Influence of the Tyrosine Kinase Inhibitors STI571 (Glivec®), Lavendustin A and Genistein on Human Mast Cell Line (HMC-1^{560}) Activation

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Abstract   The human mast cell line (HMC-1^{560}) was used to study the effects of tyrosine kinase (TyrK) inhibition on histamine release in consequence of intracellular Ca^{2+} or pH changes. This is important since the TyrK inhibitor STI571 (Glivec®) inhibits proliferation and induces apoptosis in HMC-1^{560}. HMC-1^{560} cells have a mutation in c-kit, which leads to a permanent phosphorylation of the KIT protein and their ligand-independent proliferation. The TyrK inhibitors STI571, lavendustin A and genistein decrease spontaneous histamine release in 24-h pre-incubated cells. Results are compared with those of the mast cell stabiliser cromoglycic acid, which also drops spontaneous histamine release. When exocytosis is stimulated by alkalinisation, STI571 pre-incubated cells release more histamine than non-pre-incubated cells. Alkalinisation-induced histamine release reaches still higher levels in STI571 cells with activated protein kinase C (PKC) by PMA. We do not observe modifications on histamine release in cells, treated with PKC inhibitors (rottlerin, Gf109203 or Gö6976). Lavendustin A- and genistein 24-h incubated cells behave similar to STI571 cells, whereas cromoglycic acid does not show effects after stimulation with alkalinisation. Stimulation of exocytosis with the Ca^{2+} ionophore ionomycin does not modify histamine response in TyrK inhibited cells. Ca^{2+} and pH changes are observed after long-time incubation with STI571. Results show that pH is still higher in STI571 pre-incubated cells after alkalinisation with NH_4Cl, whereas intracellular Ca^{2+} concentration remains stable. This work further strength the importance of pH_i as a cell signal and suggest that STI571 has transduction pathways in common with other TyrKs. J. Cell. Biochem. 103: 1076–1088, 2008.   © 2007 Wiley-Liss, Inc.

Key words: HMC-1; mast cells; STI571; Glivec; lavendustin; genistein; alkalinisation; intracellular pH; calcium; tyrosine kinase; protein kinase C

Mast cells are a cell type often used in signal transduction studies because they secrete their granules through a fast exocytotic process, which can be used as a functional model. Up to now, only two human mast cell lines are available: the LAD 1/2 line (Laboratory of Allergic Diseases) [Kirshenbaum et al., 2003] and the human mast cell line (HMC-1) [Butter-field et al., 1988]. HMC-1 presents a very regular growth and behaviour and there are still many unknown aspects concerning to its activation process.

HMC-1 cells express in their membrane the tyrosine kinase (TyrK) receptor KIT. KIT is a member of the type III transmembrane Tyr kinases with an extracellular domain that binds to mast cell growth factor, also known as steel factor and stem cell factor (SCF) [Linnekin, 1999; Longley et al., 1999; Heinrich et al., 2000]. The extracellular region consists of five immunoglobulin-likes binding repeats, three of them are involved in SCF-binding. Ligand binding results in dimerisation and phosphory-lation of KIT and leads to an activation of its intrinsic intracellular TyrK activity. A juxtamembrane domain and two TyrK domains, which are separated by a kinase insert, are situated in the intracellular part of KIT [Linne-kin, 1999]. Signals mediated by the extracellular

receptor influence important processes like proliferation, differentiation, migration, activation and survival of mast cells [Sundstrom et al., 2003]. Mutations in the proto-oncogene c-kit, which encodes the KIT protein, evoke a ligand independent proliferation of HMC-1 [Buchdunger et al., 2000]. HMC-1^{560} has one mutation that results in a Gly560 $\rightarrow$ Val amino acid change. HMC-1^{560,816} has the 560 mutation and one more in Asp816 $\rightarrow$ Val. These mutations lead to a constitutive activation of the KIT protein that has been found in several types of human malignant diseases [Ma et al., 2002].

The 560 mutation could be detected in cells of patients with gastrointestinal stromal tumour (GIST), cutaneous mastocytosis (CM) and in a few cases of systemic mastocytosis (SM). Mastocytosis is a mast cell disease characterised by an abnormal growth and accumulation of mast cells in one or more tissues. CM is common in children. Typical clinical and histological skin lesions and the absence of definitive signs of systematic involvement define it [Valent et al., 2001]. Cells with 816 mutations have been found in more than 80% of patients with SM. The grave disease appears commonly in adults and its diagnosis is based on multifocal histological lesions in the bone marrow or other extra-cutaneous organs. The detection of the activating c-kit point mutation at codon 816 is one diagnostic criterion of SM [Valent et al., 2001; Garcia-Montero et al., 2006]. Different mutations of KIT have a high relevance in the pathology and identification of distinct forms of SM and their modulation become major significance for its treatment and prognosis [Valent et al., 2001].

The TyrK inhibitor STI571, also known as Glivec® and imatinib, was discovered during the testing of compounds for inhibition of protein kinase C (PKC) [Buchdunger et al., 1996]. The development of a number of chemical analogs resulted in STI571, shown to be an inhibitor of platelet-derived growth factor receptor, inhibitor of v-Abl, c-Abl, Bcr-Abl and c-kit protein-TyrKs [Buchdunger et al., 2000]. STI571 acts as a competitive inhibitor of adenosine triphosphate (ATP). It binds to a portion of the ATP-binding site of the kinase in its inactivated conformation and keeps it in this frozen-like condition [Shah et al., 2006]. STI571 is successful used in mast cell diseases involving the wild-type c-kit by inhibition of the SCF-dependent kinase activation. The inhibitory

effect is still higher in mutants with Gly560Val substitution, by decreasing the autophosphorylation of the mutant KIT through inhibition of the kinase activity rather than by down-regulating expression of c-kit protein. Therefore, it is successfully used in patients with GIST [Heinrich et al., 2000]. Unfortunately, it is ineffective in cells with Asp816Val mutation that appear in the majority of aggressive SM and mast cell leukaemia [Ma et al., 2002].

The aim of this work was to study the relationship between HMC-1^{560} activation and the TyrK pathway, especially that of KIT. We used STI571 and the non-specific TyrK inhibitors, lavendustin A and genistein to investigate how they affect histamine release in consequence of changes in intracellular Ca^{2+} or pH in these cells. The availability of two cultured cell lines that differ in one mutation is an opportunity for further investigation of the influence of this mutation on signalling pathways. A better understanding could lead to more efficient drug targeting. We used HMC-1^{560} cells that appear in few patients with SM. Similar studies should be made with HMC-1^{560,816} cells that can be found in mast cell neoplasm, to clarify the effect of the 816 mutation on appointed pathways.

