increased, PKA blockade and PDEs inhibition on histamine release induced by ionomycin in HMC-1560 and HMC-1560,816 cells. HMC-1^{560} cells were incubated with 200 μM dbcAMP plus 30 μM forskolin (A), 200 μM dbcAMP plus 10 μM H89 (B), or 200 μM dbcAMP and 500 μM IBMX (C) and then stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. HMC-$1^{560,816}$ cells were incubated with 200 μM dbcAMP plus 30 μM forskolin (D), 200 μM dbcAMP plus 10 μM H89 (E), and 200 μM dbcAMP plus 500 μM IBMX (F) and then stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. Mean ± SEM of three experiments. (*) Significant differences with respect to ionomycin control.

incubated with combinations of these drugs, the inhibitory effect of dasatinib only changed when H89 was combined with dbcAMP or with rolipram. Both treatments caused a lower histamine release than the dasatinib control. When HMC-$1^{560,816}$ cells were treated with dasatinib (Fig. 7C and D) a significant decrease in histamine release induced by ionomycin was observed (53% inhibition). This effect was higher than the effect observed in HMC-1^{560} cells (16% inhibition). In HMC-$1^{560,816}$ cells treated with dasatinib the inhibitory effect of dbcAMP was lost and dasatinib effect prevailed. In these conditions, surprisingly, the inhibitory effect of rolipram remained, since a 23%

TABLE I. Summary of Effects of Modulating Drugs of cAMP/PKA/PDEs Pathway on Ionomycin-Induced Histamine Release in HMC-1^{560} and HMC-$1^{560,816}$ cells

		Effect on histamine release	
Drug	Mechanism of action	HMC-1^{560}	HMC-$1^{560,816}$
Dibutyryl cAMP 200 μM	cAMP analog	No effect	Decreases
Forskolin 30 μM	Activation of adenylate cyclase	No effect	Decreases (sum effect)
dbcAMP 200 μM + Forskolin 30 μM		No effect	Decreases
H89 10 μM	Blockage of PKA	No effect	Decreases
dbcAMP 200 μM + H89 10 μM		No effect	Decreases
Rolipram 5 μM	Inhibition of PDE4	Decreases	Decreases
dbcAMP 200 μM + Rolipram 5 μM		Decreases (like rolipram)	Decreases (sum effect)
IBMX 500 μM	Nonespecific inhibition of PDEs	Decreases	Decreases
dbcAMP 200 μM + IBMX 500 μM		Decreases (like IBMX)	Decreases (sum effect)
Imidazole 5 mM	Nonspecific activation of PDEs	Increases	Increases
dbcAMP 200 μM + Imidazole 5 mM		Increases (like imidazole)	Increases (sum effect)

