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C-Kit Mutations and PKC Crosstalks: PKC Translocates to Nucleous Only in Cells HMC^{560,816}

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ABSTRACT

The human mast cell lines HMC-1^{560} and HMC-1^{560,816} were used to study histamine release, Ca^{2+} signaling and protein kinase C (PKC) localization and expression, with phorbol 12-myristate 13-acetate (PMA). Both sublines carry activating mutations in the proto-oncogene of c-kit that cause autophosphorylation and permanent c-kit tyrosine kinase activation. Both have the Gly-560 → Val mutation but only the second carries the Asp-816 → Val mutation. In this study, it was observed that the stimulation of PKC has different effects in HMC-1^{560} and HMC-1^{560,816} and this would be related to the difference in activating mutations in both mast cell lines. PKC activation increases ionomycin-induced histamine release in HMC-1^{560}. This article demonstrates an opposite histamine response in HMC-1^{560,816} cells, even though classical PKCs are the family of isozymes responsible for this effect in both cellular lines. Furthermore, it can be observed that upon cell stimulation with PMA, primarily cytosolic PKC translocates to the nucleous in HMC-1^{560,816} cells, but not in HMC-1^{560} cell line. J. Cell. Biochem. 112: 2637–2651, 2011.

KEY WORDS: HMC-1; MAST CELLS; PROTEIN KINASE C; CYTOSOLIC Ca^{2+}; c-kit

Mast cells are tissue-based inflammatory cells produced in the bone marrow and released after their differentiation into peripheral tissues. They are abundant in skin, thymus, and lymphoid tissue as well as around blood vessels and submucosal layer of the digestive tract. Mast cells are part of the immune system and they release vascular active substances in response to danger signals of innate or acquired immunity. One of those substances is histamine. The release of this amine can lead to reactions in the skin, like erythema and edema, in the airways, like mucous secretion and cough as well as nausea, vomiting, diarrhoea, and cramping in the gastrointestinal tract. Ig-E dependent hypersensitivity or diseases of tissue disorders can evoke pathologic increases in mast cell number. The most striking increase occurs in patients with mastocytosis [Prussin and Metcalfe, 2006].

The human mast cell lines HMC-1 express in their membrane the permanent activated receptor tyrosine kinase (TyrK) c-kit [Butterfield et al., 1988]. Two activating mutations in the proto-oncogene of c-kit cause its autophosphorylation and activation of the inner TyrK and induce thereby the ligand-independent proliferation [Furitsu et al., 1993; Kitayama et al., 1995; Ma et al., 1999; Longley et al., 2001]. There are two HMC-1 sublines: HMC-1^{560} and HMC-1^{560,816}. These sublines show different behavior concerning to drug response, phenotype, and growth. Both have the Gly-560 → Val mutation but only the second carries the Asp-816 → Val mutation. The 560 mutation commonly appears in patients with cutaneous mastocytosis, whereas the 816 mutation can be found in 80% of patients with systemic mastocytosis [Valent et al., 2001].

C-kit autophosphorylation in HMC-1^{560} and HMC-1^{560,816} cells lead on to a permanent activation of other transduction pathways including the Ras-MAPK-ERK and the PI3K pathways which are involved in cell survival and proliferation [Furitsu et al., 1993; Kitayama et al., 1995; Valent et al., 2001; Sundstrom et al., 2003]. The natural ligand of c-kit is Stem Cell factor (SCF). In normal mast cells its SCF promotes proliferation and maturation [Tsai et al., 1991a,b]. Further to this, it is able to directly induce murine and

Abbreviations Used: HMC-1, human mast cell line; PKC, protein kinase C; Ca^{2+}, calcium; TyrK, tyrosine kinase.

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human mast cell degranulation and it potentiates Ig-E mediated histamine release at low concentrations. In addition, it is demonstrated that rhSCF-induced histamine release was accompanied by an increase in cytosolic Ca^{2+} in human skin mast cells, which was inhibited by PKC activation [Columbo et al., 1992, 1994; Wershil et al., 1992; Taylor et al., 1995].

PKC is a family of Ser/Thr kinases with different isoforms, subdivided into three groups. The conventional PKCs (α, β, and γ) are Ca^{2+} dependent and activated by diacylglycerol (DAG) or phorbol ester; the novel PKCs (δ, ε, η, and θ), which are Ca^{2+} independent, but require DAG or phorbol ester for their activation and the atypical isoenzymes (ζ, λ, and ι), which seem to be independent of both factors [Lessmann et al., 2006]. The activity of PKC is reversibly regulated by an autoinhibitory pseudosubstrate, which blocks the active site of the enzyme in the absence of activators [Dutil and Newton, 2000]. PKCs are involved in multiple and different biological events representative of their pivotal role in the regulation of cell metabolism which would occur subsequently to their phosphorylation and translocation from cytoskeleton to the plasma membrane [Lanuti et al., 2006]. Moreover, it has been observed that sometimes PKC is translocated from the cytoplasm to the nucleous, where it exhibits catalytic activity [Nishizuka, 1995].

Phorbol 12-myristate 13-acetate (PMA) activates PKC by linking to the DAG binding site. Unlike DAG, PMA is not metabolized and has a longer effect. Its binding activates and translocates the enzyme to the membrane where it phosphorylates subsequent enzymes like MAP-kinases, Raf-kinases, the transcription factor inhibitor, or the epidermal growth factor receptor. PKC affects a variety of important cell processes like morphology, proliferation, differentiation, apoptosis, and production of inflammatory substances in mast cells [Zhao et al., 2004; Sandler et al., 2005; Kuchler et al., 2006; Gobbi et al., 2009]. Activation of phospholipase D and PKC as well as Ca^{2+} mobilization are essential signals for degranulation of mast cells. Diversion of production of phosphatidic acid to phosphatidylbutanol by addition of 1-butanol suppressed both the translocation of diacylglyceride-dependent isoforms of PKC to the membrane and degranulation [Peng and Beaven, 2005]. For signaling to the nucleous, TyrKs associated with growth factor receptors activate MAP-kinases through a series of provocative protein–protein interactions involving Ras and Raf-1 kinases [Nishizuka, 1995].

Depending on the cellular model and the stimuli, PKC activation can increase or inhibit mast cell exocytosis [Botana et al., 1992; Alfonso et al., 2000; Pernas-Sueiras et al., 2005]; therefore the aim of this work was to determine the effect that PKC modulation induces in HMC-1^{560} and HMC-$1^{560,816}$ cells and their relationship with cellular activation.

MATERIALS AND METHODS

CHEMICALS

Ionomycin, Gö6976, GF109203X, rottlerin and chelerythrine, were from Alexis Corporation (Läufelingen, Switzerland). FURA-2 AM and Streptavidin-FITC were from Molecular Probes (Leiden, The Netherlands). Phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA), poly-L-lysine, and Anti β tubulin were from Sigma–Aldrich (Madrid, Spain). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti-PKC Clone M110, Goat Anti Mouse IgG, IgM, IgA Biotin Conjugated, Anti Histone H1, and polyvinylidene flouride (PVDF) membrane were from Millipore (Temecula). DNA Prep™ Stain was from Beckman Coulter (Fullerton, CA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from BioRad (Barcelona, Spain). Paraformaldehyde (PFA) was from Aname (Madrid, 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. The composition of this solution was (mM): Na^+ 142.3; K^+ 5.94; Ca^{2+} 1; Mg^{2+} 1.2; Cl^- 126.2; HCO_3^- 22.85; HPO_4^{2-} 1.2, SO_4^{2-} 1.2, and glucose 1 g/L.

For Ca^{2+} measurement cells were treated in the same conditions, but washed in saline solution plus 0.1%BSA. For cytometry assays cells were washed in PBS (Invitrogen).

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 $1.5–2 \times 10^6$ cells/ml were added to each tube. HMC-1 cells were pre-incubated with the phorbol ester PMA (100 ng/ml) and then stimulated with different concentrations of ionomycin (0.1, 0.5, 1, 5, 10, and 25 μM). In the experiment with PKCs inhibitors, HMC-1 cells were first incubated with Gö6979 100 nM, GF109203X 50 nM, GF109203X 500 nM [Martiny-Baron et al., 1993], rottlerin 10 μM, and chelerythrine 1 μM [Alfonso et al., 2000] for 10 min. Then, cells were incubated with PMA (100 ng/ml) and finally stimulated with ionomycin. 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 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 executed in each experiment.

Histamine release was tested with a multi-mode plate reader (Synergy™ 4, BioTek Instruments, Vermont) both in pellets and supernatants according to Shore's method [Shore, 1971]. To produce the fluorescent complex, 0.04% orthophthaldialdehyde was used and also trichloroacetic acid (14%) to avoid protein interferences in the histamine determination. 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.

