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Cross-talks between c-Kit and PKC isoforms in HMC-1^{560} and HMC-1^{560,816} cells. Different role of PKCδ in each cellular line

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ABSTRACT

The c-kit inhibitor STI571 represents one of the most important treatments for patients with mastocytosis. However, intracellular pathways modulated by this compound are not completely defined. Here, STI571 effect on Protein Kinase C (PKC) regulation is determined in HMC-1 mast cell lines. STI571 activates PKCδ isoform resulting in HMC-1^{560} apoptosis. The apoptosis observed is PKCδ-dependent, since PKCδ-silencing avoids STI571 effect. c-kit inhibition implies nuclear PKCδ translocation characterized by a clear dependence on actin cytoskeleton integrity in HMC-1^{560} cell line, but not in HMC-1^{560,816}. Therefore, PKCδ modulations can lead to a serious decrease in STI571 treatment-effectiveness.

1. Introduction

Mast cells (MCs) play a key role in the immune response, either in allergic or non-allergic inflammatory conditions [1]. The uncontrolled growth of MCs in one or more visceral organs is called mastocytosis [2–4]. This disease is usually associated with somatic gain-of-function point mutations within c-kit receptor (CD117). c-kit is a type III tyrosine kinase (TyrK) receptor expressed in MCs, hematopoietic progenitors, melanocytes, NK cells and interstitial Cajal cells [5,6]. The most common mutation is the c-kit^{D816V}, present in Human Mast Cell (HMC)-1^{560,816} line, resulting from substitution of valine-816 for aspartate [7–9]. Another important mutation is the substitution of glycine-560 for valine (V560G), present in HMC-1^{560} and HMC-1^{560,816} cell lines. The natural ligand of c-kit is the stem cell factor (SCF). Both mutations, located on c-kit receptor, imply that these sublines grow *in vitro* without SCF, since c-kit is ligand-independent phosphorylated [10–14]. STI571 compound (imatinib mesylate or Gleevec®) is a tyrosine kinase inhibitor that inhibits c-kit activity [15–19]. This compound induces HMC-1^{560} cell death, however, is ineffective against HMC-1^{560,816} proliferation [16,20].

Protein Kinase C (PKC) is a protein involved in multiple and different biological events and thus plays a crucial role in cell metabolism regulation. PKC isoforms have been subdivided into three groups: (1) classical PKCs (α, β and γ), which are calcium (Ca^{2+}) dependent and activated by diacylglycerol (DAG) or phorbol ester; (2) 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. PKC-induced cell metabolism regulation would occurs subsequently to its phosphorylation and translocation from the cytosol to the plasma membrane [21]. Therefore, PKC translocation is a response of its activation and has been described in several cellular models. For example, classical PKCs translocate to the membrane in response to an antigen stimulus in RBL-2H3 mast cell line [22,23]. However, PKC can translocates also to the nucleus depending on the stimulus [24,25]. In fact, PKC localization in the nucleus is usually associated with a role on apoptotic cell death, since PKC has been associated with this process and subsequently with caspases activation in several cellular types, including MCs [26–30].

In a previous study, the relationship between the tyrosine kinase inhibitor STI571 and PKC was study in terms of cytotoxic effect assessment on both HMC-1 cell lines [31]. Here, the effect of STI571 on PKC activated cells and the relationship between STI571-induced cell death and PKC activation will be determined in HMC-1^{560} and HMC-1^{560,816} cell lines. With the objective to clarify STI571 mechanism of action, the direct effect of this compound over PKC will be determined and PKC translocation will be assessed by measuring cytosolic, nuclear and plasma membrane PKC levels by Western blot.

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2. Materials and methods

2.1. Chemicals

STI571 was provided by Dr. Luis Escribano Mora (Centro de Estudios de Mastocitosis de Castilla la Mancha, Hospital Virgen del Carmen, Toledo, Spain). Phorbol 12-myristate 13-acetate (PMA), latrunculin A derived from *Negombata magnifica*, bovine serum albumin (BSA), Tween 20 and anti β-tubulin were from Sigma–Aldrich (Madrid, Spain). Phosphate buffered saline (PBS) was from Invitrogen (Barcelona, Spain). Anti PKCδ was from BD Biosciences (Madrid, Spain). Anti Mouse IgG was purchased from GE Healthcare (Barcelona, Spain). Anti PKC Clone M110, anti β-actin, anti Histone H1 and polyvinylidene fluoride (PVDF) membrane were from Millipore (Temecula, USA). Cell Lab ApoScreen™ Annexin V and DNA Prep™ Stain were from Beckman Coulter (Fullerton, CA, USA). Polyacrylamide gels and molecular weight marker Precision Plus Protein™ Standards Kaleidoscope™ were from Bio-Rad (Barcelona, Spain). Negative siRNA control (sc-37007) and PKCδ siRNA (sc-36253) were purchased from Santa Cruz Biotechnology (CA, USA). GeneSilencer® was from Genlantis (San Diego, CA, USA).

2.2. 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, Spain) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain) and 100 IU/mL penicillin + 100 μg/mL streptomycin (Gibco, Invitrogen, Spain) in an atmosphere containing 5% CO_2. The medium was renewed once a week.

2.3. Cell preparation

HMC-1 cells were incubated with 25 nM STI571, 100 ng/mL PMA and 200 nM latrunculin during 48 h. It is widely known that PKC activation can be induced after PMA short-incubation (10 min). However, in this study, PMA was incubated the same time as for STI571 in order to evaluate a possible PMA modulator effect. Then cells were centrifuged (1500 rpm, 5 min, 4 °C) and washed in 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.

2.4. Western blotting

2.4.1. Cytoplasmic proteins protocol

The cells were resuspended in 80 μL of lysis buffer with the following composition: 50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain). Cells were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant (cytoplasmic fraction) was collected and stored at −20 °C.