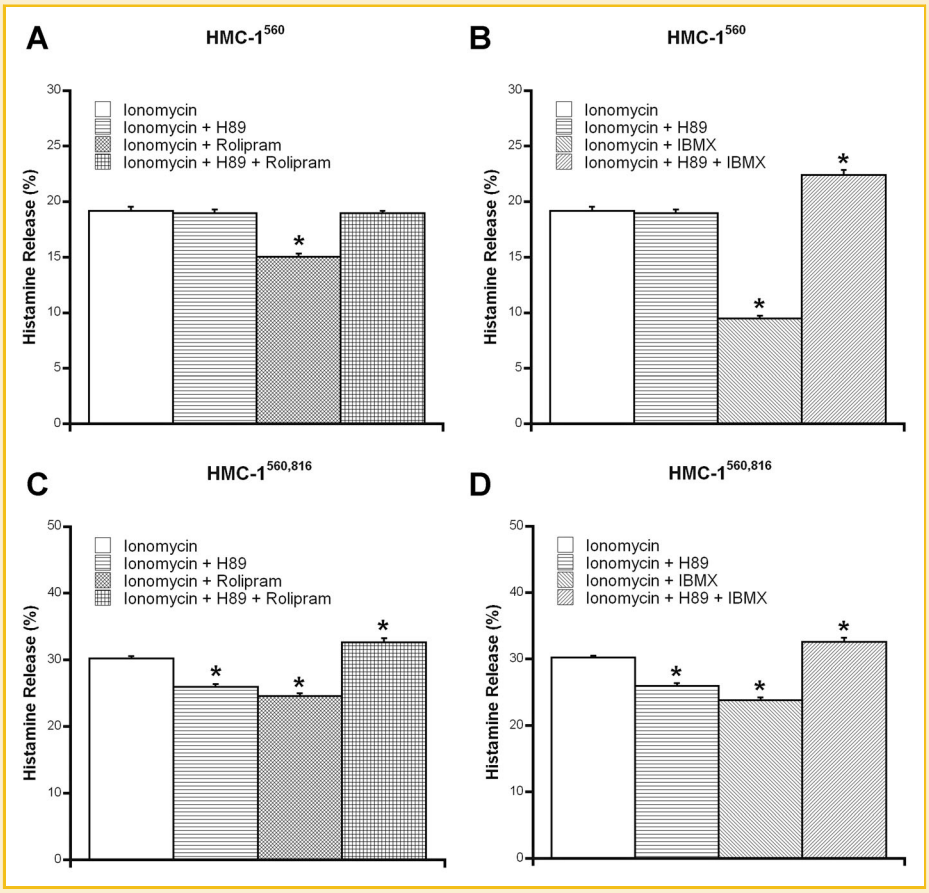

Fig. 4. Effect of the combination of PKA blockade and PDEs inhibition on histamine release induced by ionomycin in HMC-1560 and HMC-1560,816 cells. HMC-1^{560} cells were incubated with 10 μM H89 plus 5 μM rolipram (A) or 10 μM H89 plus 500 μM IBMX (B) and then stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. HMC-$1^{560,816}$ cells were incubated with 10 μM H89 plus 5 μM rolipram (C) or 10 μM H89 plus 500 μM IBMX (D) and then stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. Mean ± SEM of three experiments. (*) Significant differences with respect to ionomycin control.

inhibition of the response was observed. However, the inhibition of histamine release produced by the combined effect of IBMX and dasatinib was not increased when rolipram is present. Furthermore, the inhibitory effect of dasatinib was enhanced when cells were also incubated with H89 (87% inhibition) (Fig. 7D). When HMC-$1^{560,816}$ cells were treated with dasatinib and then incubated with combinations of these drugs, an improved inhibitory effect of dasatinib was observed, with the exception of dbcAMP plus IBMX or IBMX alone in which this effect disappeared (Fig. 7C). In addition, in HMC-$1^{560,816}$ cells treated with dasatinib, the effect of H89 in combination with rolipram or IBMX changed and ionomycin-induced histamine release was 70% inhibited with respect to untreated cells, Figure 7D.

EXPRESION OF PKA_{RIIA} AND PDE4A

Due to the different effects observed on histamine release when of PKA and PDEs were modulated in the presence of dasatinib, the levels of $PKA_{RII\alpha}$ and PDE4A in these conditions were checked (Fig. 8). PDE4A was chosen among the four subtypes of PDE4 family (PDE4A, PDE4B, PDE4C, and PDE4D) because is one of the most abundant subtypes in immune and inflammatory cells [Jin et al., 2012] and this protein is part of several protein complexes. As Figure 8E and F show, dasatinib treatment caused a significant increase in cytosolic PDE4A levels in HMC-1^{560} cells while a high decrease was observed in HMC-$1^{560,816}$ cells, 80% inhibition. Instead, ionomycin did not alter the presence of PDE4A in the cytosol fraction in any of the cellular line studied. On the other hand, dasatinib did not modify cytosolic $PKA_{RII\alpha}$ levels in HMC-1^{560} cells, although ionomycin significantly decreased these levels (Fig. 8G). As for HMC-1^{560} cells, ionomycin treatment of HMC-$1^{560,816}$ cells decreased $PKA_{RII\alpha}$ levels but when these cells were treated with dasatinib plus ionomycin the levels of $PKA_{RII\alpha}$ were even lower, Figure 8H.

In summary, these results support that cAMP/PKA/PDEs signaling pathway participates in cellular activation of HMC-1^{560} and HMC-^{1560,816} cells but in a very different fashion. Furthermore, the c-kit receptor is closely related to this pathway.

DISCUSSION

In previous reports it has been described that cAMP has an inhibitory effect on exocytosis [Alm, 1984] and that the role of the cAMP transduction pathway is key in the activation of mast cells [Alfonso