MEASUREMENT OF CYTOSOLIC FREE Ca^{2+}

HMC-1 cells were loaded with FURA-2 AM (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 40×—immersion UV—Fluor objective). Addition of drugs was made by aspiration and addition of fresh bathing solution to the chamber. Cytosolic Ca^{2+} concentrations were obtained from the images collected by fluorescence equipment (Life Sciences Resources). The light source was a 175W xenon lamp, and the used wavelengths were selected with filters. For FURA-2 AM, 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].

WESTERN BLOTTING

Cytoplasmic proteins protocol. Cells were incubated first with PMA (100 ng/ml) (10 min) and then with ionomycin (10 μM) (10 min). Afterwards, cells were centrifuged and washed twice with saline solution. After the last centrifugation, pellets were resuspended in lysis buffer specific for cytoplasmic proteins. The composition of this buffer was 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM DTT, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A, and 1 mg/ml bezamidine. The determination of protein concentration was carried out using Bradford assay and BSA as protein standard. For separating proteins according to their molecular weight sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) procedure was used. 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. The cytoplasmic proteins were transferred to a PVDF membrane, which was blocked with 0.25% non-fat dry milk and then it was incubated for 10 min with anti-PKC Clone M110 (1:1,000, Millipore). After two washes with washing buffer (PBS + 0.1%Tween), the membrane was incubated for 10 min with the secondary antibody, Anti-Mouse IgG conjugated with horseradish peroxidase (GE Healthcare). A chemiluminescence detection kit (SuperSignal® West Pico; Pierce) was used to determine the levels of protein expression. Relative protein expression was calculated in relation to β-tubulin expression for each experiment. To chemiluminescence measures Diversity GeneSnap software (Syngene) was used. The experiments were carried out three times by duplicate.

Nuclear fractionation protocol. Control and treated cells were centrifuged and washed twice with saline solution. Pellet was resuspended in 500 μl of buffer containing 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, 2.5 mM PMSF, 40 mg/ml aprotinin, 4 mg/ml leupeptin, 5 mM NaF, 1 mM Na_3VO_4, 1 mg/ml pepstatin A, and 1 mg/ml bezamidine at pH 7.9. Cells were centrifuged at 3,000 rpm for 10 min (4°C), the supernatant was kept out, and pellet was resuspended on ice in 374 μl of buffer which contains 5 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v) at pH 7.9 and then 26 μl of 4.6 M NaCl was added. Samples were sonicated four times for 10 s. Assay tubes were left on ice for 30 min and centrifuged at 24,000*g* (Beckman J2-21) for 20 min at 4°C. Finally, the supernatant was aliquoted and stored at −20°C. Relative protein expression was calculated in relation to Histone H1 expression.

FLOW CYTOMETRY

About 20×10^6 cells/ml were washed with saline solution and centrifuged at 1,500 rpm, 5 min at 4°C. Samples were incubated with PMA (100 ng/ml) and then with ionomycin (10 μM), as for Western blotting assays. Then the cells were fixed with 4%PFA for 15 min at room temperature and permeabilized using PBS+ 5%BSA+ 0.1%Triton X-100. The cells were incubated for 12 h in the presence of anti PKC Clone M110 (1:1,000, Millipore). After PBS wash, primary antibody was detected with FITC-conjugated secondary antibody (Goat Anti Mouse IgG, IgM, IgA Biotin Conjugated, 1:1,000, Millipore). For propidium iodide staining, the Coulter® DNA Prep™ Stain (Beckman Coulter) was used for 2 min at 37°C. Images files of 10,000 events were collected for each sample using the ImageStream imaging flow cytometer (Amnis Corporation, Seattle, WA) and analyzed using IDEAS software (Amnis Corporation). Negative control of each condition was done and no signal was observed.

STATISTICAL ANALYSIS

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

RESULTS

To determine the involvement of PKC in HMC-1 activity, several experiments studying histamine release, cytosolic Ca^{2+} levels, and PKC activation were carried out. First, the effect of PMA treatment in the histamine release induced by ionomycin was studied in HMC-1 cells. As Figure 1A shows, in HMC-1^{560} cells 100 ng/ml PMA induces a statistically significant increase in the histamine release at 0.5, 1, and 5 μM ionomycin concentrations. However, in HMC-$1^{560,816}$ cells, the pre-incubation with PMA significantly decreased ionomycin-induced histamine release in concentrations up to 10 μM of the ionophore (Fig. 1B). Several PKCs inhibitors were used with the aim of determining which class of PKCs is involved in this effect. The inhibition of Ca^{2+}-dependent isozymes (or classical PKCs) was induced by Gö6976 100 nM and GF109203X 50 nM; moreover, GF109203X 500 nM was used to inhibit both classes of PKCs: classical PKCs and Ca^{2+}-independent isozymes (or novel PKCs). PKCδ was inhibited by incubation with rottlerin 10 μM. Finally, chelerythrine 1 μM was used as a nonspecific PKC inhibitor. In these experiments 100 ng/ml PMA and two concentrations of

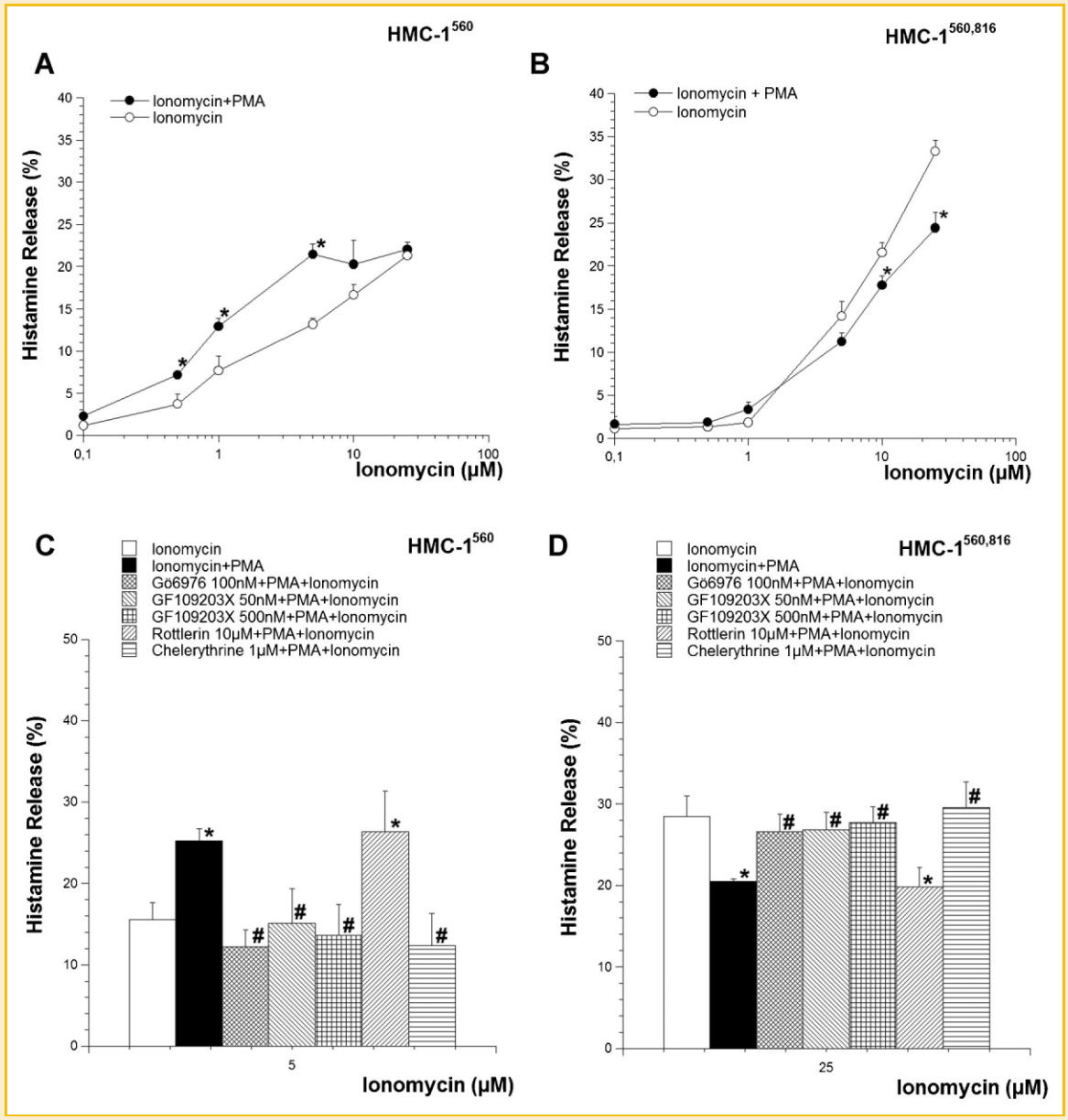

Fig. 1. Effect of PKC stimulation and inhibition on histamine release induced by ionomycin in HMC-1^{560} and HMC-$1^{560,816}$ cells. A: HMC-1^{560} cells and (B) HMC-$1^{560,816}$ cells were pre-incubated with PMA 100 ng/ml and afterwards stimulated with ionomycin. C: HMC-1^{560} cells and (D) HMC-$1^{560,816}$ cells were pre-incubated during 10 min with Gö6976 100 nM, GF109203X 50 nM, GF109203X 500 nM, rottlerin 10 μM, and chelerythrine 1 μM, then cells were stimulated with PMA 100 ng/ml and finally with ionomycin. Incubations were carried out into a bath at 37°C for 10 min. Mean ± SEM of three experiments. *Significant differences between ionomycin and PMA pre-treated cells. #Significant differences between PKC inhibitors pre-treated cells and PMA pre-treated cells.