2.4.2. Nuclear proteins protocol

The cells were resuspended in 500 μL of buffer containing: 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 5% Triton X-100, 1X complete protease inhibitor (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain) at pH 7.9. Cells were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was kept out and the 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 26 μL of 4.6 M NaCl were added. Samples were sonicated four times (10 s). Assay tubes were refrigerated on ice for 30 min and centrifuged at 13,500 rpm for 20 min at 4 °C. Finally, the supernatant was collected and stored at −20 °C.

2.4.3. Membrane proteins protocol

The cells 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 (Roche, Spain) and 1X phosphatase inhibitor cocktail (Roche, Spain). Samples were shaken and sonicated for 1 min and then refrigerated on ice for 20 min. Then cells were sonicated 10 s/three times and then centrifuged (12,000 rpm, 20 min, 4 °C). Supernatant (cytosolic fraction) was discarded and samples were resuspended in lysis buffer. Samples were sonicated 10 s/3 times and then centrifuged (12,000 rpm, 20 min, 4 °C). Finally, the supernatant was stored at −20 °C.

Once the proteins have been lysated, following the three protocols previously described, protein concentration determination was carried out by using Bradford assay and BSA as protein standard. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) procedure was used to separate proteins according to their molecular weight. Proteins were transferred to a PVDF membrane blocked with 0.25% non-fat dry milk and then it was incubated for 10 min with anti-PKC clone M110 (1:1000, Millipore), or anti PKCδ (1:1000, BD Biosciences). After three washes with washing buffer (PBS + 0.1% Tween 20), 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 Femto; Pierce) was used to determine protein expression levels. Relative protein expression was calculated in relation to β-actin (0.3:1000, Millipore) for cytosolic and membrane proteins. For experiments with latrunculin, relative protein expression was calculated in relation to β-tubulin (0.3:1000, Sigma Aldrich). Histone H1 expression (1:1000, Millipore) was used to calculate relative protein expression for nuclear proteins.

2.5. PKCδ-nucleus co-localization by flow cytometry

HMC-1^{560} cells were treated with 25 nM STI571 and 200 nM latrunculin for 48 h. Then cells were fixed with 4% PFA for 20 min at room temperature and permeabilized using PBS + 5%BSA + 0.1%Triton X-100 for 1 h 30 min at room temperature. Then cells were incubated for 12 h in the presence of anti PKCδ (1:1000, BD Biosciences). After PBS wash, primary antibody was detected with FITC-conjugated secondary antibody (Goat Anti Mouse IgG, IgM, IgA Biotin Conjugated, 1:1000, Millipore). For propidium iodide staining, the Coulter DNA Prep™ Stain (Beckman Coulter) was used for 2 min at 37 °C. ImageStream imaging flow cytometer (Amnis Corporation, Seattle, WA) was used in this study. A total of 10,000 events were collected per sample and IDEAS software (Amnis Corporation) was used for analysis.

2.6. Apoptotic and necrotic cell death determination by flow cytometry

Apoptosis was detected by Annexin-V-FITC/PI staining using the Cell Lab ApoScreen™ Annexin V kit. The cells were incubated with 25 nM STI571 for 48 h. Then cells were prepared exactly following manufacturer's instructions assay and Amnis Corporation IS-100 flow cytometer was used.

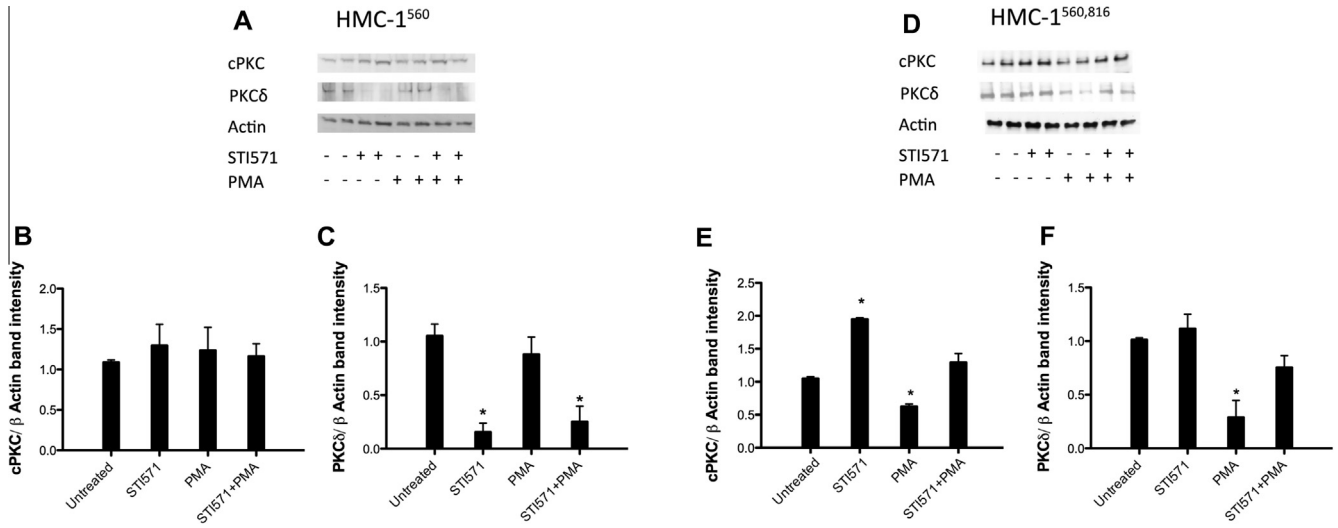

Fig. 1. Effect of STI571 treatment and PKC activation on cytosolic cPKC and PKCδ levels in HMC-1^{560} and HMC-$1^{560,816}$ cell lines. (A and D) Show a representative experiment of each condition in HMC-1^{560} and HMC-$1^{560,816}$, respectively. (B and C) Represent cytosolic cPKC and PKCδ levels for HMC-1^{560} cell line, whereas (E and F) represent cytosolic cPKC and PKCδ levels for HMC-$1^{560,816}$ cell line. Protein levels are represented in relation with β-actin cytosolic levels. Mean ± SEM of three experiments. (*) Significant differences between untreated and treated cells.