METHODS

Chemicals

Ammonium chloride (NH$_4$Cl) was from Panreac (Barcelona, Spain); rottlerin, ionomycin, Gö6976, GF109203X, genistein and lavendustin A were from Alexis Corporation (Läufelfingen, Switzerland); 2,7-bis (carboxyethyl)-5(6)carboxyfluorescein-acetoxymethylester (BCECF AM) and FURA-2 AM were from Molecular Probes (Leiden, The Netherlands). Phorbol 12-myristate 13-acetate (PMA), Cromoglycic acid and MTT (3-[4,5-dimethyldiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (Madrid, Spain). STI571 was provided by Dr. Luis Escribano Mora (Servicio de Hematología, Hospital Ramón y Cajal, Madrid).

Cell Cultures

HMC-1 cells were kindly provided by Dr. J. Butterfield (Mayo Clinic, Rochester, MN) and maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were

expanded weekly and not more than 40 passages.

Cell Preparation

For histamine release assays, cells were centrifuged (1,500 r.p.m., 5 min, 4°C) and washed twice with saline solution (1,000 r.p.m., 5 min, 4°C). 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; glucose 1 g/L.

For Ca^{2+} and pH measurements cells were treated in the same conditions, but washed in saline solution plus 0.1% bovine serum albumin (BSA).

The incubation medium was equilibrated with CO_2 prior to use. During the experiments, pH (7.20) was maintained constant by bubbling CO_2. Experiments were carried out at least three times, by duplicate, both for histamine release assays and Ca^{2+} and pH measurements.

Cell Incubation

Freshly prepared concentrated solution (6.2 μl) of each drug (40 times concentrated) were added to the incubation medium to attain a final volume of 150 μl and pre-incubated. 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. Incubations were carried out in a bath at 37°C for 10 min.

The incubations were stopped by immersing the tubes in a cold bath. After centrifugation at 2,300 r.p.m. for 10 min, 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 every experiment.

Histamine Release Assays

Histamine release was tested with a spectrofluorometer, (Fluoroskan II, Labsystems, Barcelona, Spain) in both pellet (non-released histamine) and supernatant (released histamine) according to Shore's method [Shore, 1971]. To form the fluorescent complex 0.04% orthophthaldialdehyde was used, also trichloroacetic acid (14%) to avoid protein interferences in the histamine release determination. To ensure total histamine, pellets were sonicated for 60 s in 0.2 ml of 0.1 N HCl. In NH_4Cl experiments, histamine release was measured only in pellets,

since this compound interferes with the fluorescent complex. Results shown were expressed as the percentage of released histamine from the total histamine content.

Cell Viability

After exposure to different concentrations of STI571 during 24 h in culture medium, cells were centrifuged (1,500 r.p.m., 5 min, 4°C). The pellets were resuspended in saline solution with MTT (250 μg/ml) and incubated at 37°C for 30 min in darkness.

After washing twice with saline solution cells were sonicated in water for 60 s. The coloured formazan salt was measured at 595 nm in a spectrophotometer plate reader.

Measurement of Cytosolic Free Ca^{2+} and Intracellular pH

HMC-1 cells were loaded with FURA-2 AM (0.2 μM) and BCECF AM (0.05 μM) in a bath at 37°C, for 10 min. After this time, loaded cells were washed with saline solution (1,000 r.p.m., 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. Intracellular pH and cytosolic Ca^{2+} concentration 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. For FURA-2 AM, the excitation wavelengths were 340 and 380 nm, with emission at 505 nm; for BCECF AM the excitation was performed at 440 and 490 nm, with 530 nm for emission. The calibration of the fluorescence values versus intracellular Ca^{2+} was made according to the method of Thomas et al. [1979] and of fluorescence values versus pH as per Grynkiewicz et al. [1985]. In brief, a calibration curve was obtained with four known values of pH, measuring the fluorescence ratio obtained in the presence of nigericin, in a K^+ solution, for each pH value.

Statistical Analysis

Results were analysed using the Student's t-test for unpaired data. A probability level of

0.05 or smaller was used for statistical significance. Results were expressed as the mean $\pm$ SEM.

RESULTS

Following to our previous work in HMC-1[560] cells [Pernas-Sueiras et al., 2005, 2006a,b], we checked the effect of TyrK inhibitors on histamine release induced by alkalinisation and ionomycin.