ionomycin were used to stimulate cells, 5 μM for HMC-1^{560} and 25 μM for HMC-$1^{560,816}$. As Figure 1C shows, the significant increase in ionomycin-induced histamine release due to PMA pre-treatment in HMC-1^{560} cells is avoided by Gö6976, GF109203X, and chelerythrine pre-treatments, while PKCδ inhibition does not block this increase. On the other hand, in HMC-$1^{560,816}$ cells (Fig. 1D) the significant decrease in ionomycin-induced histamine release due to PMA treatment was prevented by Gö6976, GF109203X, and chelerythrine pre-treatments while Rottlerin pre-treatment does not block PMA effect. In this cell line, another concentration of ionomycin was used, 10 μM, and similar results were obtained (data not shown).

In order to clarify the opposite behavior of each cellular model in histamine release, the next group of experiments focuses on studying the effect of PKC activation in cytosolic Ca^{2+} levels. In HMC-1^{560} cells, ionomycin 0.1 μM induces a small increase (around 20 nM) of cytosolic Ca^{2+} levels at the addition point of ionomycin, Figure 2A. The same figure shows that PMA alone did not affect cytosolic Ca^{2+} levels; by contrast, when cells were pre-treated with PMA prior to ionomycin, cytosolic Ca^{2+} levels were significantly

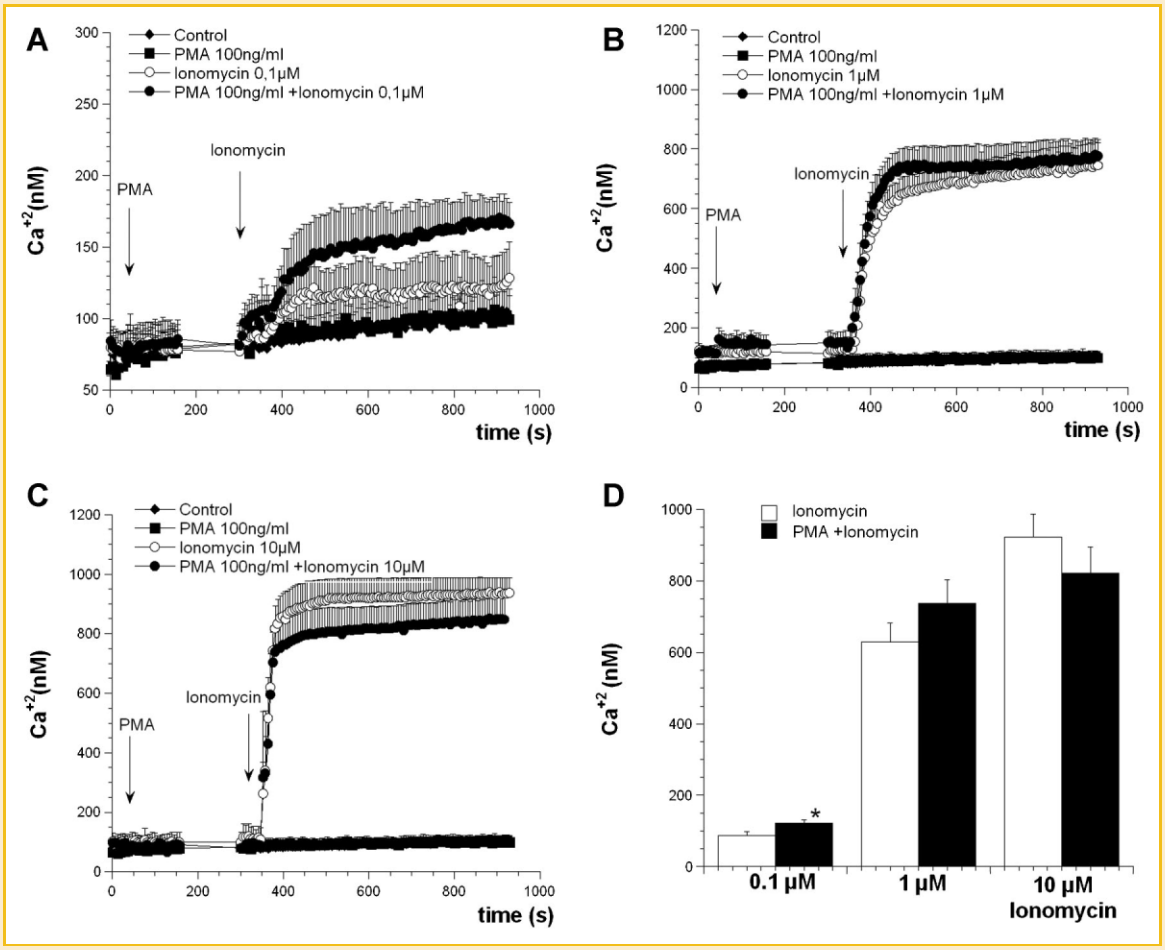

Fig. 2. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-1^{560} cells in a saline solution with calcium. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from extracellular Ca^{2+} influx. Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

higher. This increase was kept for 10 min. The effect of PMA was also evident using 1 μM of ionomycin, Figure 2B, since cytosolic Ca^{2+} levels were up from 600 to 700 nM, even though the increase disappears after 5 min. However, when ionomycin 10 μM was used, cytosolic Ca^{2+} levels reached in PMA + ionomycin-treated cells were lower than in ionomycin-treated cells, Figure 2C. These variations in cytosolic Ca^{2+} levels at the addition point of ionomycin are summarized in Figure 2D. In HMC-$1^{560,816}$ cells, an increase (not significant) in the cytosolic Ca^{2+} levels was observed at the addition point of ionomycin (0.1 μM) (Fig. 3A). The same figure shows that PMA alone did not increase the levels of this ion. However, when both drugs are present an increase in cytosolic Ca^{2+} levels is observed. This effect was kept and increased after 10 min. The effect was significantly higher when cells were preincubated with PMA and ionomycin 1 μM, Figure 3B, and also in the presence of 10 μM ionomycin, Figure 3C. Figure 3D summarizes all these results in HMC-$1^{560,816}$ cells at the addition point of ionomycin. In summary, in HMC-1^{560} cells the treatment with PMA + ionomycin induces a significant increase in cytosolic Ca^{2+} levels when ionomycin 0.1 μM was used; when higher concentrations are used no increase or even a lower levels in cytosolic Ca^{2+} are observed (Fig. 2D). On the contrary in HMC-$1^{560,816}$ cells, PMA + ionomycin treatment induces a significant increase in Ca^{2+} levels at any concentration checked (Fig. 3D).