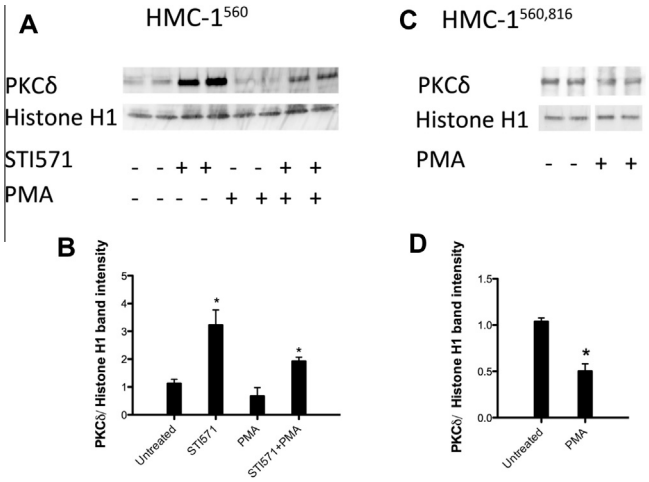

Fig. 2. Effect of STI571 treatment and PKC activation on nuclear PKCδ levels in HMC-1^{560} and HMC-$1^{560,816}$ cell line. (A and C) Show a representative experiment of each condition in HMC-1^{560} and HMC-$1^{560,816}$, respectively. (B) HMC-1^{560} and (D) HMC-$1^{560,816}$ nuclear PKCδ expression was represented in relation with Histone H1 protein band intensity. Mean ± SEM of three experiments. (*) Significant differences between untreated and treated cells.

2.7. Transfection by lipid-based method

GeneSilencer® (Genlantis) was composed by the transfection reagent and the diluent. Manufacturer's instructions were followed carefully. On the day of transfection two solutions were prepared. On the one hand, solution A, composed by: diluent, FBS/Antibiotic-free IMDM medium and PKCδ siRNA. Control siRNA (sc-37007) was used as negative control for evaluating siRNA off-target effects. On the other hand, solution B was composed by transfection reagent diluted in FBS/Antibiotic-free IMDM medium. Solutions A and B were mixed and incubated for 5 min at room temperature. HMC-1^{560} cells were incubated in a total volume of 500 μL in FBS/Antibiotic-free IMDM medium. Cellular concentration was 2×10^6/ml. 500 μL of IMDM (supplemented with 20% FBS and Penicillin/Streptomycin 2x) were added to the HMC-1^{560} cells after 5 h transfection. 19 h after cells were incubated with 25 nM STI571 for 48 h.

2.8. Statistical analysis

Results were analyzed using the ANOVA and 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.

3. Results

Several experiments were carried out in order to determine STI571-induced cell death relationship with PKC activity in HMC-1 cell lines. First, STI571 effect over PKC expression was determined by Western blot. Fig. 1A and B shows that STI571, PMA and STI571 + PMA treatments do not modify cytosolic classical PKC (cPKC) levels in HMC-1^{560} cell line. The expression of the PKC isoform PKCδ, widely related with cell death and with DNA damage through caspase-3 apoptotic pathway, was also studied [32–35]. Contrary to STI571 effects previously observed in cPKC isoforms, STI571 treatment induces a decrease of cytosolic PKCδ levels, from 1 to 0.2 ratio intensity values (Fig. 1A and C). This negative STI571 effect prevails even after PMA treatment, whereas PMA alone does not modify cytosolic PKCδ levels. This experiment was also performed in the HMC-$1^{560,816}$ cell line. In this case, STI571 induces a significant increase of cPKC levels (protein levels are two-fold higher than in untreated cells), whereas PMA treatment causes a decrease of cPKC expression (Fig. 1D and E). However, no modifications were observed after STI571 + PMA treatment. In addition, STI571 incubation does not modify cytosolic PKCδ levels in HMC-$1^{560,816}$ cells (Fig. 1D and F), while PMA induces a significant decrease of cytosolic PKCδ expression (ratio intensity values fall down from 1 to 0.3). Besides, STI571 prevents this negative PMA effect. Therefore it seems that both cPKC and PKCδ move from the cytosol to other localizations after HMC-1 cells treatment.

Since PKCδ translocation to the nucleus is frequent after apoptotic stimulation, the next step was to determine nuclear PKCδ levels. As Fig. 2A and B shows, PKCδ translocates to the nucleus after STI571 treatment in HMC-1^{560} cell line. Nuclear levels significantly increase from 1 to 3 (ratio intensity values). Besides, PKC activation with PMA does not have any effect by itself and does not prevent nuclear PKCδ translocation (Fig. 2A and B). In HMC-$1^{560,816}$ cell line, PMA induces a significant decrease on nuclear PKCδ levels (Fig. 2C