After 10 min of incubation with one of the TyrK inhibitors STI571 (10 µM), lavendustin A (1 µM) and genistein (10 µM), we stimulated the cells with different concentrations of NH_4Cl or the Ca^{2+} ionophore ionomycin [Pernas-Sueiras et al., 2005]. Neither alkalinisation (Fig. 1A–C)
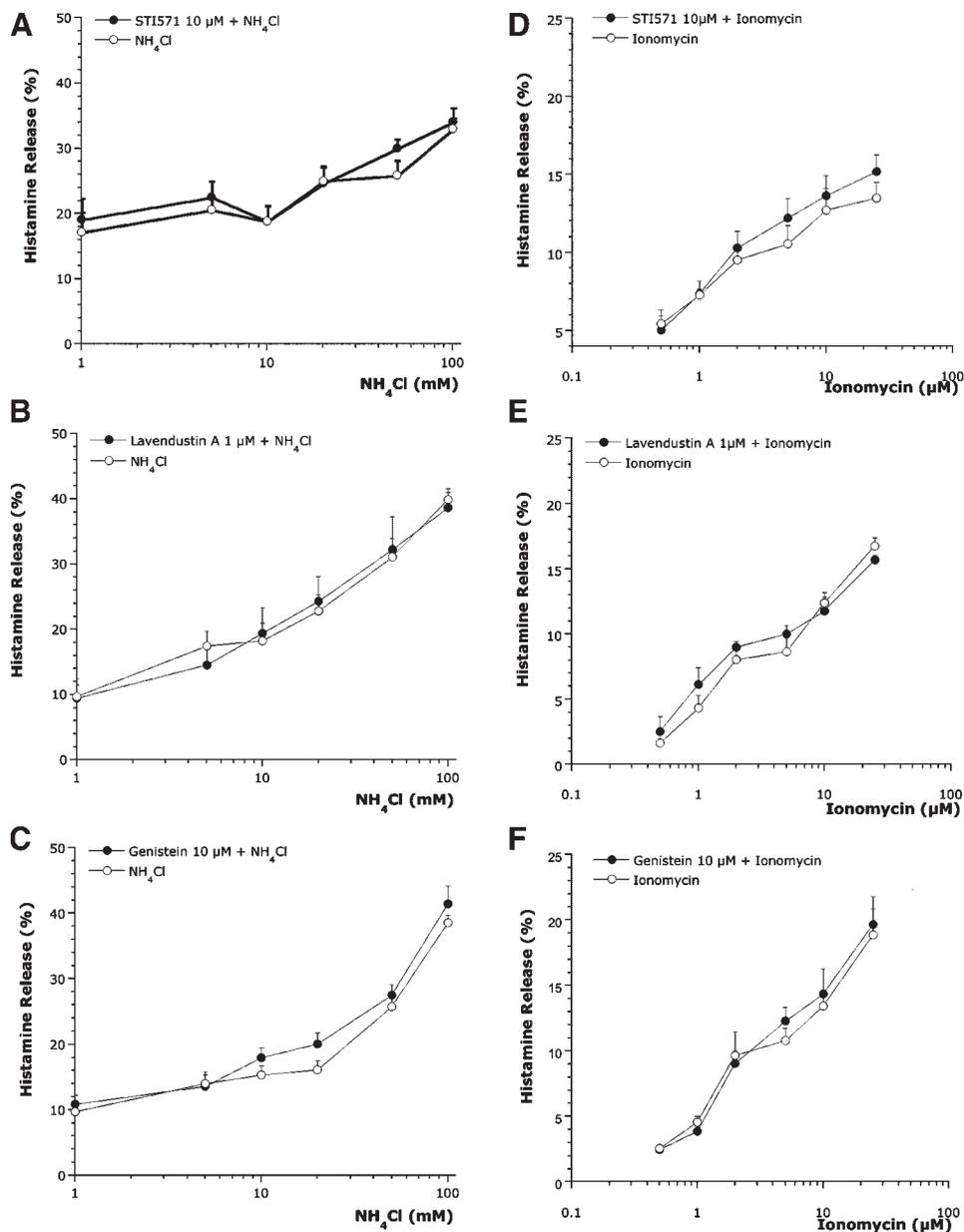
Fig. 1. Effect of TyrK inhibition on histamine release. HMC-1[560] cells were pre-treated during 10 min in saline solution with STI571 (10 µM; **A,D**), lavendustin A (1 µM; **B,E**), genistein (10 µM; **C,F**) and afterwards stimulated with different concentrations of NH_4Cl (A–C) o ionomycin (D–F). Mean $\pm$ SEM of four experiments.

nor stimulation with ionomycin (Fig. 1D–F) induced any change in histamine release.

Next, we investigated the time dependence of TyrK inhibition in HMC-1[560]. We tested STI571 viability by MTT assays in cells 24 h incubated with different concentrations of the drug. Our results confirmed those of other studies that HMC-1[560] cell died with increased drug presence [Heinrich et al., 2000; Akin et al., 2003] (Fig. 2). From this graphic, we selected the concentration 25 nM where 70% of the cells survived but STI571 still had an effect.

We incubated HMC-1[560] cells 24 h with this concentration and a control without drug in culture medium. Afterwards histamine assays were realised as described above. Our results in Figure 3A show that spontaneous histamine release was significantly decreased in STI571 (25 nM) 24 h pre-incubated cells. We obtained similar results with cells pre-treated with lavendustin A (1 μM; Fig. 3B) and genistein (1 μM; Fig. 3C), two unspecific TyrK inhibitors. Hereon, histamine response of cromoglycic acid (100 μg/ml) pre-incubated cells was checked. Its salt sodium cromoglycate prevents mast cell degranulation [Theoharides et al., 1980; Edwards and Howell, 2000]. When this drug was present during 24 h spontaneous histamine release was also decreased (Fig. 3D).

Furthermore, histamine release assays were made with NH_4Cl (50 mM) to compare the TyrK inhibited cells with control cells, since alkalinisation is known to stimulate histamine release in this cell line [Pernas-Sueiras et al., 2005]. As Figure 4A shows, it is surprising that STI571

pre-incubated cells released more histamine after alkalinisation. HMC-1[560] cells pre-treated with lavendustin A (1 μM; Fig. 4B) and genistein (1 μM; Fig. 4C) showed similar behaviour, whereas cromoglycic acid did not affect histamine release induced by alkalinisation (Fig. 4D).

The Ca^{2+} ionophore ionomycin is another stimulus that induces histamine release in HMC-1[560] [Pernas-Sueiras et al., 2005]. STI571 pre-incubation on further stimulation with ionomycin (2 μM), Figure 4E, did not change histamine response compared to ionomycin treatment only. In the same way, the other TyrK inhibitors lavendustin A (Fig. 4F) and genistein (Fig. 4G) did not show differences when comparing pre-treated cells with control cells after ionomycin stimulation. In addition, cromoglycic acid, Figure 4H, did not affect histamine release induced by the Ca^{2+} ionophore.

To check if the increased histamine release was a consequence of intracellular Ca^{2+} or pH changes, we observed both parameters in TyrK inhibited cells. HMC-1[560] cells were pre-incubated with STI571 (25 nM) during 24 h like in histamine release assay experiments. We performed the experiments in Ca^{2+}-free medium and restored the ion later on. After obtaining a baseline, we added NH_4Cl (50 mM). As Figure 5A shows, cytosolic pH increased at the addition point of the compound and slowly decreased afterwards. After adding Ca^{2+} (1 mM) to the medium, we could not observe any other pH_i changes. As the same figure shows, pH increase was higher in STI571 pre-incubated cells than in not treated cells. The difference was statistically significant. The intracellular Ca^{2+} concentration remained stable during the whole experiment (Fig. 5B).