Since in the presence of PMA plus ionomycin each cellular line reached different cytosolic Ca^{2+} levels, the effect of PMA on Ca^{2+}-pools depletion and Ca^{2+}-influx from extracellular media was checked. Ionomycin (0.1 μM) induced Ca^{2+} release from intracellular pools (Fig. 4A). This effect was not modified in the presence of PMA. However, when Ca^{2+} is restored to the medium, the presence of PMA increased Ca^{2+} influx (from 300 to 450 nM). Figure 4B shows that the addition of ionomycin (1 μM) induces a cytosolic Ca^{2+} increase due to Ca^{2+}-pools depletion. When Ca^{2+} was restored to the extracellular medium, levels of this ion increased to values about 700 nM. The treatment with PMA prior to ionomycin did not affect neither Ca^{2+}-pools depletion nor extracellular Ca^{2+} influx. When ionomycin 10 μM was added, PMA increased (not significant) Ca^{2+}-pools depletion and inhibited cytosolic Ca^{2+} recovery to basal values. When Ca^{2+} is restored to the medium, no differences between ionomycin and PMA + ionomycin treated cells

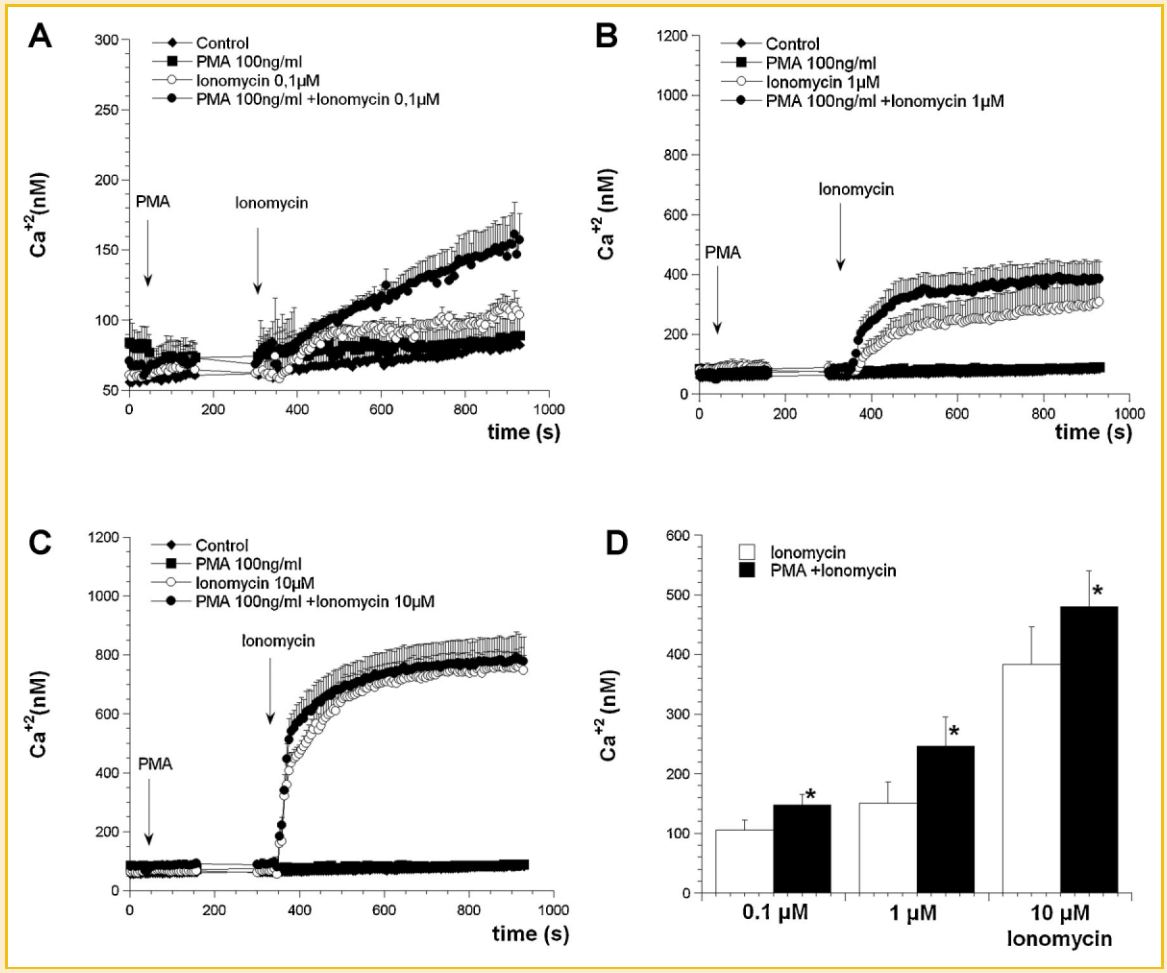

Fig. 3. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-$1^{560,816}$ cells in a saline solution with calcium. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from extracellular Ca^{2+} influx. Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

were observed (Fig. 4C). Figure 5 shows the results obtained in HMC-$1^{560,816}$ cells. Figure 5A shows the cytosolic Ca^{2+} increase at the addition point of ionomycin (0.1 μM), due to the depletion of intracellular Ca^{2+}-pools (125 nM). An increase of 225 nM in cytosolic Ca^{2+} levels was observed when Ca^{2+} was restored to the extracellular medium. In cells treated with PMA alone no effect in cytosolic Ca^{2+} levels was observed. When cells were pre-treated with PMA prior to ionomycin (0.1 μM), Ca^{2+} pools depletion was 20 nM lower (125–105 nM) while Ca^{2+} influx was significantly higher (350–490 nM). The decreasing effect of PMA in intracellular-pools depletion was more evident using 1 μM of ionomycin (47 nM lower than with ionomycin alone), Figure 5B, whereas the enhancing effect on Ca^{2+} influx disappeared. The inhibition of pools depletion by PKC activation was even more notable using 10 μM of ionomycin (86 nM); Figure 5C. In these conditions, when Ca^{2+} was restored to the extracellular medium, the influx of this ion reached the same values than in control cells (ionomycin treated). In summary, in HMC-1^{560} cells PKC activation does not modify Ca^{2+}-pools depletion induced by ionomycin, Figure 4D, while, in HMC-$1^{560,816}$ cells, PMA treatment induces a significant inhibition of ionomycin effect in Ca^{2+}-pools, Figure 5D. After PMA pre-treatment, Ca^{2+}-influx is significantly increased when 0.1 μM ionomycin is used both in HMC-1^{560} and HMC-$1^{560,816}$ cells, while the influx is not affected when the inophore concentration is increased, Figures 4E and 5E.

Different effects either on Ca^{2+} fluxes or on histamine released on HMC-1^{560} and HMC-$1^{560,816}$ cells were observed as consequence of PKC activation; therefore the levels of this protein in the presence of ionomycin, PMA, and Ca^{2+} were checked. A representative experiment for each condition studied is represented in Figure 6A–D. Figure 6E–H shows cytosolic PKC levels (average of band intensity of three experiments). In HMC-1^{560} cells PMA and/or ionomycin treatments did not modify cytosolic PKC levels either in the presence or absence of Ca^{2+} in the extracellular medium (Fig. 6E,G). However, in HMC-$1^{560,816}$ cells, PMA decreased significatively cytosolic PKC levels (Fig. 6F). This decrease was also observed in a Ca^{2+}-free medium and when cells are incubated with ionomycin (Fig. 6H). These results were complemented with the study of nuclear PKC levels. A representative experiment for each condition is shown in Figure 7A–D and Figure 7E–H shows nuclear PKC levels (average of