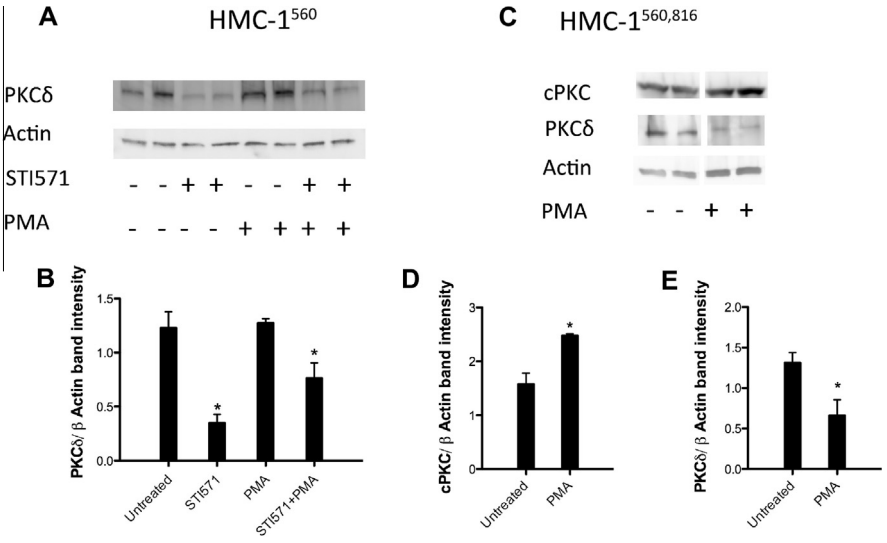

Fig. 3. Effect of STI571 treatment and PKC activation on plasma membrane cPKC and PKCδ levels in HMC-1^{560} and HMC-$1^{560,816}$ cell line. (A and C) Show a representative experiment of each condition in HMC-1^{560} and HMC-$1^{560,816}$, respectively. HMC-1^{560} (B) and HMC-$1^{560,816}$ (E) plasma membrane PKCδ expression was represented in relation with β-actin plasma membrane expression. (D) Plasma membrane cPKC expression in relation with β-actin in HMC-$1^{560,816}$ cell line. Mean ± SEM of three experiments. (*) Significant differences between untreated and treated cells.

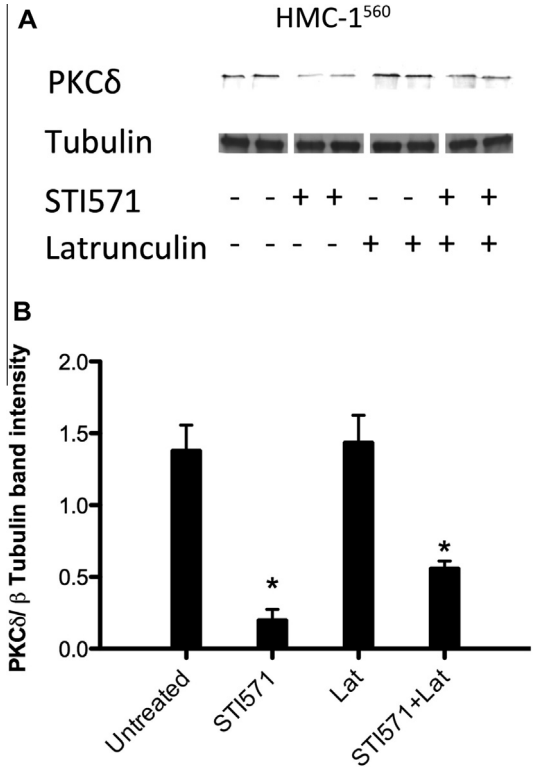

Fig. 4. Effect of STI571 on cytosolic PKCδ levels in HMC-1^{560} cell line-latrunculin incubated. (A) Is a representative experiment of each condition. (B) Represents cytosolic PKCδ levels for HMC-1^{560} cell line. Mean ± SEM of three experiments. (*) Significant differences between untreated and treated cells.

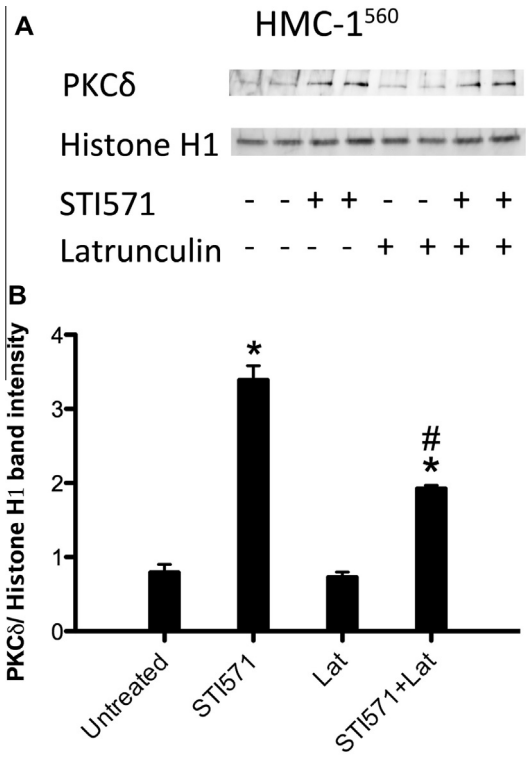

Fig. 5. Effect of STI571 on nuclear PKCδ levels in HMC-1^{560} cell line-latrunculin incubated. (A) Representative experiment of each condition. (B) Nuclear PKCδ expression was represented in relation with Histone H-1 band intensity. Mean ± SEM of three experiments. (*) Significant differences between untreated and STI571-treated cells. (#) Significant differences between STI571 and STI571 + latrunculin treated cells.

and D). Therefore, nuclear PKCδ levels are increased after STI571 treatment in HMC-1^{560} cells. Nuclear cPKC levels were not analyzed since in a previous paper we have demonstrated that cPKC isoforms do not translocate to the nucleus [25].

Plasma membrane is one of the most common subcellular spaces for PKC translocation. Therefore, PKCδ levels were also studied in this localization [36]. As Fig. 3A and B shows, STI571 does not cause PKCδ membrane translocation, since protein levels are significantly diminished. Besides, STI571 + PMA co-treatment induces a significant decrease of PKCδ membrane levels, whereas PMA does not have any effect by itself. As Fig. 3C and D shows, PMA increases cPKC levels on plasma membrane in HMC-$1^{560,816}$ cell line. However, PKC activator diminishes PKCδ levels (Fig. 3C and E), as happens in the cytosol and in the nucleus.