The same experiment was performed by adding ionomycin (0.1 μM) instead of NH_4Cl. As expected, the cytosolic Ca^{2+} increased at the ionomycin addition point because intracellular reservoirs were depleted (Fig. 5D). Thereupon the Ca^{2+} concentration steadily decreased until Ca^{2+} (1 mM) was added to the external medium. Cytosolic Ca^{2+} rapidly rose through the fast influx of the cation. As Figure 5C shows, the intracellular pH remained stable until Ca^{2+} addition. After that we could observe a lightly, not significantly alkalinisation in ionomycin stimulated cells. However, no significant differences between STI571 treated and control cells were seen neither in intracellular Ca^{2+} nor in

Fig. 2. HMC-1[560] viability after 24 h incubation with STI571. Different concentrations of STI571 were added in culture medium and cells were incubated for 24 h at 37°C. Cell viability was checked by MTT test. Control cells with non-STI571 treatment, were used as 100% viability and ethanol treatment as 100% dead, in each experiment. Mean ± SEM of four experiments.
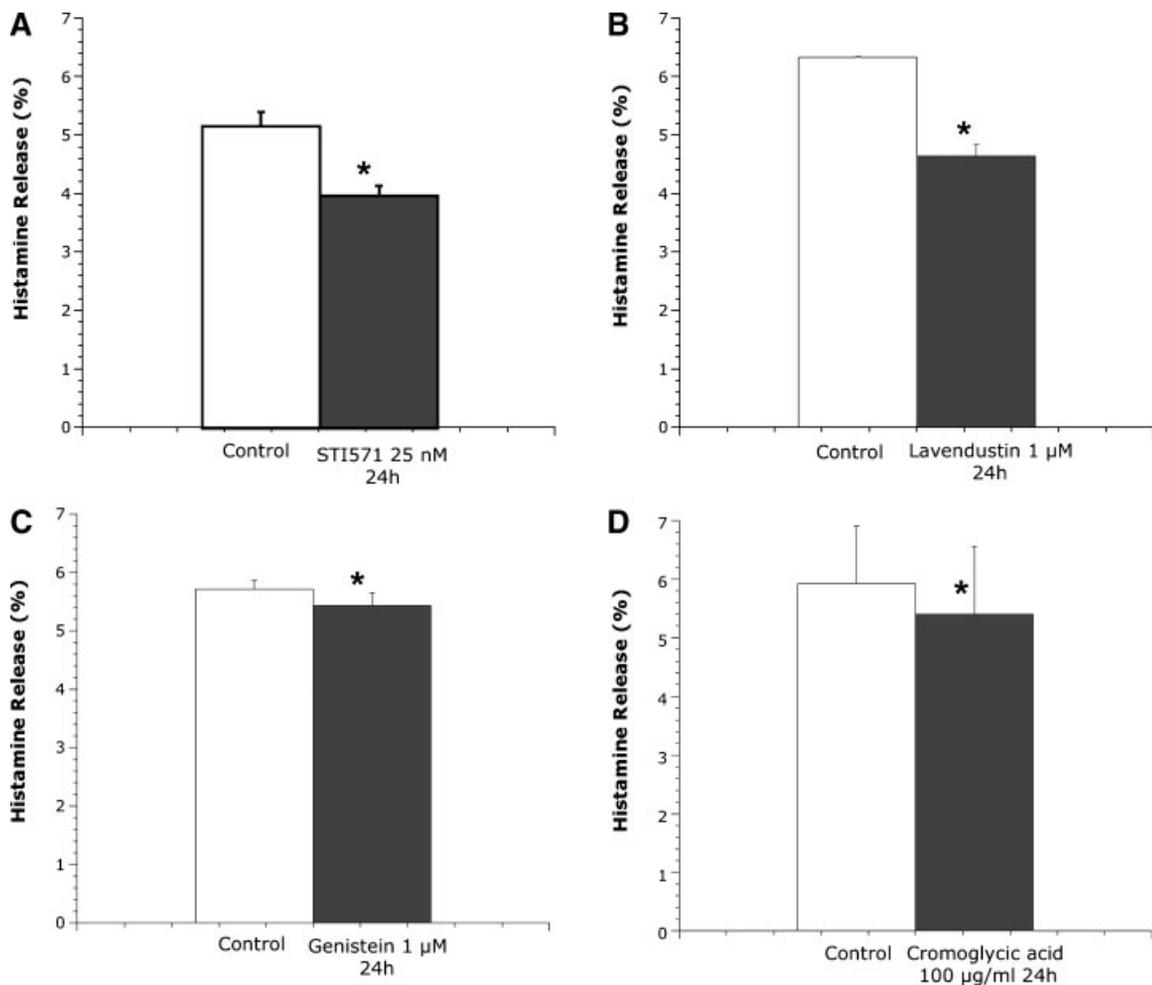
Fig. 3. Effect of TyrK inhibitors and cromoglycic acid on HMC-1^{560} spontaneous histamine release after 24 h of incubation. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM; **A**), lavendustin A (1 µM; **B**), genistein (1 µM; **C**) or cromoglycic acid (100 µg/ml; **D**) in culture medium. Histamine release was checked after 10 min of incubation in saline solution (37°C). Mean ± SEM of four experiments. * Significant differences between control and drug pre-incubated cells.

pH changes after ionomycin stimulation (Fig. 5C,D).

From previous studies, we know that PKC stimulation by the phorbol ester PMA induces release of histamine after NH$_4$Cl stimulation [Pernas-Sueiras et al., 2006a]. As Figure 6A shows, STI571 significantly increased alkalinisation-induced histamine release after treatment with PMA (100 ng/ml). Pre-treatment of HMC-1^{560} with lavendustin A (Fig. 6B) or genistein (Fig. 6C) had the same effect. Cromoglycic acid (Fig. 6D) did not affect histamine response.

In contrast to alkalinisation, STI571 did not change the histamine release in ionomycin-stimulated cells in combination with PMA (Fig. 6E). Neither lavendustin A, Figure 6F,

nor genistein, Figure 6G, had effects. Figure 6H shows that the mast cell stabiliser cromoglycic acid did not affect histamine release.

Accordingly, we checked intracellular Ca^{2+} and pH profiles when PKC was stimulated. As Figure 7A shows, pH increased after NH$_4$Cl (50 mM) addition to a Ca^{2+}-free medium. When Ca^{2+} (1 mM) was restored to the medium, no effect was observed on the descending pH. We could see a slightly increased pH in STI571 pre-incubated cells, although that was not statistically significant. During the whole experiment, in both, STI571 pre-incubated and control cells, Ca^{2+} levels remained stable (data not shown).