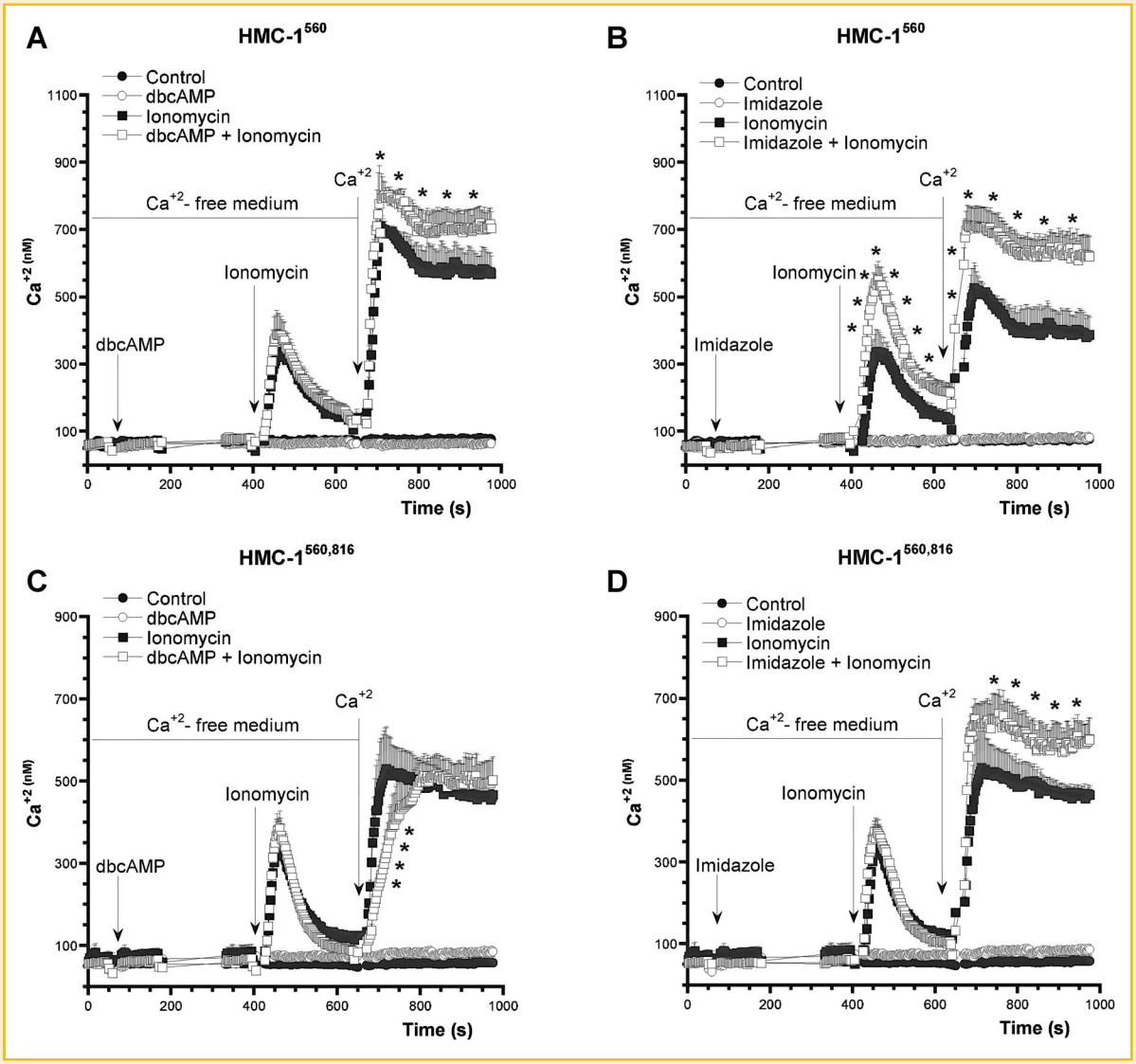

Fig. 5. Effect of cAMP increased, PDEs inhibition or activation on cytosolic Ca2+ levels of HMC-1560 and HMC-1560,816 cells. Ca^{2+} was removed from the extracellular medium to check intracellular pools contribution. HMC-1^{560} cells were incubated with 200 μM dbcAMP (A) or 5 mM imidazole (B) and then stimulated with 2.5 μM ionomycin. HMC-$1^{560,816}$ cells were incubated with 200 μM dbcAMP (C) or 5 mM imidazole (D) and then stimulated with 2.5 μM ionomycin. 1 mM Ca^{2+} was added again at the end of each experiment to restore external Ca^{2+} conditions. Mean ± SEM of three experiments. (*) Significant differences with respect to ionomycin control.

TABLE II. Summary of Effects of Modulating Drugs of cAMP/PKA Pathway on Cytosolic Ca^{2+} Concentration in HMC-1^{560} and HMC-$1^{560,816}$ Cells

		Effect on cytosolic Ca^{2+}concentration			
		HMC-1^{560}		HMC-$1^{560,816}$	
Drug	Mechanism of action	Ca^{2+} pools	Ca^{2+} influx	Ca^{2+} pools	Ca^{2+} influx
dbcAMP 200 μM	cAMP analog	No effect	Increases	No effect	Delay
Rolipram 5 μM	Inhibition of PDE4	No effect	No effect	No effect	No effect
IBMX 500 μM	Nonespecific inhibition of PDEs	No effect	No effect	No effect	No effect
Imidazole 5 mM	Nonspecific activation of PDEs	Increases	Increases	No effect	Increases