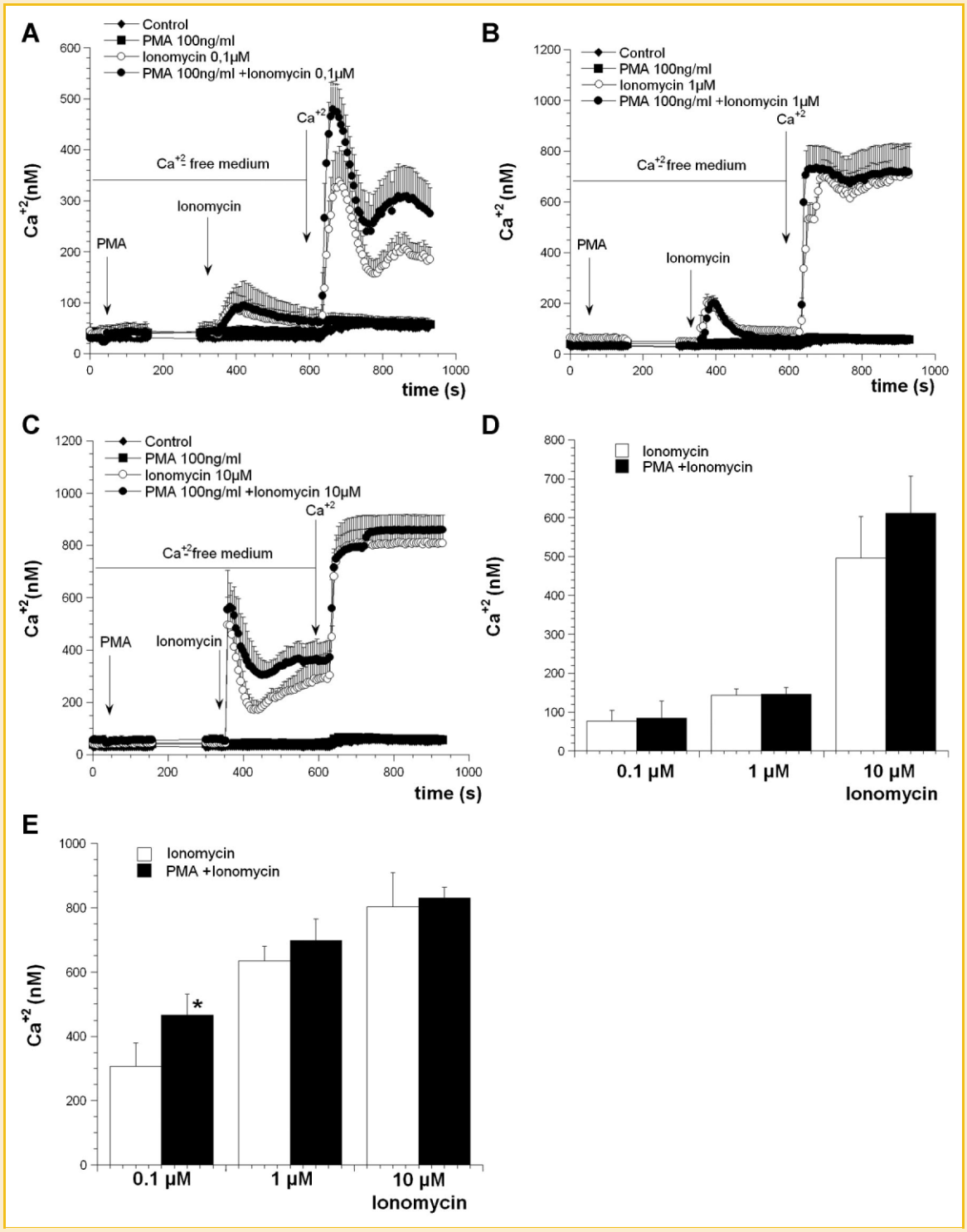

Fig. 4. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-1^{560} cells in a calcium-free saline solution. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from intracellular Ca^{2+} reservoir depletion or extracellular Ca^{2+} influx (E). Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

band intensity of three experiments). In HMC-1^{560} cells PMA and PMA + ionomycin treatments did not modify nuclear PKC levels, both in a saline solution with or without Ca^{2+} (Fig. 7E,G). However, in HMC-$1^{560,816}$ cell line the results were opposite. Surprisingly, when cells were treated with PMA and PMA + ionomycin, nuclear PKC levels (Fig. 7F) were not modified in a saline solution with Ca^{2+}. However, the phorbol ester increased significantly nuclear PKC expression in a Ca^{2+}-free saline solution (Fig. 7H). In these

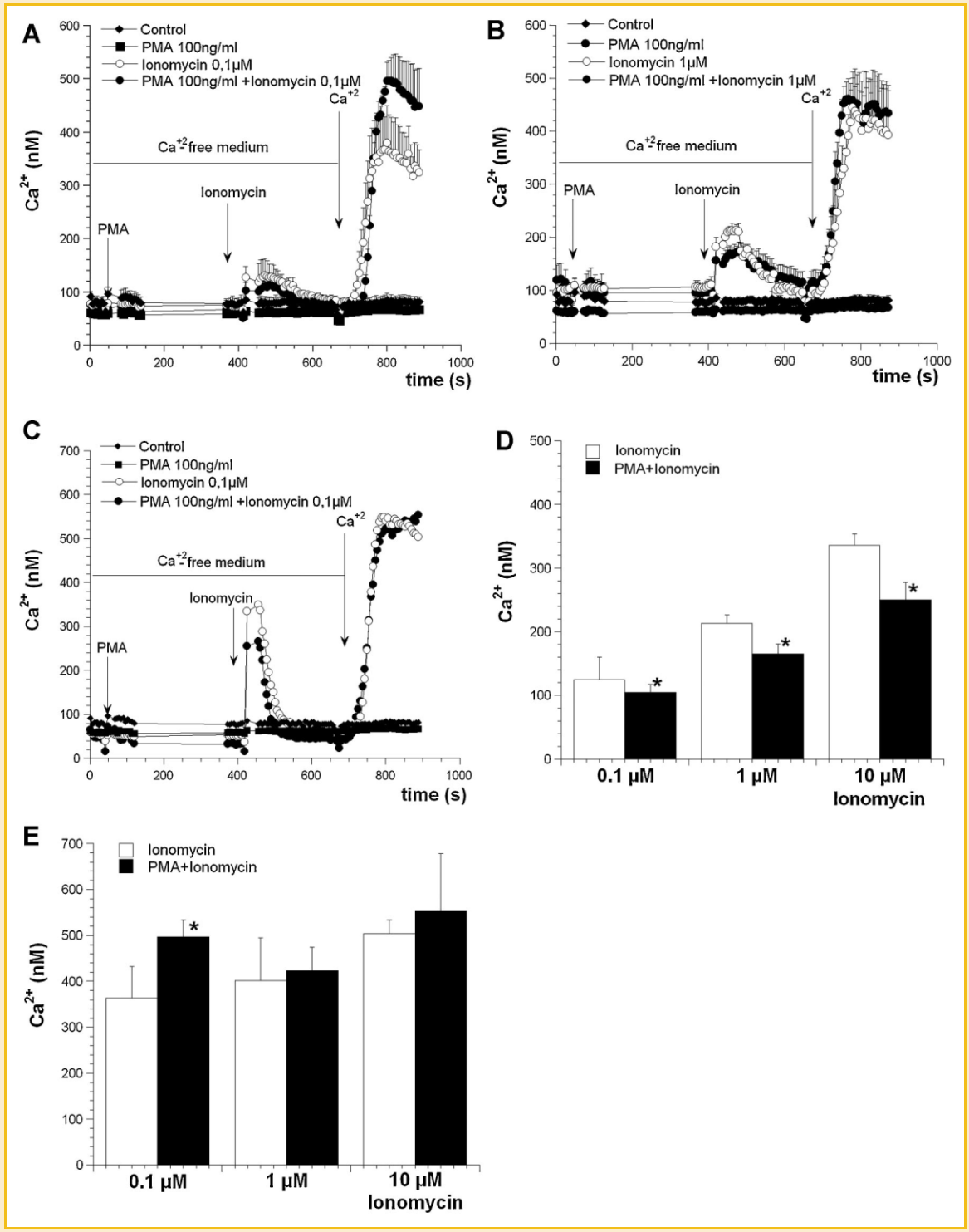

Fig. 5. Effect of PKC activation and ionomycin on cytosolic Ca^{2+} levels of HMC-$1^{560,816}$ cells in a calcium-free saline solution. Cytosolic Ca^{2+} profile of cells that were pre-incubated with PMA 100 ng/ml plus (A) ionomycin 0.1 μM or (B) ionomycin 1 μM or (C) ionomycin 10 μM was added. D: Summary of results shown in (A–C) from intracellular Ca^{2+} reservoir depletion or extracellular Ca^{2+} influx (E). Mean ± SEM of four experiments. *Significant differences between ionomycin and PMA pre-treated cells.

conditions, protein expression levels in the presence of PMA are threefold higher than in control or ionomycin-treated cells.

To confirm nuclear PKC localization, several image-flow cytometry experiments were designed in the conditions before described. Double stained methodology was used in order to check the co-localization of nucleous (propidium iodide) and PKC (fluorescein). To quantify the extent of nuclear PKC localization, the degree of pixel intensity correlation between PKC and nuclear