In those experiments of ionomycin in combination with PMA, no changes were observed in
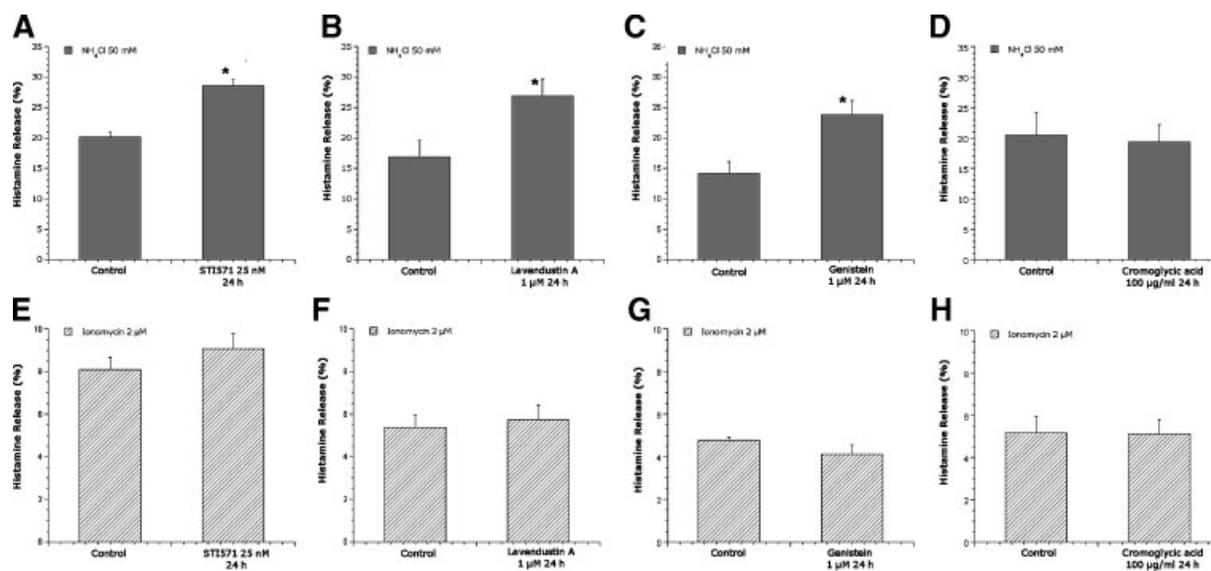
Fig. 4. Effect of TyrK inhibitors and cromoglycic acid on alkalinisation- and ionomycin-induced histamine release in HMC-1[560] cells. Cells pre-incubated with STI571 (25 nM; **A**), lavendustin A (1 µM; **B**), genistein (1 µM; **C**) or cromoglycic acid (100 µg/ml; **D**) during 24 h were stimulated with NH₄Cl (50 mM) during 10 min in saline solution (37°C). Cells pre-incubated with STI571 (25 nM; **E**), lavendustin A (1 µM; **F**), genistein (1 µM; **G**) or cromoglycic acid (100 µg/ml; **H**) during 24 h were stimulated with ionomycin (2 µM) during 10 min in saline solution (37°C). Mean ± SEM of four experiments. * Significant differences between control and drug pre-incubated cells.

pH$_i$ at the point where drug was added (data not shown). Results of cytosolic Ca^{2+} changes in Figure 7B show an expected increase after adding ionomycin (0.1 µM) and a subsequent drop. The Ca^{2+} (1 mM) restoration to the external medium induced another but higher increase in intracellular Ca^{2+} concentration due to the extracellular entry of the cation.

Furthermore to proof the influence of PKC inhibition we performed histamine assays in STI571 (25 nM) 24 h pre-incubated cells using the PKC inhibitors Gö6976 (100 nM), GF109203X (500 nM) and rottlerin (10 µM). We could not find any significant alteration between TyrK blocked and control cells, neither after alkalinisation (Fig. 8A–C) nor after stimulation with ionomycin (Fig. 8D–F).

DISCUSSION

In previous works in HMC-1[560], we studied transduction pathways that have influence in intracellular Ca^{2+} and pH as activation signals for release of histamine. The object of this study was to characterise signalling pathways mediated by TyrKs. This is important since the KIT TyrK inhibitor STI571 is able to induce apoptosis and to inhibit proliferation in cells with Gly560 → Val amino acid change in KIT.

We wanted to clarify especially the influence of TyrK-activity on exocytotic process.

In HMC-1[560], KIT is permanently phosphorylated and SCF independently activated. It is involved in a variety of intracellular signalling pathways like that of phosphatidylinositol-3′-kinase (PI3K) and mitogen activated protein kinases (MAPK) [Sundstrom et al., 2003]. Furthermore, it is suggested that JAK/STAT and Scr play an important role in the proliferation process induced by KIT in HMC-1 like it has been shown in other cell lines [Linnekin, 1999]. To modulate these processes we inhibited the catalytic centre of the intracellular KIT TyrK with STI571, which acts as a competitive inhibitor of ATP [Shah et al., 2006]. Subsequently connected pathways as those mentioned above are blocked. The efficacy of the drug shows its use in GIST [Ma et al., 2002] and the viability test in this work and other studies [Heinrich et al., 2000]. HMC-1[560] cells died with increased drug presence.

All these studies were long-term studies. When we incubated the cells for 10 min with STI571 or the two unspecific TyrK inhibitors lavendustin A and genistein, we could not observe any change in histamine release.