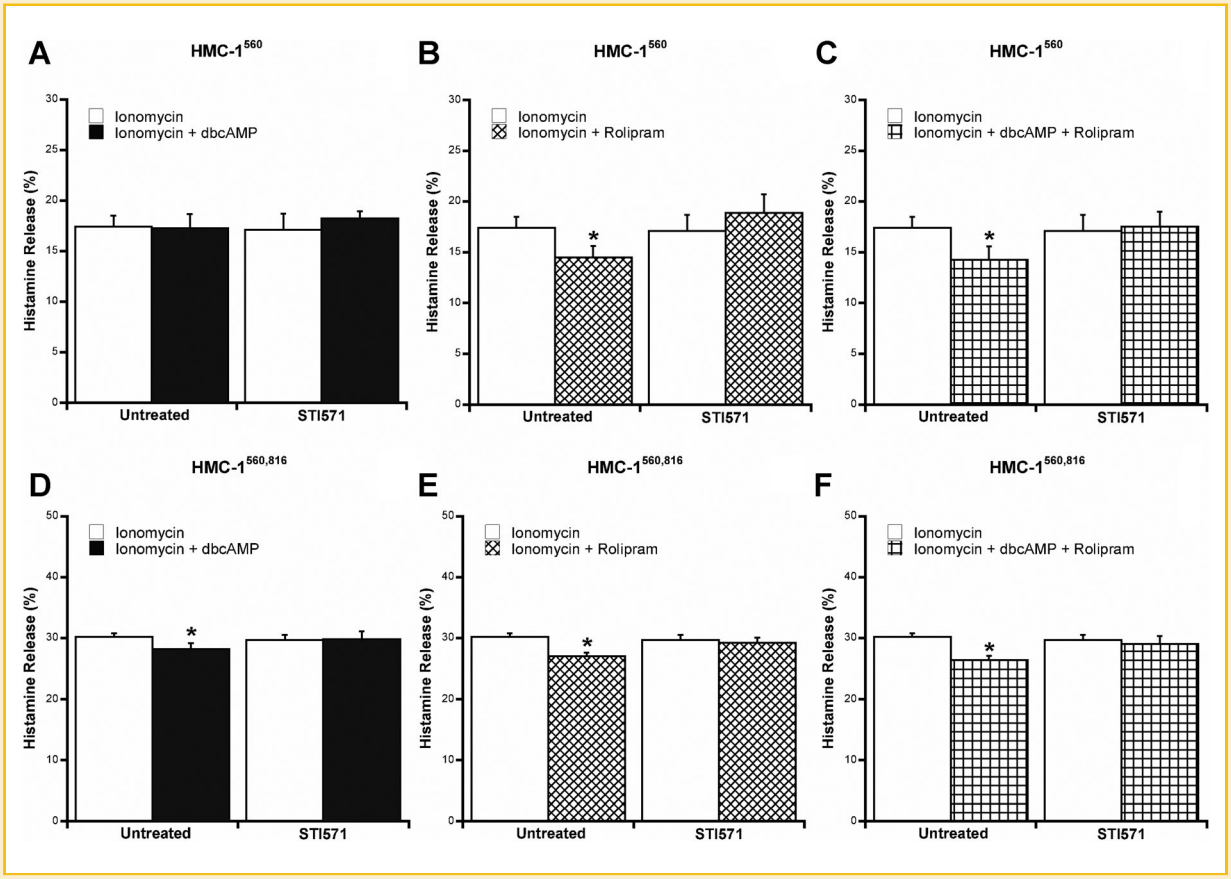

Fig. 6. Effect of STI571 on ionomycin-induced histamine release in HMC-1560 and HMC-1560,816 cells. HMC-1^{560} cells were treated for 24 h with 25 nM STI571 and then incubated with 200 μM dbcAMP (A), 5 μM rolipram (B), or the combination of both drugs (C) and then stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. HMC-$1^{560,816}$ cells were treated for 24 h with 25 nM STI571 and then incubated with 200 μM dbcAMP (D), 5 μM rolipram (E) or the combination of both drugs (F) and then stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. Mean ± SEM of three experiments. (*) Significant differences with respect to ionomycin control.

et al., 2000]. In HMC-1^{560} cells it has been suggested that the increase intracellular cAMP levels does not modify histamine release induced by ionomycin [Pernas-Sueiras et al., 2006], but its effect was unknown in HMC-$1^{560,816}$ cells. Earlier studies have also confirmed that PDEs inhibitors cause a lower response in rat mast cell [Alfonso et al., 1995] and many treatments have been proposed for asthma based on the effect of these enzymes. Moreover, there are several differences between both lines such as the survival of HMC-$1^{560,816}$ cells after STI571 treatment, the smaller size of HMC-$1^{560,816}$ and the translocation of PKC to the nucleus in HMC-$1^{560,816}$ cells but not in HMC-1^{560} upon phorbol 12-myristate 13-acetate (PMA) stimulus [Sundstrom et al., 2003; Gleixner et al., 2006; Lober et al., 2008; Tobio et al., 2011]. In this paper, we study the influence of the cAMP/PKA/PDEs pathway on histamine release in HMC-1^{560} and HMC-$1^{560,816}$ cells. The same drugs were used in the two cellular lines to know how they behave with the same signal modulation. Previous