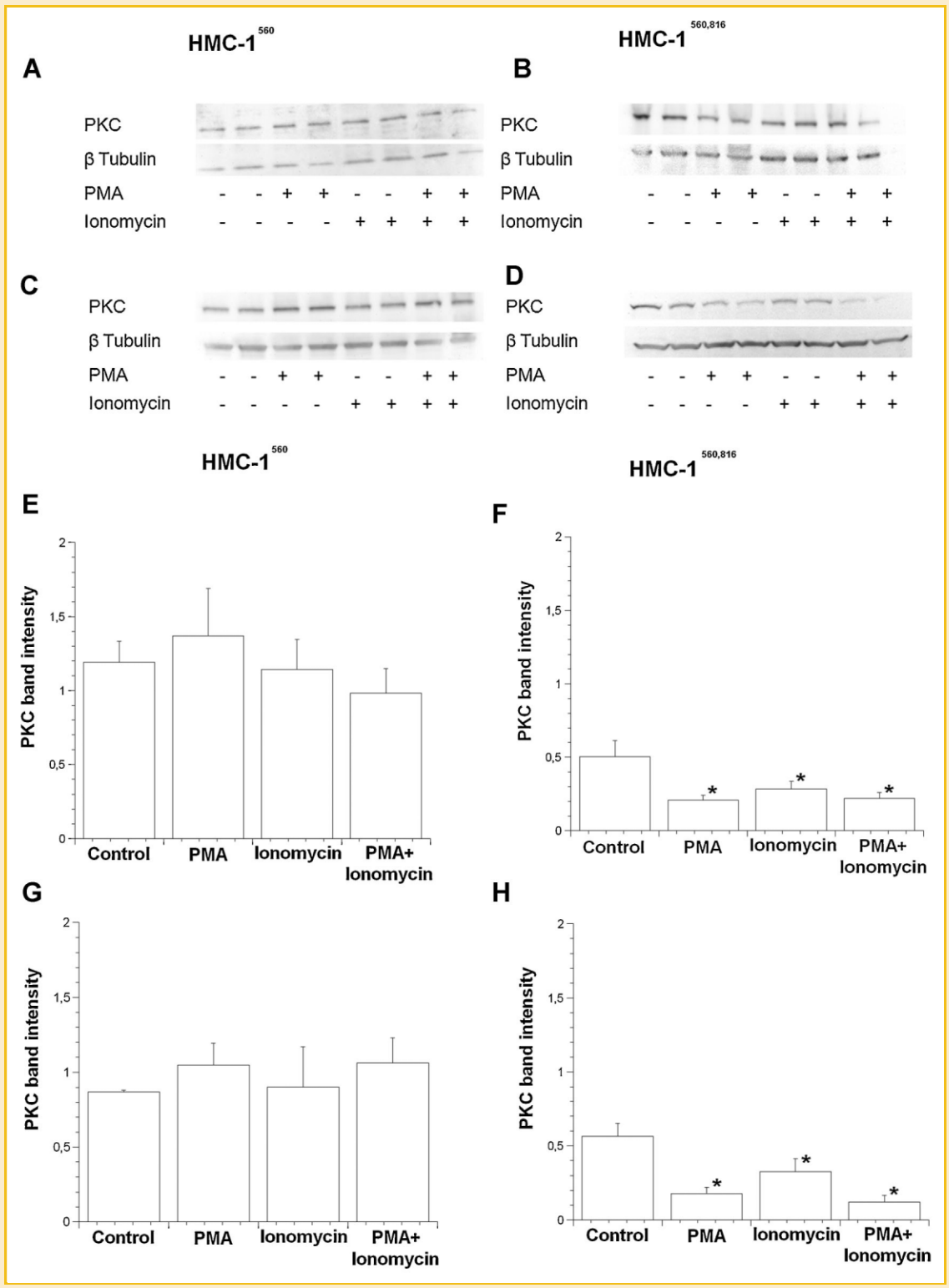

Fig. 6. Effect of PKC activation and Ca^{2+} presence on cytosolic PKC expression in HMC-1^{560} and HMC-$1^{560,816}$ cells. Mean of the ratio of the PKC/Tubulin band intensity. Cells were incubated during 10 min with PMA 100 ng/ml and afterwards stimulated with ionomycin 10 μM for 10 min. At the top of the figure are represented an experiment of each condition. A: HMC-1^{560} and (B) HMC-$1^{560,816}$ corresponding to experiments that were carried out in a saline solution with Ca^{2+}, whereas (C) HMC-1^{560} and (D) HMC-$1^{560,816}$ corresponding to experiments that were carried out in a Ca^{2+}-free saline solution. Cytosolic PKC expression was measured in both cell lines, HMC-1^{560} (E) and HMC-$1^{560,816}$ (F). The same experiment was carried out in a saline solution without Ca^{2+}. G: From HMC-1^{560} cell line and (H) from HMC-$1^{560,816}$ cells. Mean ± SEM of three experiments.

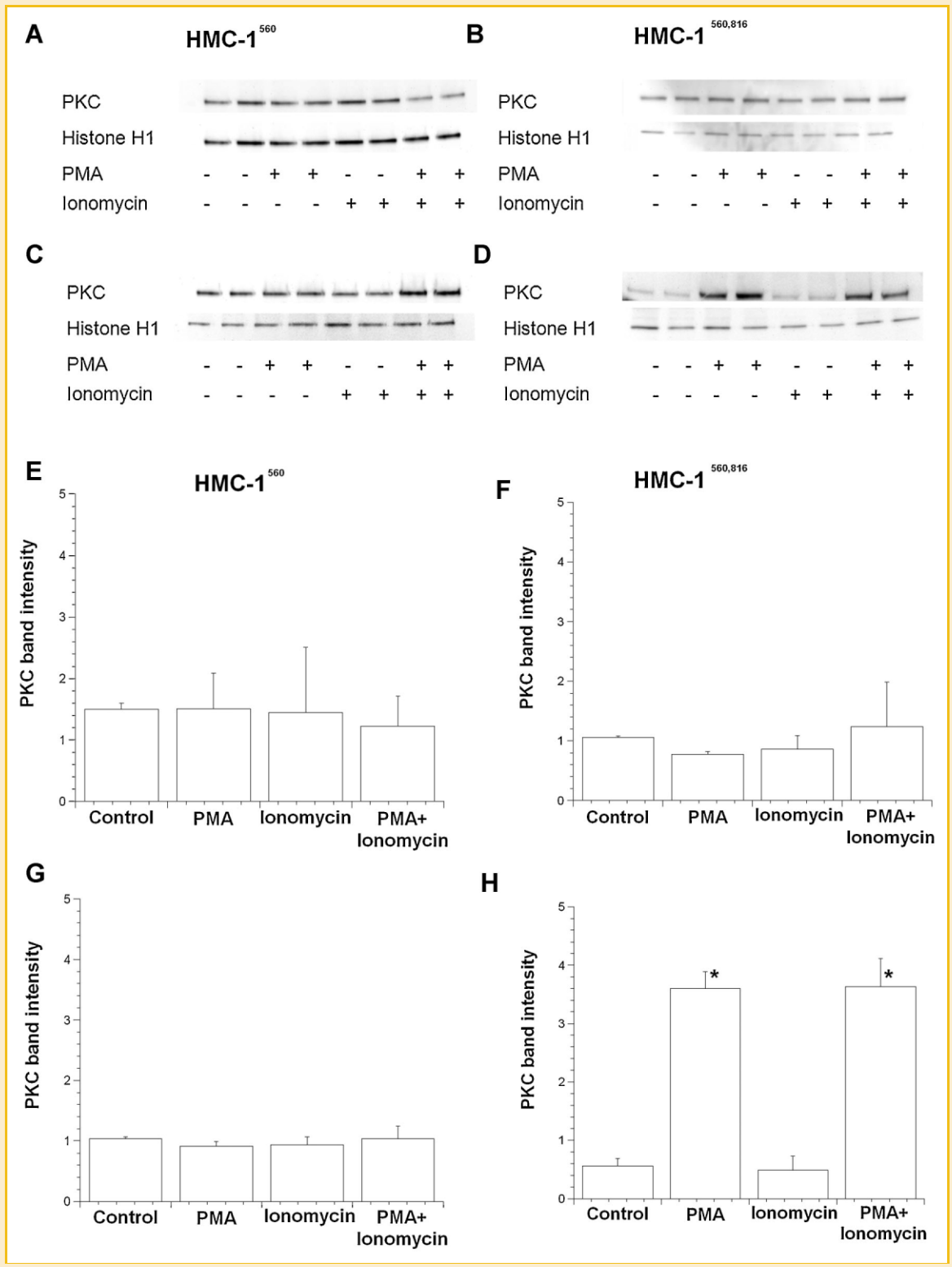

Fig. 7. Effect of PKC activation and Ca^{2+} presence on nuclear PKC expression in HMC-1^{560} and HMC-$1^{560,816}$ cells. Mean of the ratio of the PKC/Histone band intensity. Cells were incubated during 10 min with PMA 100 ng/ml and afterwards stimulated with ionomycin 10 μM for 10 min. At the top of the figure are represented an experiment of each condition. A: HMC-1^{560} and (B) HMC-$1^{560,816}$ corresponding to experiments that were carried out in a saline solution with Ca^{2+}, whereas (C) HMC-1^{560} and (D) HMC-$1^{560,816}$ corresponding to experiments that were carried out in a Ca^{2+}-free saline solution. Cytosolic PKC expression was measured in both cell lines, HMC-1^{560} (E) and HMC-$1^{560,816}$ (F). The same experiment was carried out in a saline solution without Ca^{2+}. G: From HMC-1^{560} cell line and (H) was from HMC-$1^{560,816}$ cells. Mean ± SEM of three experiments.