Tyrosine kinase and PKC are both related with actin cytoskeleton. On the one hand, STI571 apoptotic effect is associated with actin cytoskeleton alteration in HMC-1^{560} cell line, accompanied by actin filamentous aggregation and actin polymerization decrease [37]. On the other hand, it was described that PKC isoforms binding to F-actin enhances isozymes activity [38]. In addition to F-actin, PKC isoforms can be associated with other cytoskeletal proteins as vimentin and cytokeratins [39]. In order to determine if actin cytoskeleton participates in STI571-induced nuclear PKCδ translocation, latrunculin effect was studied in cytosolic and nuclear fractions. First, latrunculin, which affects actin cytoskeleton by sequestering (monomeric) G-actin [40], effect was checked in the presence of STI571. As Fig. 4A and B shows, latrunculin partially inhibits STI571 effect over PKCδ cytosolic expression, since PKCδ levels up from 0.2 to 0.5 ratio intensity values. Moreover, latrunculin does not have any effect by itself. At nuclear level, latrunculin partially inhibits PKCδ translocation induced by STI571, since intensity values fall from 3 to 1.8 (Fig. 5A and B). To confirm these results, nuclear PKCδ translocation was determined in HMC-1^{560} cell line by using a double-staining technique (FITC for PKC and propidium iodide for nucleus). As expected, STI571 induces nuclear PKCδ translocation (Fig. 6). The similarity of both dyes (FITC and propidium iodide) is significantly higher in the presence of STI571 (Fig. 6B). The percentage of nuclear PKCδ translocated cells in STI571 treated cells is 87% (Fig. 6E). Latrunculin does not produce any effect by itself (Fig. 6C and E). However, the percentage of translocated cells decreases from 87% to 30% when STI571 is incubated in the presence of latrunculin. These results are consistent with those obtained by Western blot.

It is well known that STI571 has cytotoxic effect against HMC-1^{560}, however the pathways activated by STI571 and the role of PKCδ are still poorly understood. We therefore next investigated the effect of STI571 on HMC-1^{560} cells by using the Annexin V-FITC technique. This method allows us to determine live, apoptotic and necrotic cells distribution. As Fig. 7A and C shows in control conditions (untreated cells) that the percentage of viable cells is 70%, while the rest, 30%, are necrotic cells. After STI571 treatment, 40% of the total are live cells, and apoptotic and necrotic populations are the 39% and 15%, respectively (Fig. 7B and C). Next, PKCδ role on apoptosis induced by STI571 was studied by PKCδ silencing in HMC-1^{560} cell line. The first step was to evaluate PKCδ silencing method effectiveness, by using a tested siRNA PKCδ [41–44]. As Fig. 8A and B shows, PKCδ cytosolic expression significantly decreases (from 0.46 to 0.16) in PKCδ-silenced cells. Thus, a silencing around 65% is obtained. Then, STI571 cytotoxic effect was determined in PKCδ-silenced cells (Fig. 9). Surprisingly, apoptotic cell death induced by this tyrosine kinase inhibitor disappears in PKCδ-silenced cells, since apoptotic population significantly falls down from 40% to 15% and the live population increases in the same percentage (Fig. 9B and C). Therefore these data indicate that STI571 mechanism of action is dependent of PKCδ.

4. Discussion

Results shown in this paper demonstrate that c-kit inhibition is clearly linked to PKC activity. In the HMC-1^{560} cell line, long-term PKC activation does not induce a decrease neither on cPKCs nor PKCδ cytosolic levels. This observation is in accordance with a previous study in which PKC was only activated for 10 min and no