works have proposed that histamine release is correlated with increased intracellular Ca^{2+} and decreased cAMP levels [Izushi and Tasaka, 1989; Botana and MacGlashan, 1994; Takei and Endo, 1994]. On the other hand, it is known that ionomycin is a Ca^{2+} ionophore that can only lead to exocytosis in saline solution [Pernas-Sueiras et al., 2005]. Therefore, histamine release induced by ionomycin occurs when Ca^{2+} is restored to the extracellular medium and Ca^{2+} influx is activated. Our results demonstrate that in HMC-1^{560} cells the increase of cAMP mediated by forskolin or dbcAMP does not modify the release of histamine induced by ionomycin, although PDEs inhibitors and activators inhibit and activate histamine release, respectively. Therefore, the histamine release is not modulated by cAMP levels in HMC-1^{560} cells and PDEs effect is independent of cAMP presence. On the other hand, dbcAMP increased Ca^{2+} influx but this effect does not modify the exocytosis. Instead, the inhibition on histamine release caused by PDEs inhibitors does not match the changes in Ca^{2+} levels, whereby the effect of these inhibitors is independent of Ca^{2+} signaling. In HMC-$1^{560,816}$ cells, when cAMP concentration has been increased and PDEs have been inhibited or stimulated an additive effect of the drugs was observed. Then, exocytosis in these cells is modulated by cAMP and PDEs, and these enzymes may have an independent effect of cAMP. In HMC-$1^{560,816}$ cells the treatment with dbcAMP caused a delay in Ca^{2+} influx; in this case, the increase of Ca^{2+} might be associated with the lower release or may be it is not relevant because cytosolic Ca^{2+} reached the same levels. As in HMC-1^{560} cells, PDEs inhibitors do not affect cytosolic