From the viability test, we selected the concentration 25 nM of STI571 that allowed
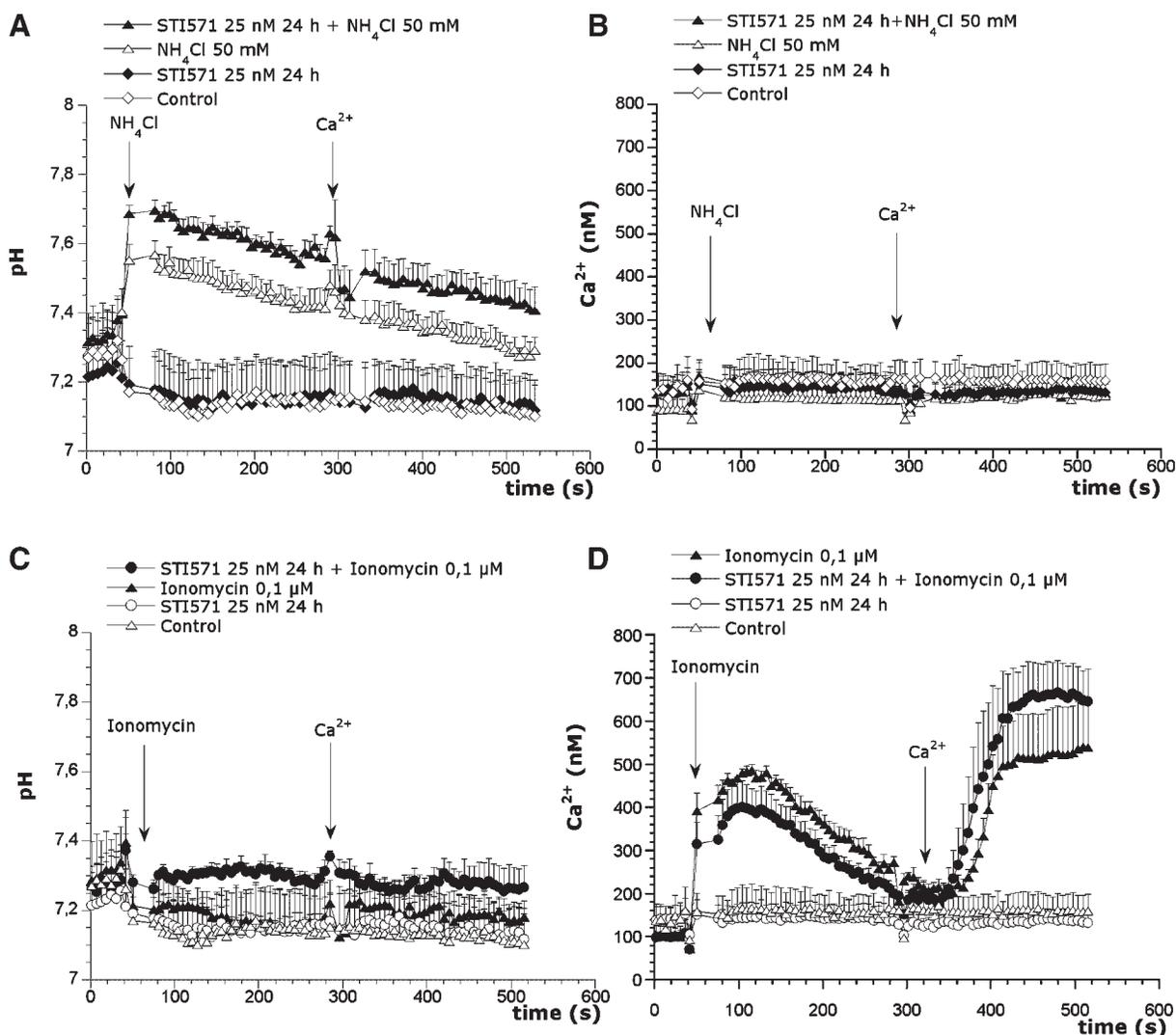
Fig. 5. Effect of STI571 on intracellular Ca^{2+} and pH levels in HMC-1^{560} cells. Cells were pre-incubated for 24 h in presence of STI571 (25 nM) in culture medium. Cytosolic pH and Ca^{2+} changes were observed after NH_4Cl (50 mM; **A,B**) and ionomycin (0.1 µM) addition (**C,D**). The first arrow indicates drug addition and second that extracellular Ca^{2+} (1 mM) was restored. Mean ± SEM of four experiments.

70% of the cells to survive but still showed an effect. We expected that histamine release would decrease after 24-h pre-incubation with STI571. Our results confirmed a lower spontaneous histamine response. We made similar experiments with lavendustin A and genistein, since these drugs are reported to diminish histamine release in human basophiles [Tedeschi et al., 2000]. Lavendustin A and genistein affected spontaneous histamine release of HMC-1^{560} cells like STI571. We suggested, this might be due to the block of above-mentioned TyrK connected transduction pathways as PI3K and JAK/STAT.

To rule out the possible role of the TyrK inhibitors as membrane stabilisers, we treated the cells for 24 h with cromoglycic acid, a drug often used in treatment of diseases with high transmitter release such as different kind of allergies, asthma and mastocytosis [Shin et al., 2004]. Its mechanism of action was thought to be the stabilisation of the mast cell membrane and subsequent the prevention of transmitter release [Theoharides et al., 1980; Edwards and Howell, 2000]. Further on the drug was shown to inhibit the activation of human neutrophils, eosinophils and monocytes in vitro and to reduce IgE production [Kimata et al., 1991;
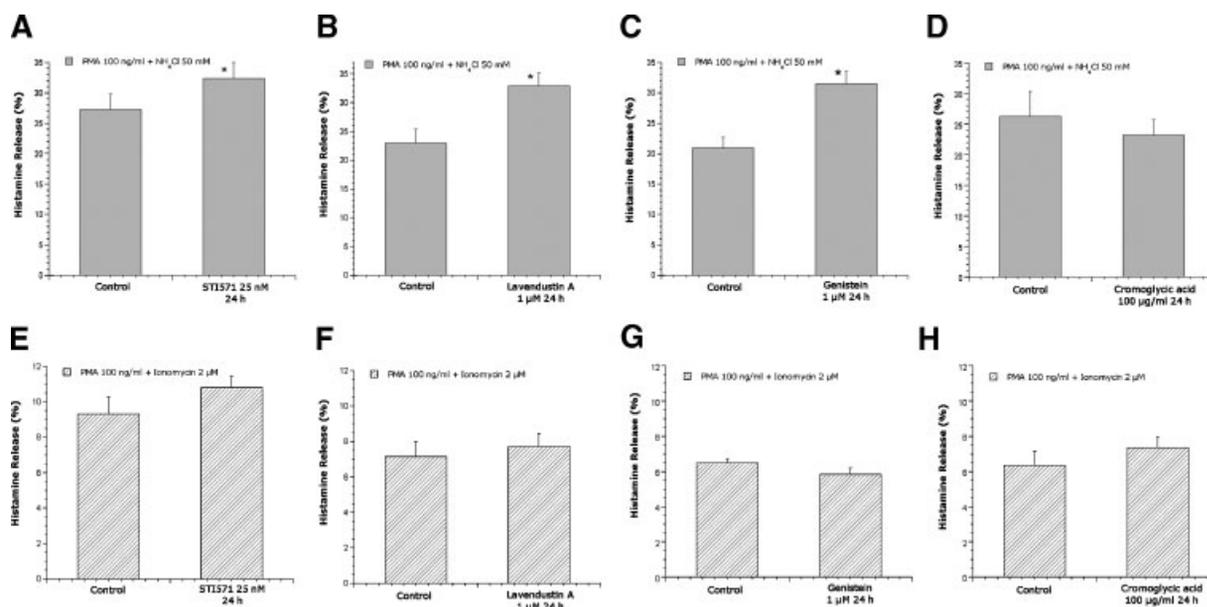
Fig. 6. Effect of PKC activation and TyrK inhibition o cromoglycic acid incubation on alkalinisation and ionomycin induced histamine release in HMC-1[560]. Cells pre-incubated with STI571 (25 nM; **A**), lavendustin A (1 μM; **B**), genistein (1 μM; **C**) or cromoglycic acid (100 μg/ml; **D**) during 24 h were pre-treated with PMA (100 ng/ml) 10 min and afterwards stimulated with NH_4Cl (50 mM) during 10 min in saline solution (37°C).