images was analyzed. Two features, the correlation coefficient (ρ) and logarithmic transformation of ρ (Similarity), were calculated with the following equations: $\rho = \mathrm{Cov}(X,Y)\backslash\sigma_x\sigma_y$, $\mathrm{Similarity} = \ln((1+\rho)\backslash(1-\rho))$. Figures 8 and 9 show the images and histograms obtained from a representative experiment. These histograms represent the percentage of translocated cells in the experiment. In this analysis, a large score to positive values indicates a great degree of similarity between FITC and propidium iodide channels and thus PKC translocation; specifically in this study high similarity was visualized at values higher than 3. In addition, the median of the entire "Live" population was shown in the upper left-hand corner of the histograms. The image galleries show four representative cells in brightfield illumination (gray) adjacent to a composite image that represents an overlay of the images from the two fluorescent channels (fluorescein and propidium iodide). At the bottom, the bars plot represents the mean of three experiments. In HMC-1^{560} cells, no co-localization was observed at any condition tested (Fig. 8E), in addition the percentage of cells with translocated PKC is lower than 5%. However, as Figure 9E shows, in HMC-1^{560,816} cells, PMA treatment induces PKC translocation (nucleous co-localization) in approximately 28% of the cells. These results agree with the increase in nuclear PKC levels observed in Figure 7H.

DISCUSSION

Mast cells express in their membrane the tyrosine kinase receptor c-kit, which is involved in important processes like proliferation, activation, and maturation. The mechanism of down-regulation of the c-kit receptor by the kit ligand was investigated in earlier studies. After binding to the c-kit receptor, kit ligand was internalized and

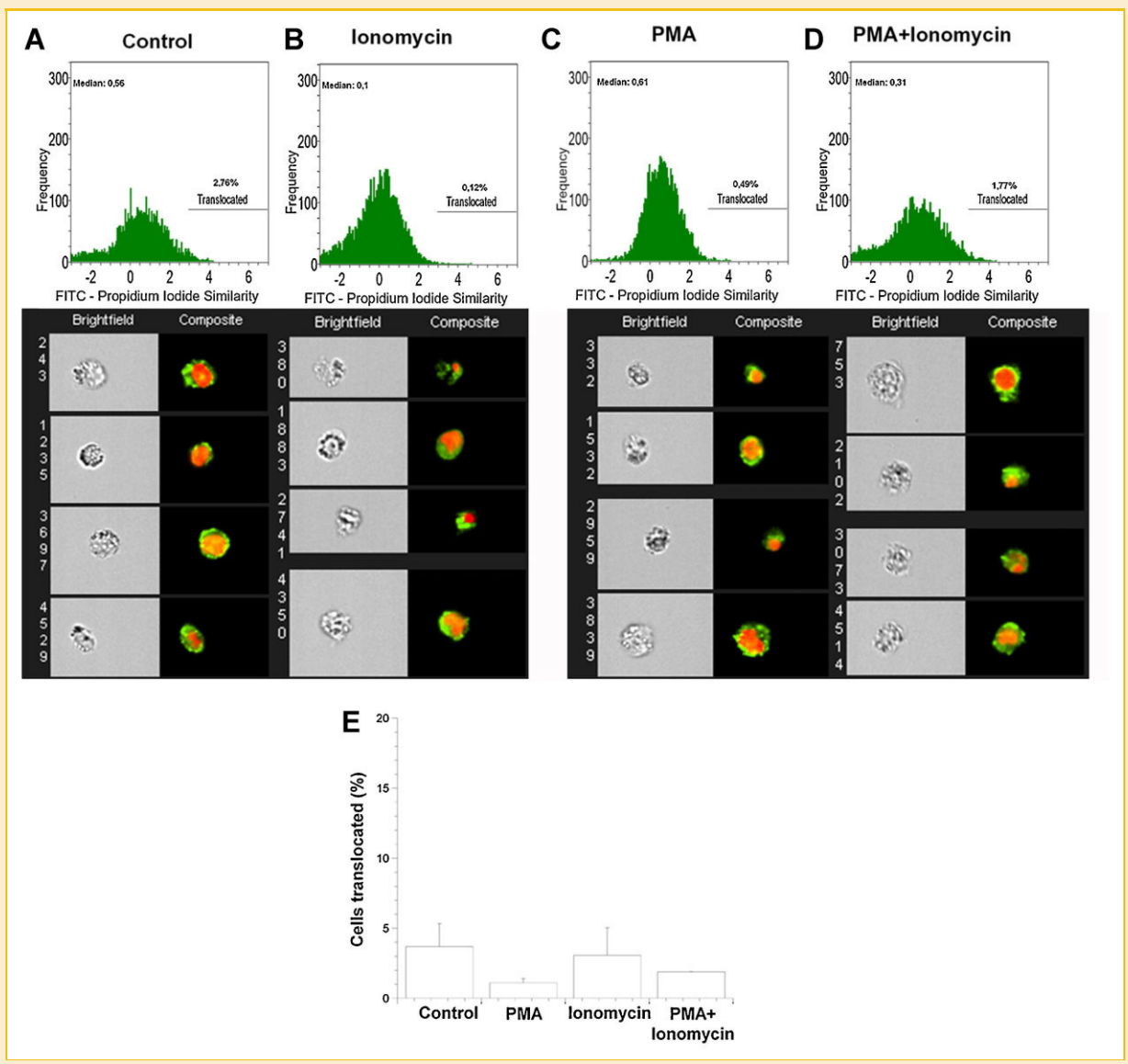

Fig. 8. Analysis of propidium iodide/fluorescein co-localization. Flow cytometry experiments of HMC-1^{560} cell line. This figure shows representative histograms of each condition, control cells (A), PMA treated cells (C), ionomycin treated cells (B) and PMA + ionomycin treated cells (D). Below the histograms a sampling of cell images corresponding with the four conditions is observed. E: Represents the percentage of translocated cells (mean ± SEM of three experiments).