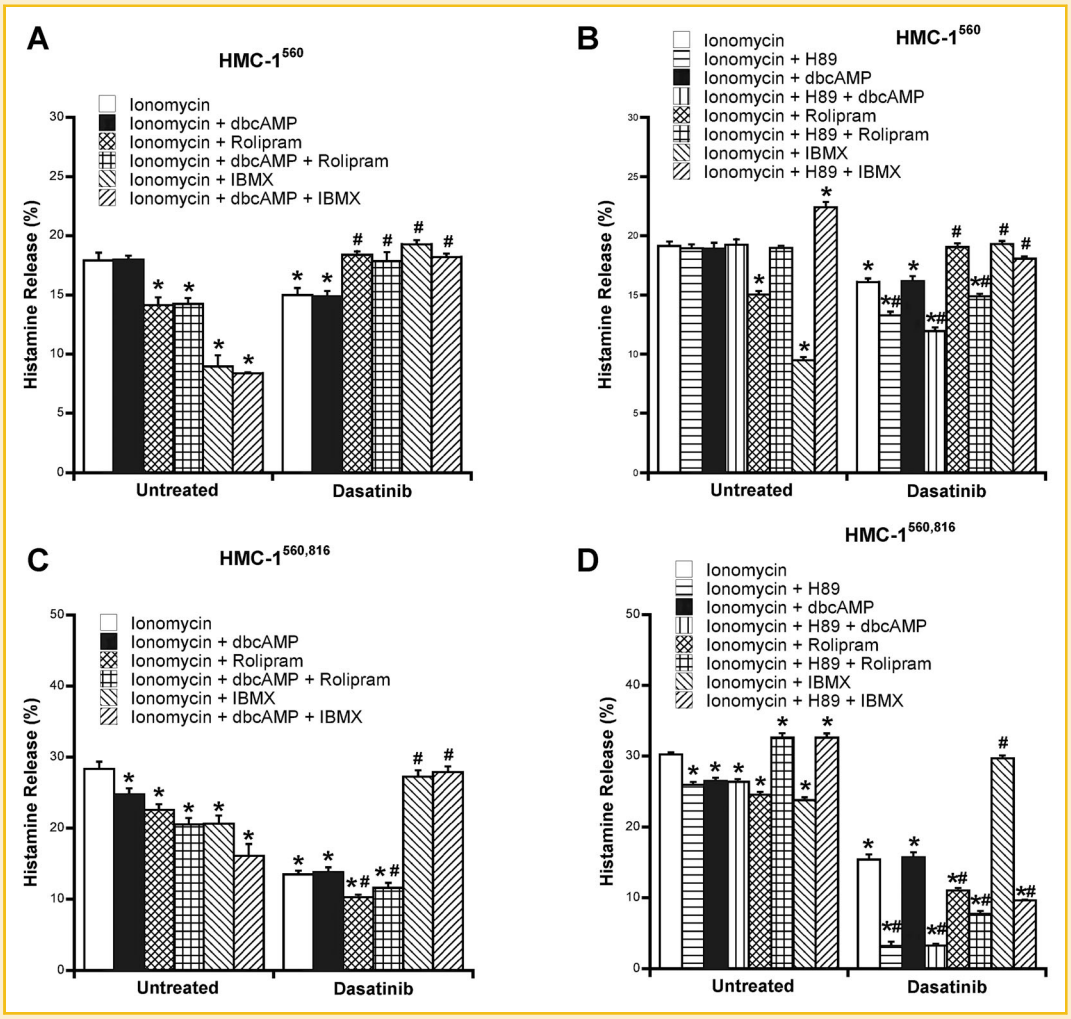

Fig. 7. Effect of dasatinib on ionomycin-induced histamine release in HMC-1560 and HMC-1560,816 cells. HMC-1^{560} cells (A and B) and HMC-$1^{560,816}$ cells (C and D) were treated for 24 h with 8 μM and 10 μM dasatinib respectively and then incubated with 200 μM dbcAMP, 5 μM rolipram, 200 μM dbcAMP plus 5 μM rolipram, 500 μM IBMX, 200 μM dbcAMP plus 500 μM IBMX, 10 μM H89, 10 μM H89 plus 200 μM dbcAMP, 10 μM H89 plus 5 μM rolipram or 10 μM H89 plus 500 μM IBMX and stimulated with 25 μM ionomycin for 10 min in saline solution at 37°C. Mean ± SEM of three experiments. (*) Significant differences with respect to ionomycin control. (#) Significant differences with respect to dasatinib control.

Ca^{2+} levels but inhibit histamine release, so its effect is independent of Ca^{2+} signaling. In both lines, PDEs stimulation with imidazole causes an increase of Ca^{2+} influx. This effect on Ca^{2+} is related to the increase of histamine release, however this is an unspecific PDEs stimulator and might affect another target and therefore cause an exocytosis increase. In summary, the histamine release is modulated by PDEs in HMC-1^{560} and HMC-$1^{560,816}$ cells but cAMP is only involved in the modulation of exocytosis of HMC-$1^{560,816}$ cells and these effects are not related with variations in intracellular Ca^{2+} levels.

On the other hand, we revealed that HMC-$1^{560,816}$ cells have a very low cAMP basal level compared with cells with one mutation. We propose that this difference may be the cause of the different response that both lines show with respect to the cAMP/PKA/PDEs pathway modulation. In this regard, there may be a threshold of cAMP beyond which HMC-1 cells stop responding. In this sense, the lack of response to rising cAMP concentration in HMC-1^{560} cells could be because the basal level of this nucleotide already exceeds the response threshold.

PKA involvement in exocytosis process has been studied in several cell types including neurons [Szaszak et al., 2008] but there are few studies of this protein in mast cells. Our results show that PKA blockade does not modify histamine release induced by ionomycin in HMC-1^{560} cells. However, it involves a reduction in histamine release in cells with two mutations, which is similar to that obtained in dbcAMP-treated cells and dbcAMP plus H89-treated cells. With these results it is not possible to know if cAMP participates in exocytosis through PKA and/or independently. It is also important to be careful in the interpretation of the results due to the nonspecific effect of some reagents, although they are widely used for the study of this pathway [Diaz-Munoz et al., 2012; Du et al., 2012; Zhang et al., 2012]. The effects of PKA-independent cAMP, such as the activation of cyclic nucleotide-ion channel dependent and the activation of