Cells pre-incubated with STI571 (25 nM; **E**), lavendustin A (1 μM; **F**), genistein (1 μM; **G**) or cromoglycic acid (100 μg/ml; **H**) during 24 h were pre-treated with PMA (100 ng/ml) for 10 min and afterwards stimulated with ionomycin (2 μM) during 10 min in saline solution (37°C). Mean ± SEM of four experiments. * Significant differences between control and drug pre-incubated cells.

Loh et al., 1994]. An interesting approach was that cromolyn might inhibit histamine secretion by phosphorylation of a mast cell protein, which is involved in exocytosis regulation. PKC inhibitors and a cation ionophore blocked this phosphorylation [Correia et al., 1996; Wang et al., 1999]. We found as well less histamine release in unstimulated cells treated with cromoglycic acid. However, results of experiments with cromoglycic acid differed of those of the TyrK-inhibitors after stimulation with NH_4Cl or ionomycin. Therefore, we discarded our hypothesis that STI571 could act in a cromolyn like manner.

In HMC-1[560], histamine release increases after stimulation with NH_4Cl simultaneously with an increase of intracellular pH, and at the same time Ca^{2+} levels remain stable [Pernas-Sueiras et al., 2005]. In rat peritoneal mast cells [Wan et al., 2005] and human basophiles [Tedeschi et al., 2000], TyrK inhibition leads to a decrease of histamine release after stimulation. Surprisingly STI571 pre-treated HMC-1[560] cells released more histamine after alkalinisation than control cells. As far as we know, this is the first time that describes that TyrK inhibition increased histamine release in human mast cells.

We could not explain the stimulation of exocytosis after alkalinisation with the KIT kinase inhibition, because as previous mentioned, inhibition should occur downstream the activation process. Therefore, we performed experiments with the unspecific TyrK inhibitors, lavendustin A and genistein in the same fashion as with STI571. Especially genistein has been tested earlier to decrease histamine release after stimulation in different mast cell models [Tedeschi et al., 2000; Wan et al., 2005]. However, alkalinisation induced histamine release rose after 24-h treatment with both drugs. This suggests that STI571 has transduction pathways in common with other Tyr kinases, at least as evidenced with lavendustin A and genistein in this cell line.

To investigate this behaviour, intracellular Ca^{2+} and pH levels were observed in STI571 pre-incubated and control cells. Our results showed that in pre-treated cells pH_i significantly increased more after alkalinisation. This matches with our previous statements that alkalinisation induces histamine release in this
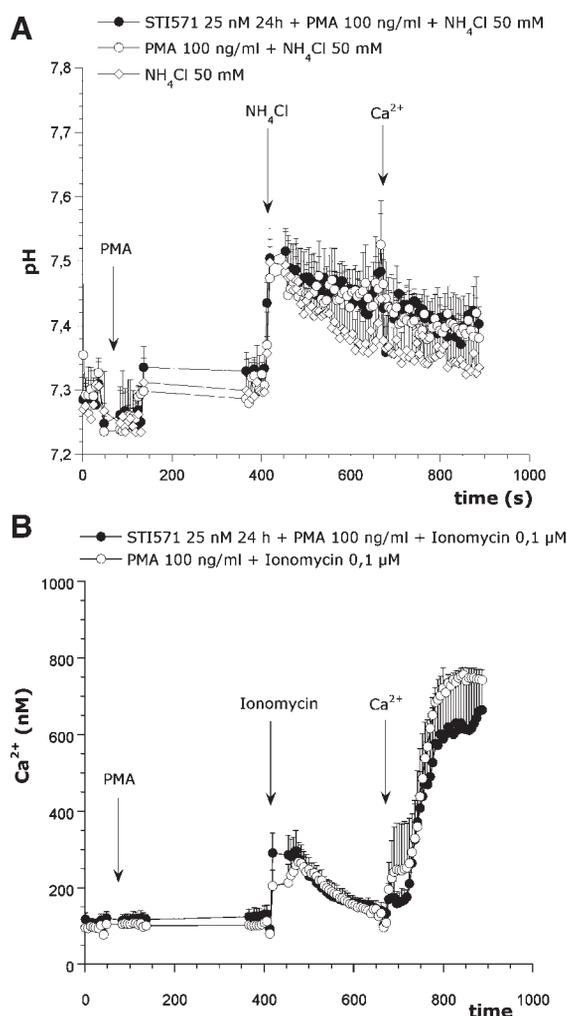
Fig. 7. Effect of PKC activation and STI571 on alkalinisation-modulated intracellular pH and Ca^{2+} levels in HMC-1[560] cells. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. NH_4Cl (50 mM) was added and cytosolic pH changes were observed (**A**). Cytosolic Ca^{2+} changes were observed after ionomycin 0.1 μM addition (**B**). The first arrow indicates the addition of PMA (100 ng/ml), the second arrow indicates stimulator addition and the third that extracellular Ca^{2+} (1 mM) was restored. Mean ± SEM of three experiments.

cell line [Pernas-Sueiras et al., 2005]. Observing intracellular Ca^{2+} in the same experiments, we did not find any difference in STI571 and control cells. This suggests once more that pH is a stimulating signal to induce exocytosis in HMC-1[560] and that STI571 influences somehow this signalling pathway. It has been described in cardiomyocytes that ATP is a strong activator of Src/Tyr kinases and of the anion Cl^-/HCO_3^- exchanger. TyrK activation results in intracellular acidosis [Puceat et al., 1998; de la Rosa et al., 2001]. Src TyrK inhibition by genistein

leads to an inhibition of Cl^-/HCO_3^- exchanger and this prevents ATP-induced acidification. It is necessary to bear in mind that STI571 inhibits the ATP binding site of KIT and that KIT is connected to other Tyr kinases. It might be that TyrK inhibition increases intracellular pH by inhibiting the anion Cl^-/HCO_3^- exchanger, similarly as in cardiomyocytes, what results in increased histamine release after alkalinisation. Alkalinisation induced by TyrK inhibition could be part of the initiation of the apoptotic process that induces STI571 in these cells. Further studies should be done, including apoptosis activation experiments, to investigate the impact of alkalinisation in cellular death.