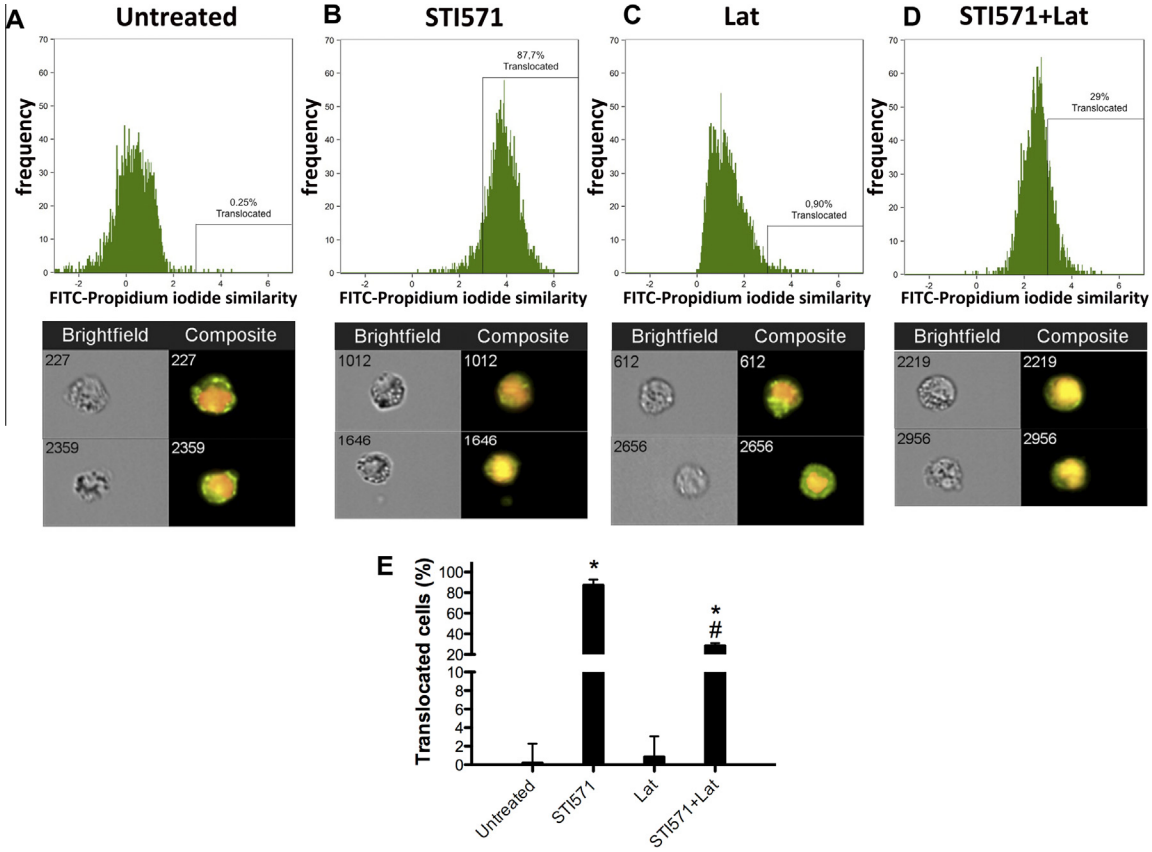

Fig. 6. Determination of PKCδ-nucleus co-localization by flow cytometry in HMC-1^{560} cell line. (A, B, C and D) Show a representative histogram of each condition. Two cells of each condition in brightfield and composite (FITC and propidium iodide) channels are shown below the histograms. The mean ± SEM of three experiments is represented in Fig. 6E. (*) Significant differences between untreated and treated cells. (#) Significant differences between STI571 and STI571 + latrunculin treated cells.

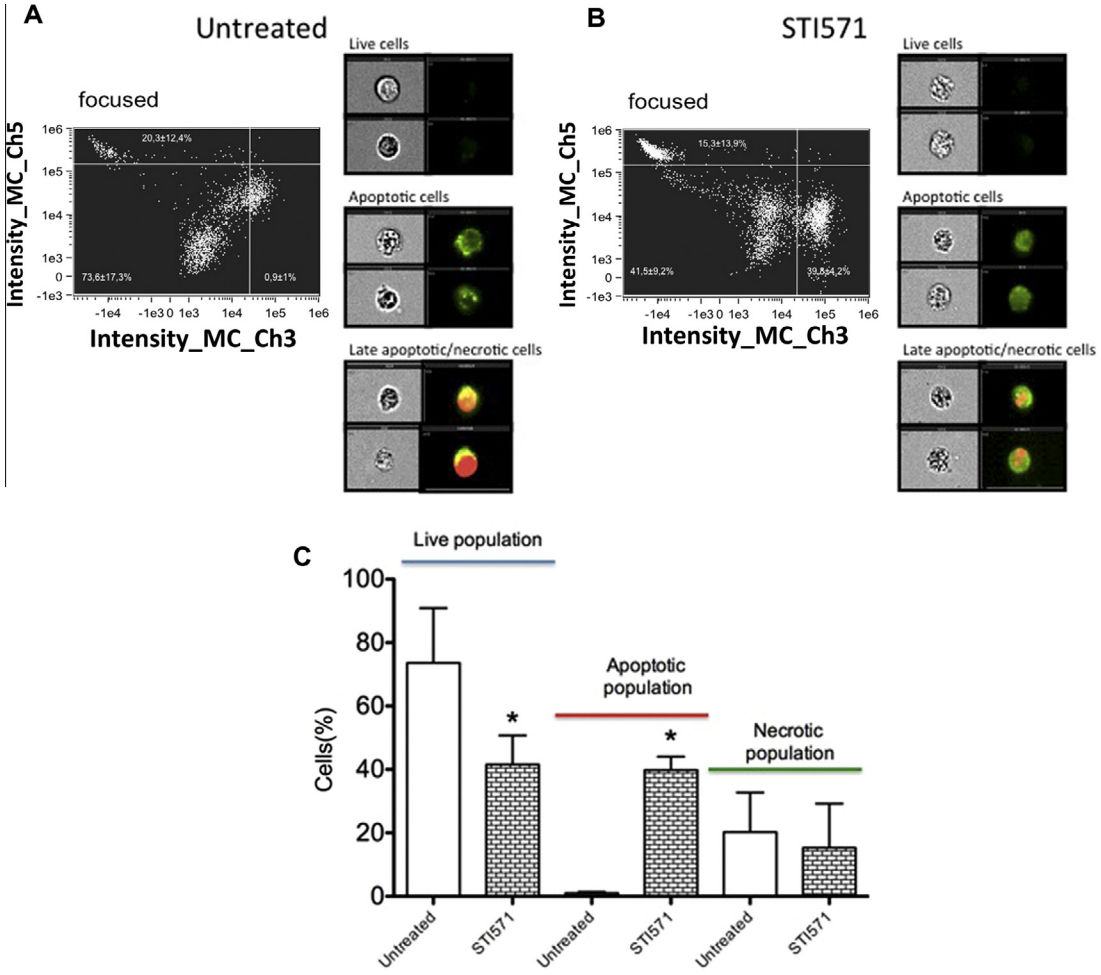

Fig. 7. Determination of live, apoptotic and necrotic HMC-1^{560} cell line treated with 25 nM STI571. Live (botton left panel of each graph), apoptotic (botton right panel of each graph) and late apoptotic/necrotic (upper panel of each graph) cells were detected by Annexin-V-FITC/PI staining. Graphs represent one of three repeated experiments: (A) untreated cells and (B) STI571-treated cells. The mean ± SEM of three experiments is represented in Fig. 7C. (*) Represents significant differences between untreated and treated cells.