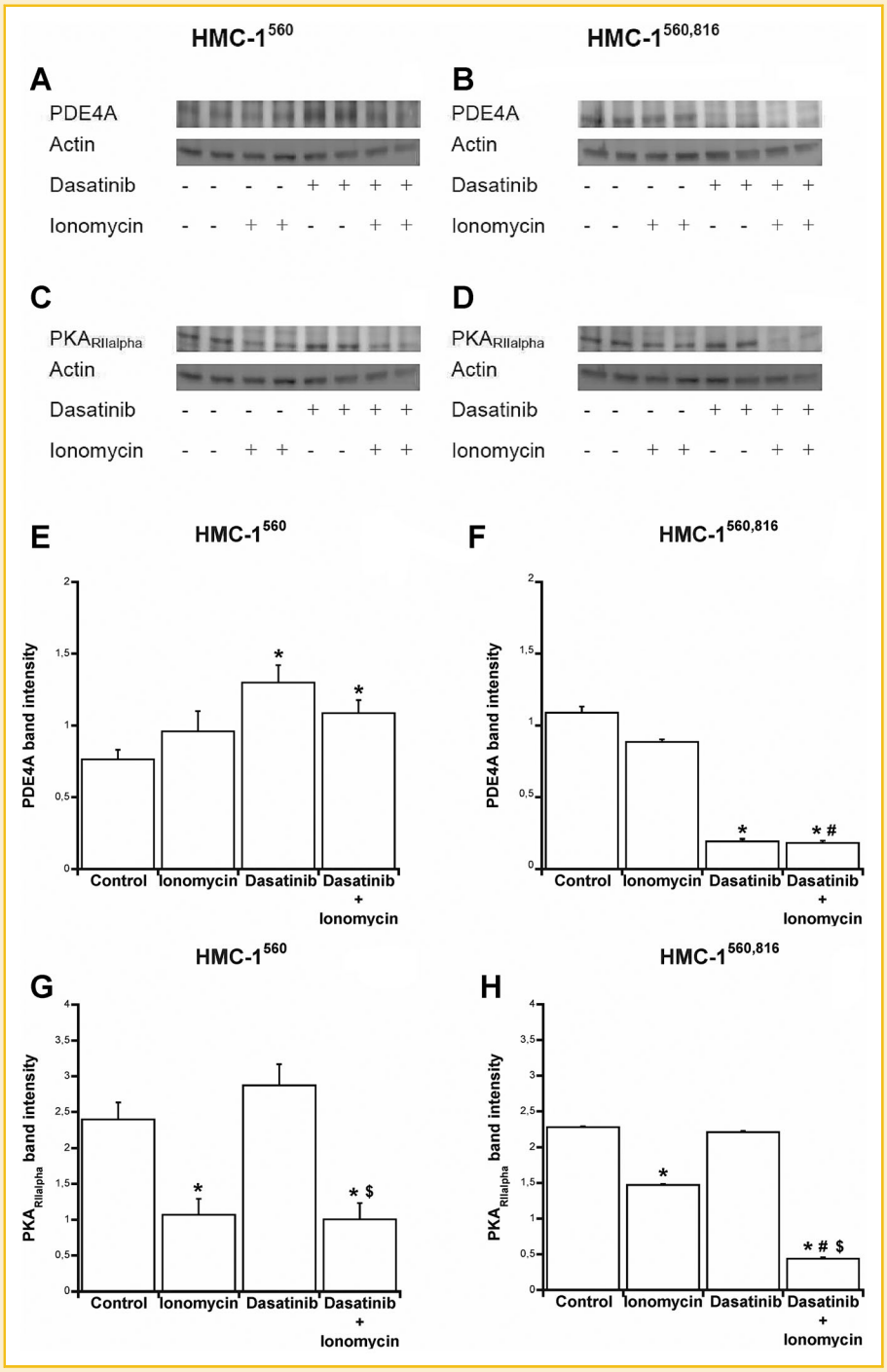

Fig. 8. Effect of dasatinib and cellular activation on cytosolic PKARIIα and PDE4A expression in HMC-1560 and HMC-1560,816 cells. Cells were treated for 24 h with dasatinib (8 μM for HMC-1^{560} and 10 μM for HMC-$1^{560,816}$ cells) and afterwards incubated with 25 μM ionomycin at 37°C for 10 min. At the top of the figure is represented an experiment of each condition. A and C: HMC-1^{560} cells experiments. B and D: HMC-$1^{560,816}$ cells experiments. Mean of the ratio of the $PKA_{RII\alpha}$/Actin band intensity and PDE4A/Actin band intensity ± SEM of three experiments Cytosolic PDE4A expression: HMC-1^{560} cells (E) and in HMC-$1^{560,816}$ cells (F). Cytosolic $PKA_{RII\alpha}$ expression: HMC-1^{560} (G) and HMC-1 560,816 cells (H). (*) Significant differences with respect to control cells. (#) Significant differences with respect to cells treated with ionomycin. ($) Significant differences with respect to cells treated with dasatinib.

guanine nucleotide exchange factors for the Epac have already been widely studied [Bos, 2003; Kopperud et al., 2003; Gloerich and Bos, 2010]. Furthermore, the regulatory role of cAMP on exocytosis by coordinating both PKA-dependent and PKA-independent mechanisms in secretory cells like exocrine cells, endocrine, or neurons has already been reported [Seino and Shibasaki, 2005]. We reported in an earlier paper that human lymphocytes show PKA-independent pathways that link cAMP and Ca^{2+} [de la Rosa et al., 2001], but

further studies are needed to confirm if cAMP is able to act independently of PKA in HMC-$1^{560,816}$ cells. We demonstrate the existence of a relationship between PKA effect and PDEs effect on ionomycin-induced histamine release. Surprisingly, PKA blockade prevents the inhibitory effect of rolipram and IBMX on histamine release activated by ionomycin. In fact, in HMC-$1^{560,816}$ cells the inhibition of PKA while PDEs or PDE4 are inhibited, results in a stimulation of ionomycin-induced exocytosis. These results suggest that PKA and PDE effects are interrelated in both cellular lines and could be linked within a protein complex in which its functions are modulated. It is well known the existence of a scaffolding protein called AKAP (A Kinase Anchoring Protein) that is able to recruit different types of proteins and enzymes, form a multivalent protein complex and thus integrate spatially constituents of different signaling pathways [Tasken and Aandahl, 2004; Carnegie et al., 2009].