Cytosolic Ca^{2+} concentrations play a role in exocytotic processes since the Ca^{2+} ionophore ionomycin induces histamine release by modulating intracellular Ca^{2+} concentration [Pernas-Sueiras et al., 2005, 2006b]. Therefore, similar experiments using ionomycin instead of NH_4Cl were performed. Whereas histamine release was significantly elevated after alkalinisation in STI571 pre-treated cells, the increase after ionomycin stimulation was not significantly. Results obtained in the microscope with ionomycin in STI571 pre-incubated cells confirmed those of histamine release assays. There were no significant differences in Ca^{2+} and pH changes between STI571 and control cells.

These results demonstrate that STI571 influences exocytosis of HMC-1[560] by a pH-, but not by a Ca^{2+}-dependent pathway. To underpin this statement we further studied the modulation of PKC, since PMA induced an increased histamine release in NH_4Cl and ionomycin stimulated cells [Pernas-Sueiras et al., 2006a]. Alkalinisation induced exocytosis took place without modulating cytosolic Ca^{2+} levels in cells pre-treated with PMA. We demonstrated that a long-term pre-incubation with one of the TyrK inhibitors induced a significantly higher release of histamine in PKC stimulated cells after alkalinisation. STI571 pre-incubated cells showed a slightly higher initial pH after NH_4Cl addition. Even if the difference was not significant, we suggest that this is once more a reason for the increased histamine release of STI571 pre-incubated cells. We could not find differences observing intracellular Ca^{2+} concentrations. Pre-treated cells with activated PKC, stimulated by ionomycin did not show
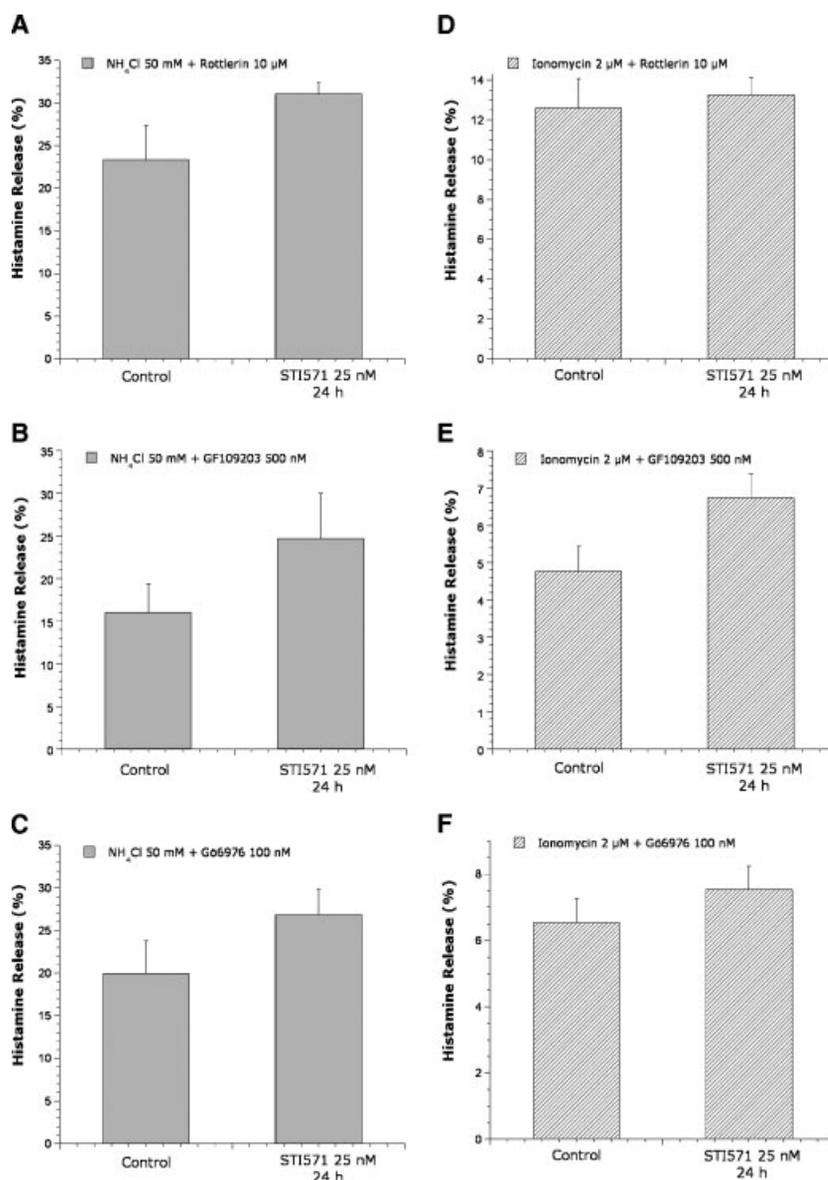
Fig. 8. Effect of PKC inhibition and STI571 on alkalinisation or ionomycin induced histamine release in HMC-1[560]. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with rottlerin (10 µM), Gö6976 (100 nM), GF109203X (500 nM) for 10 min and afterwards stimulated with NH$_4$Cl (50 mM; **A–C**) or with ionomycin (2 µM; **D–F**) during 10 min in saline solution (37°C). Mean ± SEM of four experiments.

differences neither concerning to histamine release nor to intracellular pH and Ca^{2+} changes. Results of STI571 pre-incubated and control cells, when PKC was inhibited by rottlerin, GF109203X or Gö6976, did not differ from each other concerning to histamine release.

Nevertheless, the fact that PMA-increased alkalinisation induced histamine release in TyrK inhibited cells let us suggest that there exists a connection between PKC and TyrK pathways in HMC-1[560].

Our results demonstrate that the TyrK inhibitors STI571, lavendustin A and genistein inhibit in this cellular model similar pathways. They are able to influence intracellular pH levels, and this leads in the case of HMC-1[560] to an increased histamine release after alkalinisation. Furthermore like in previous studies, we show the importance of intracellular pH in HMC-1[560] and that can be by itself enough signal to activate exocytosis [Alfonso et al., 2000; Pernas-Sueiras et al., 2005]. Our study may have potential implications to modify the

effect on the clinical use of STI571. It should be noticed that by decreasing the amount of HMC-1^{560} cells by using STI571, surviving cells could be more reactive after their stimulation.

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