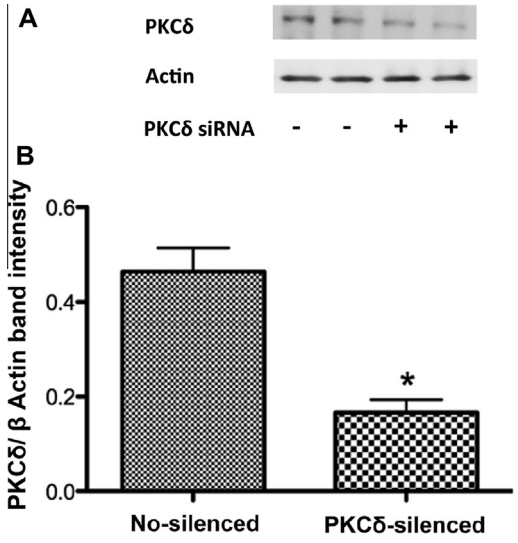

Fig. 8. Determination of PKCδ silencing efficiency by Western blot analysis in HMC-1^{560} cell line. (A) Shows a representative image of each condition. (B) Mean ± SEM of three experiments. Cytosolic PKCδ levels were represented respect to cytosolic β-actin expression. (*) Significant differences between no-silenced and PKCδ-silenced cells.

modifications on cPKC levels were observed [25]. Surprisingly, PMA treatment induces plasma membrane cPKCs translocation in the HMC-1^{560,816} cell line. This opposite PMA effect observed in both HMC-1 cell lines could be related with PKC capacity of c-kit phosphorylation (at two specific serine residues) previously described [45–47]. In this sense, the PKC activation induced by PMA can produces c-kit phosphorylation in a different way in each HMC-1 line and as consequence different cPKCs levels. Also, it was observed that c-kit inhibition induces nuclear PKCδ translocation in HMC-1^{560} cell line, which is accompanied with a decrease on cytosolic and membrane levels. Moreover, it has been demonstrated that PKCδ translocation to the nucleus takes place through actin cytoskeleton, since PKCδ translocation is lower after actin disruption. This fact could indicate that apoptotic effect due to c-kit inhibition is actin cytoskeleton-dependent. Specifically, it has been described that apoptotic effect induced by c-kit inhibition is accompanied with actin filamentous instability in colon adenocarcinoma cells [37]. Also, it was observed that nuclear PKCδ translocation induced by c-kit inhibition predominates even after PKC activation. Besides, STI571 effect over other different kinases, including PKCε and protein tyrosine kinase 2 (PyK2), has been previously described in T98G malignant glioma cells [20]. Based on these findings, these kinases might be implicated as targets for the drug.