From experiments performed inhibiting the c-kit receptor, we demonstrated that cAMP/PKA/PDEs signaling pathway is associated with this receptor. Previous studies in HMC-1 cells have clearly confirmed that c-kit autophosphorylation lead on to permanent activation of other transduction pathways including the Ras-MAPK-ERK and the PI3K pathways [Furitsu et al., 1993; Kitayama et al., 1995; Valent et al., 2001]. Likewise, inhibitors of receptor c-kit such as STI571 and dasatinib inhibits other tyrosine as BCR-ABL kinase or platelet-derived growth factor receptor [Giles et al., 2009; Lindauer and Hochhaus, 2010]. Some authors have claimed that STI571 did not affect the HMC-$1^{560,816}$ cells due to a conformational change in c-kit that prevented the drug binding [Heinrich et al., 2000; Ma et al., 2002; Akin et al., 2003; Roskoski, 2003], subsequently our group observed that STI571 did have an effect in cells with two mutations since it affected the spontaneous release of histamine in the same way than in HMC-1^{560} cells, but after a long-time incubation [Lober et al., 2008]. Furthermore, it is known that STI571 does not affect ionomycin-induced histamine release in any of the cellular lines. In this paper, the inhibitory effect of dasatinib on histamine release activated by ionomycin in HMC-1^{560} and HMC-$1^{560,816}$ cells has been shown. Other works have confirmed this effect on anti-IgE or allergen-induced histamine release in basophils [Kneidinger et al., 2008]. In contrast to STI571, the transduction pathways regulated by dasatinib are linked with ionomycin and therefore with intracellular Ca^{2+} concentration. These data agree with the fact that dasatinib regulates more tyrosine kinases and has a more potent effect than STI571 [Condorelli and Genazzani, 2010; Kitagawa et al., 2013]. Moreover, it has been found that the inhibitory effect of cAMP on ionomycin-induced exocytosis disappears in HMC-$1^{560,816}$ cells both with STI571 treatment and dasatinib treatment, while these treatments in cells with one mutation do not alter the effect of cAMP which continues without causing any change in release of histamine. Therefore, the c-kit receptor does not seem to be functionally linked with the cAMP in HMC-1^{560} cells but it is highly related in HMC-$1^{560,816}$ cells. The inhibition of the c-kit causes cells with two mutations to behave as the cells carrying a single mutation when cAMP is increased, as the inhibition of histamine release evidenced in dbcAMP-treated cells in two-mutation cells is ablated. This finding supports our theory about the posible cAMP-independent effect of PDEs on histamine release in HMC-$1^{560,816}$ cells because when c-kit is inhibited, dbcAMP has no effect on the release yet PDEs inhibitors continue inhibiting it. On the other hand, it was observed that STI571 treatment ablates the inhibitory effect of rolipram in both cellular lines, whereas the dasatinib treatment does not change the effect of rolipram in HMC-$1^{560,816}$ cells, but the effect disappears in HMC-1^{560} cells. By contrast, IBMX inhibitory effect disappears in both cellular lines. Thus, it can be confirmed that the effect of PDEs on histamine release is functionally linked to the c-kit receptor in HMC-1^{560} and HMC-$1^{560,816}$ cells. Likewise, it is suggested that PDE4 appears to play a more important role than other PDEs families in HMC-1^{560} cells because the c-kit blockage prevents the inhibitory effect of rolipram and IBMX. Instead, in HMC-$1^{560,816}$ cells the PDE4 seems less relevant because rolipram inhibitory effect is the same when c-kit is activated or inhibited. Regarding PKA, the H89 treatment improved the inhibitory effect of dasatinib in both cellular lines. Surprisingly, when in HMC-1^{560} cells the PKA is blocked and at the same time PDE4 is inhibited, the inhibition induced by dasatinib is potentiated, but if H89 is combined with IBMX the dasatinib effect disappears. These findings support the theory that in cells with one mutation PDE4 has a preponderant regulatory function. In contrast, the same treatments in HMC-$1^{560,816}$ cells similarly improved the inhibitory effect of dasatinib. All these data support the overall conclusion that PKA and PDEs are functionally related to the c-kit receptor.

Finally, cytosolic $PKA_{RII\alpha}$ and PDE4A levels were studied and it was found that the $PKA_{RII\alpha}$ presence is related to intracellular Ca^{2+} concentration and the PDE4A presence with c-kit receptor both in HMC-1^{560} and HMC-$1^{560,816}$ cells. It may be suggested that cellular activation mediated by ionomycin leads to a decrease of $PKA_{RII\alpha}$ synthesis. Moreover, $PKA_{RII\alpha}$ and PDE4A levels significantly decrease in HMC-$1^{560,816}$ cells treated with dasatinib plus ionomycin and perhaps these proteins are translocated to the cellular membrane or to the nucleus. In HMC-1^{560} cells the same may happen but it is not as evident because high levels of PDE4 are detected in the cytosol, although this can be due to a synthesis increase. This fact agrees with the possible important role of PDE4 in HMC-1^{560} cells. A more deeply study of the presence of these proteins in membrane and nucleus is required to better understand its role. Also more experiments should be done in order to conclude what family and subtype of PDEs and if some MAPK or any AKAP may be involved in the modulation of histamine release by c-kit receptor in HMC-1 cells.

In summary, in this paper it has been demonstrated for the first time the different involvement of cAMP/PKA/PDEs signaling pathway on HMC-1^{560} and HMC-$1^{560,816}$ activation, the cAMP-independent effect of PDEs in HMC-1^{560} cells and a relationship between PKA and PDEs in HMC-1 cells. Likewise, it has been observed that the c-kit receptor is part of cAMP/PKA/PDEs signaling pathway and therefore, it is also involved in cellular activation.

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