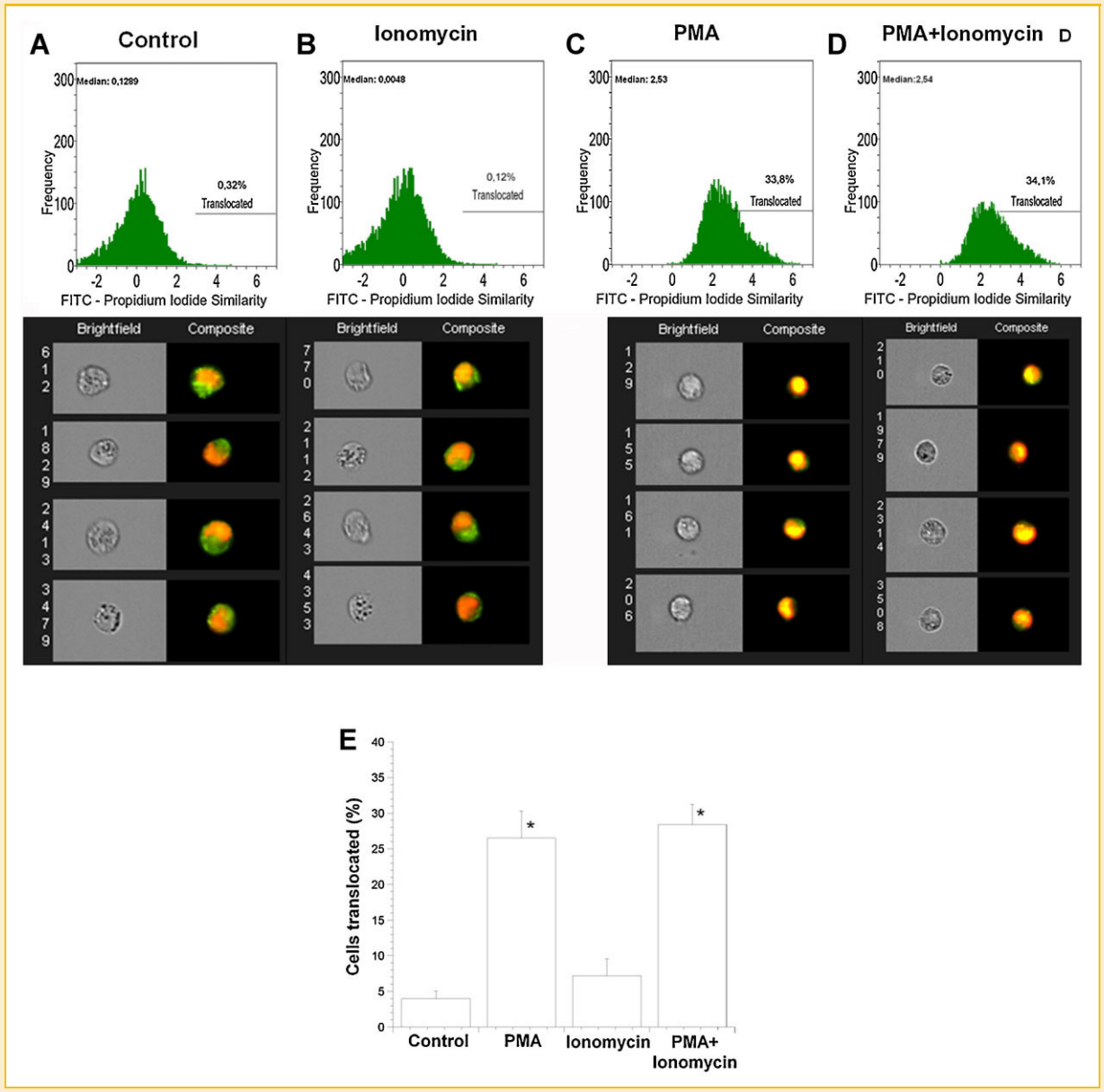

Fig. 9. Analysis of propidium iodide/fluorescein co-localization. Flow cytometry experiments of HMC-$1^{560,816}$ cell line. This figure shows representative histograms of each condition, control cells (A), PMA treated cells (C), ionomycin treated cells (B) and PMA + ionomycin treated cells (D). Below the histograms a sampling of cell images corresponding with the four conditions is observed. E: Represents the percentage of translocated cells (mean ± SEM of three experiments).

accelerated c-kit receptor degradation. Moreover, c-kit receptor transmodulation was produced by the PKC activator PMA and by the Ca^{2+} ionophore ionomycin [Yee et al., 1993]. One pathway to limit c-kit levels proceeds through the internalization and ubiquitin-assisted degradation upon binding of its ligand SCF. This mechanism is rapid and requires an intact kinase activity [Babina et al., 2006]. In addition, it has been described that the activation of c-kit induces or forces mast cell exocytosis and PKC stimulation by PMA down-regulates c-kit expression in mast cell membrane [Columbo et al., 1992, 1994; Wershil et al., 1992; Taylor et al., 1995]. On the other hand, HMC-1^{560} and HMC-$1^{560,816}$ differ in activating mutations in the proto-oncogene c-kit, which cause in both sublines autophosphorylation and permanent activation of the inner TyrK and activate thereby subsequent pathways [Furitsu et al., 1993; Kitayama et al., 1995; Ma et al., 1999; Longley et al., 2001; Sundstrom et al., 2003]. Therefore, PKC has an essential role in c-kit down-regulation. The opposite effect than PKC activation induces in histamine release and calcium fluxes in HMC-1^{560} and HMC-$1^{560,816}$ cells may be related to the activating mutations of c-kit. Ionomycin stimulates histamine release in HMC-1^{560} cells and PKC activation increases exocytosis and Ca^{2+} influx and also cytosolic Ca^{2+} levels. By contrast, the PKC activation negatively affected ionomycin-induced histamine release in HMC-$1^{560,816}$ cells and also intracellular Ca^{2+}-pools depletion while cytosolic Ca^{2+} levels

are increased. These results were surprising and inspired further investigation about the effect of PKC in both HMC-1 sublines. The negative effect of PKC on histamine release in HMC-$1^{560,816}$ cells might be partly regulated by Ca^{2+}-pools depletion, since this was significantly decreased at any ionomycin concentrations after PMA pre-incubation. An earlier study suggested the existence of a feedback mechanism that uses PKC to limit the increase in intracellular Ca^{2+} levels [Barajas et al., 2008]. Results shown in the present article demonstrate that PMA treatment increases cytosolic Ca^{2+} levels in both mast cell lines; however phorbol ester treatment has different effects on ionomycin-induced histamine release and in pools depletion. The increase in histamine release after PKC activation in HMC-1^{560} cell is probably due to the increase in cytosolic Ca^{2+} levels. In HMC-$1^{560,816}$ cells the decrease in histamine release is related to a lower pools depletion. From these results, it can be concluded that PKC acts as a negative regulator of exocytosis in HMC-$1^{560,816}$ cells, but not in HMC-1^{560} cell line. Since calcium levels are increased in both cellular lines, it can be further pointed that either Ca^{2+} has an inhibitory effect in HMC-$1^{560,816}$ line or PKC has a Ca^{2+} independent activity. Data shown in this study confirm that in both cell lines (HMC-1^{560} and HMC-$1^{560,816}$), the effect of PMA on ionomycin-induced histamine release is mediated by classical PKCs. For this reason, when this family of PKCs is inhibited no effect on histamine release is observed. Moreover, it can be affirmed that PKCδ is not involved in the effect of PMA on ionomycin-induced histamine release.

The different effects on histamine release and Ca^{2+} levels in both cell lines can be also related to PKC expression and localization. Some studies have shown that PKC isoforms could be translocated to the membrane or nucleous depending on cellular model. In Mz-ChA-1 cells stimulated with (R)-(alpha)-(−)-methylhistamine dihydrobromide (RAMH), phosphorylation of PKC and mitogen-activated protein kinase isoforms were measured. RAMH induced a shift in the localization of PKCalpha expression from the cytosolic domain into the membrane region of Mz-ChA-1 cells [Francis et al., 2009]. In addition, in NIH 3T3 cells PMA treatment induces the tight association of PKC to the nucleous [Thomas et al., 1988]. Besides, translocation of PKCα to the nucleous in response to PMA was reported in many studies [Eldar et al., 1992; Maissel et al., 2006]. In summary, PKC translocation to the membrane or to the nucleous, when the protein is activated, might be correlated with PKC isoforms and cell lines studied. Furthermore, in this paper it was observed that PKC translocation was different in both mast cell lines, since in HMC-$1^{560,816}$ cells PKC is completely translocated to the nucleous in 28% of the cells but in the other cell line, HMC-1^{560}, PKC activation did not imply their nuclear translocation. For this, PKC activation would down-regulate their cytosolic expression in HMC-$1^{560,816}$ cell line in Ca^{2+}-free medium. In this study it can be stated that PMA and PMA + ionomycin treatments induce the translocation of PKC to the nucleous in a Ca^{2+}-free saline solution in HMC-$1^{560,816}$ cell line. It is necessary to point out that in this cellular line the expression of cytosolic PKC decreases when cells are activated. In Ca^{2+}-free medium, the decrease in cytosolic PKC levels matches the increase in nuclear localization of PKC.

The results obtained show that PKC activation implies a decrease in cytosolic PKC levels that was matched with an increase in nuclear PKC levels. However, this translocation did not occur in a saline solution with calcium. The presence of Ca^{2+} is not needed for the nuclear translocation and the protein would be translocated to another place like plasma membrane [Kimata et al., 1999]. Moreover, ionomycin treatment induces a decrease in cytosolic PKC levels in the absence of Ca^{2+}, but this effect was not accompanied with an increase in nuclear PKC levels. The previous work describes that PKC is translocated into cell membrane along with an increase in intracellular Ca^{+2} concentrations [Izushi and Tasaka, 1992]. In HMC-1^{560} cells PKC activation induces the same effects than in normal mast cells [Friis and Johansen, 1996]. However in HMC-$1^{560,816}$ cells, PKC activation inhibits histamine release and Ca^{2+}-pools depletion. Moreover, in HMC-1^{560} cells PKC activation does not affect cytosolic PKC expression whereas in HMC-$1^{560,816}$ cells PKC activation affects PKC localization and cytosolic expression.

The results shown in this article show for the first time the difference of PKC localization in HMC-1^{560} and HMC-$1^{560,816}$ cell lines when they were activated with PMA. The findings obtained demonstrate that PKC is translocated to the nucleous in 28% of the HMC-$1^{560,816}$ cell line population and that this translocation does not occur in HMC-1^{560}. PKC translocation to the nucleous could be related to the inhibition of pools depletion and histamine release in HMC-$1^{560,816}$ cell line, since all these effects occur in a Ca^{2+}-free saline solution. However, PKC translocation did not happen in HMC-1^{560} cell line and PMA effect on ionomycin-induced histamine release is opposite. Unlike HMC-$1^{560,816}$ cells, in cells with one mutation PMA treatment did not affect calcium pools' depletion. The crosstalks between the two mutations and the final control of PKC remain to be defined. Furthermore, it would be interesting to know the characteristics of the cells that have completely translocated PKC and open a new research line that includes the study of nuclear PKC effect. Finally, the effect of this translocation to the nucleous should be studied in subsequent publications.

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