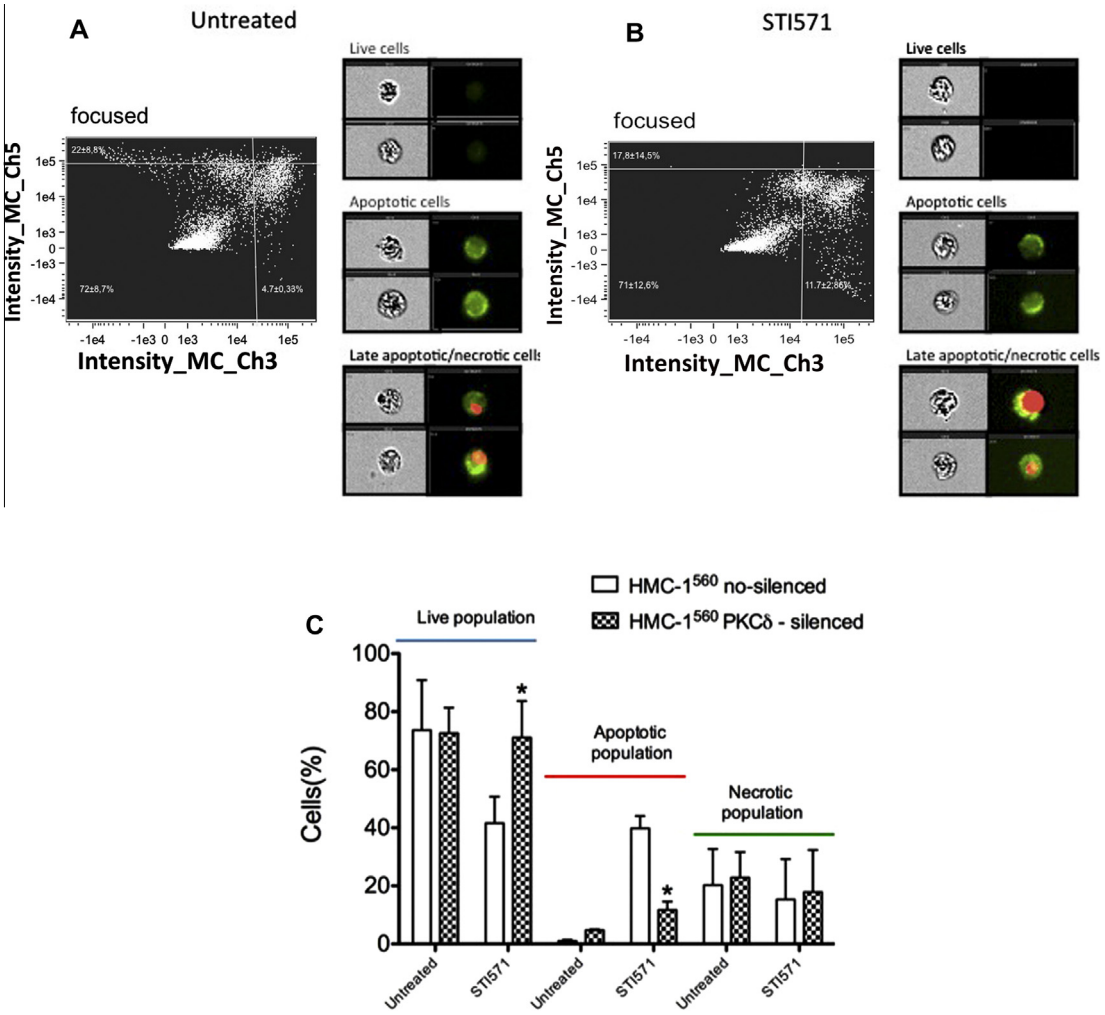

Fig. 9. Determination of live, apoptotic and necrotic population in PKCδ-silenced HMC-1^{560} cells treated with 25 nM STI571. Live (botton left panel of each graph), apoptotic (botton right panel of each graph) and late apoptotic/necrotic (upper panel of each graph) cells were detected by Annexin-V-FITC/PI staining. Graphs represent one of three repeated experiments: (A) untreated cells and (B) STI571-treated cells. The mean ± SEM of three experiments is represented in Fig. 9C. (*) Statistical significance difference between no-silenced and PKCδ-silenced HMC-1^{560} cells.

In HMC-$1^{560,816}$ cell line, STI571 has an opposite effect in PKC levels, since increases cPKCs but does not have any effect on PKCδ expression. The positive effect of STI571 over cPKC expression might be related with the fact that c-kit is an excellent activator of cPKCs through phospholipase C γ activation, leading to DAG release, Ca^{2+} and PKC activation [48]. In the HMC-$1^{560,816}$ cell line, cPKC levels increase induced by STI571 can be related with a decrease in the spontaneous histamine release and with an increase in the histamine released after cellular alkalinisation previously described [49]. Therefore, STI571 enhancer effect over cPKC cytosolic expression might be related with HMC-$1^{560,816}$ activation. On the other hand, PKC activation induces membrane cPKC translocation in these cells, which is in agreement with cytosolic cPKC levels decrease. However, cytosolic cPKC levels are not modified after PMA and STI571 simultaneous addition. This fact might indicate a possible competitive action between both compounds (PMA and STI571), whereas under these conditions cellular activation is not affected [49]. This relationship does not exist in HMC-1^{560} cell line, since tyrosine kinase inhibition does not have any effect over cPKC isoforms and only PKCδ isoform is modified. Moreover, in HMC-$1^{560,816}$ cell line, PMA induces a decrease of both cytosolic cPKC and PKCδ levels. In this case, cPKC isoforms translocate to the membrane when they are activated. It is important to note that the plasma membrane is a subcellular localization very common when this kinase is activated [36,50]. On the other hand, PKCδ isoform does not translocate to the plasma membrane or to the nucleus, in fact, membrane PKCδ levels decrease when PMA is added. Hence, this suggests an intense down-regulation of this isoform in the HMC-$1^{560,816}$ cell line. Besides, cytosolic PKCδ expression regulation when this kinase is activated was also described in liver tumor, suggesting an inhibitory role on tumor cell proliferation [51]. In HMC-$1^{560,816}$ cell line, PKCδ expression down-regulation is not only observed in the cytosol but also in plasma membrane and nuclear fractions. It has been previously described that PMA (30 min) induces protein translocation from the cytosol to the membrane and also increases cell immunological response of RBL-2H3 cells. However, PKC activation for (18 h) induces a complete absence of degranulation and the absence of cytosolic PKC is observed, which is in accordance with the present results [52]. The opposite effect of PKC activation in cPKC and PKCδ cytosolic expression in both cellular lines can explain the contrary effect on the cellular activation, quantified as histamine release, previously described [25,31].

Results obtained in the present work confirm that long-term PMA exposure implies the degradation of a specific isoform, PKCδ, in HMC-$1^{560,816}$ cell line. Taking into account that PKC has a central

role in mast cell activation and degranulation, these results might indicate some specific role of PKCδ in HMC-$1^{560,816}$ cell line activation that is not present in the other cell line HMC-1^{560} [49,53–56]. On the contrary, nuclear PKCδ translocation is closely related with cell death in HMC-1^{560} cells. As it was described in other studies PKCδ translocates from the cytoplasm to the nucleus in response to apoptotic stimuli, STI571 in our case [17]. Thus, our results confirm that apoptosis induced by c-kit inhibition takes place through PKCδ translocation. In this sense, several intracellular changes might be occur in STI571-induced cell death related to nuclear PKCδ translocation, since caspase-3, mitogen activated protein kinases (MAPKs) and PARP are proteins also involved in STI571-apoptotic cell death [57,58]. It is important to note that actin cytoskeleton plays an important role in this translocation. Therefore, this study demonstrates that apoptotic cell death induced by c-kit inhibition is clearly PKCδ-dependent in HMC-1^{560} cell line. In this sense, the apoptotic effect induced by STI571 is three-fold lower when PKCδ expression is abolished. For this, it can be stated that PKCδ plays an essential role in apoptotic cell death STI571-induced. This conclusion is of potential relevance, and should be bear in mind in patients with STI571 treatment, since any factor or secondary effect that could down-regulate PKCδ might diminish three fold the apoptotic cell death induced by the tyrosine kinase inhibitor STI571.

Hence, results showed in this paper clearly demonstrate that apoptotic cell death induced by c-kit inhibitor STI571 is PKCδ-dependent in the HMC-1^{560} cell line. Also, PKCδ isoform translocates to the nucleus in response to c-kit inhibition and actin cytoskeleton integrity is essential to achieve this translocation in this cell line. However, in HMC-$1^{560,816}$ cell line, c-kit inhibitor STI571 increases cPKC levels and does not affect PKCδ expression. In this cellular line PKCδ seems to be down-regulated.

Authors' contributions

The design of the experiments has been done by Araceli Tobío, Amparo Alfonso and Luis M. Botana. The experiments, data collection, analysis and interpretation as well as statistical analysis and the writing of the manuscript have been done by Araceli Tobío. The manuscript has been supervised by Amparo Alfonso and Luis M. Botana.

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