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STI571 (Glivec®) Affects Histamine Release and Intracellular pH After Alkalinisation in HMC-$1^{560, 816}$

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Abstract The human mast cell line (HMC-$1^{560, 816}$) was used to study the effect of the tyrosine kinase inhibitor STI571 (Glivec®) on exocytosis, intracellular Ca^{2+} and pH changes, because STI571 inhibits the proliferation of HMC-1^{560} and induces its apoptosis. This drug does not have these effects on HMC-$1^{560, 816}$. Exocytosis in HMC-$1^{560, 816}$ cells can be stimulated by alkalinisation with NH_4Cl as well as with ionomycin. Surprisingly 24-h pre-incubation with STI571 decreases spontaneous histamine release of HMC-$1^{560, 816}$ cells, but increases the histamine response after alkalinisation and not after ionomycin-stimulation. After addition of NH_4Cl, pH_i has a higher increase in STI571 pre-incubated cells, without changing intracellular Ca^{2+} concentration. Activation of PKC in combination with tyrosine kinase inhibition increases also histamine release in HMC-$1^{560, 816}$ cells. Strangely, STI571 pre-incubated cells with PKC inhibited by rottlerin show the same effects. In these cells, cytosolic pH increases more than in control cells. This is the first report of STI571 effect in HMC-$1^{560, 816}$ cells. It seems that different pathways modulate signals for proliferation and exocytosis. STI571 does not only inhibit KIT TyrK, but may also influence cytosolic pH after alkalinisation in both cell lines, HMC-1^{560} and HMC-$1^{560, 816}$, and this ends in induced histamine release. This work is important since HMC-$1^{560, 816}$ cells are reported in 80% of aggressive systemic mastocytosis cases and the understanding of some signalling pathways involved in mast cell response could facilitate drug targeting. J. Cell. Biochem. 103: 865–876, 2008.

Key words: HMC-1^{560}; HMC-$1^{560, 816}$; mast cells; STI571; Glivec; alkalinisation; intracellular pH; calcium; tyrosine kinase; protein kinase C

Human mast cells (HMC-1^{560} and HMC-$1^{560, 816}$) can be found in patients with different kinds of mastocytosis. Both cell lines express the receptor TyrK KIT in their membrane. KIT is composed of an extracellular and an intracellular part. In the extracellular part the ligand-binding site is situated with five immunoglobulin likes regions, three of them are involved in ligand binding. The natural ligand of the KIT receptor is the stem cell factor (SCF). SCF binding results in proliferation and in inhibition of apoptosis [Linnekin, 1999]. The intracellular part of the receptor TyrK KIT consists of a juxtamembrane region and two Tyr kinases separated by a kinase insert. In HMC-1 cells KIT is constitutive activated [Sundstrom et al., 2003]. This activation is caused by mutations in the proto-oncogene c-kit, what encodes the KIT protein. These lead to an amino acid exchange in the protein, on the one hand in Gly-560 → Val (HMC-1^{560}), and on the other Asp-816 → Val (HMC-$1^{560, 816}$). The change in the position 560 occurs in the juxtamembrane region of KIT and results in the permanent ligand independent activation of KIT in both cell lines. The mutation is common in various human malignant diseases, including gastrointestinal stromal tumour and cutaneous

Abbreviations used: HMC-1, human mast cell line; TyrK, tyrosine kinase; SCF, stem cell factor; Ca^{2+}, calcium; NH_4Cl, ammonium chloride.

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mastocytosis [Shah et al., 2006]. HMC-$1^{560, 816}$ cells carry both mutations. The Asp-816 → Val change occurs in the intracellular part of KIT and modifies the conformation of the TyrK [Ma et al., 2002; Yavuz et al., 2002; Akin et al., 2003].

Mastocytosis is a mast cell disease characterized by an abnormal growth and accumulation in one or more tissues [Valent et al., 2001]. Systemic mastocytosis almost only occurs in adults and is associated with an activating mutation in codon 816 of c-kit. The 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 systemic mastocytosis. The mutation could be identified in more than 80% of systemic mastocytosis cases [Akin and Metcalfe, 2004; Garcia-Montero et al., 2006]. Especially in its aggressive variants and in mast cell leukaemia, the 816 mutation of KIT seems to play an important role [Valent et al., 2001]. Even if this mutation appears in grave diseases, there is not much known about activating routes in HMC-$1^{560, 816}$ cells.

The TyrK inhibitor STI571 (Glivec®) is very successfully used in cells that carry the 560 mutation. The drug binds with a high affinity to the inactive conformation of the intracellular kinase of the receptor and keeps it in this condition. STI571 acts as a competitive inhibitor of adenosine triphosphate (ATP) on its binding site [Shah et al., 2006]. Thereby it is able to inhibit proliferation and to induce apoptosis of the permanent activated cells [Heinrich et al., 2000]. Unfortunately, it is ineffective in cells with Asp816Val mutation that are in the majority of aggressive systemic mastocytosis and mast cell leukaemia [Valent et al., 2001; Ma et al., 2002]. This behaviour towards STI571 is due to its confirmation change that prevents drug binding on the TyrK receptor.

In both cell lines, it is demonstrated that the constitutive KIT phosphorylation activated other transduction pathways like that of phosphatidylinositol-3′-kinase (PI3K) and mitogene activated protein kinase (MAPK). JAK/STAT and Scr signalling pathways are also associated with KIT and thereby involved in cell survival and proliferation. They are considered to be as well permanent activated in both HMC-1 lines [Linnekin, 1999; Sundstrom et al., 2003].

We have previously observed that long-term pre-incubation of HMC-1^{560} cells with STI571 affects histamine release (manuscript in press). Non-stimulated cells released less histamine, which is attributable to the inhibition of subsequent pathways that are connected with KIT. Cells stimulated by alkalinisation increased their transmitter release. We concluded that STI571 could act as well on other tyrosine kinases than KIT and that proliferation and exocytotic processes have different transduction pathways.

To evidence our thesis, in this work we performed histamine assays with STI571 in HMC-$1^{560, 816}$ cells that different authors have described to be resistant to this drug. We stimulated them with NH_4Cl and the Ca^{2+} ionophore ionomycin and compared the results with those of HMC-1^{560}.

The aim of this work was to study the influence of the KIT mutation in signalling pathways in two HMC-1 sublines. This is important since these cells appear in different kinds of mastocytosis. Therefore, a better understanding of both cellular lines and the knowledge of signalling pathways involved in their activation could lead to a more efficient drug targeting.

METHODS

Chemicals

Ammonium chloride (NH_4Cl) 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^{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) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were expanded weekly.

Cell Preparation

For histamine release assays, cells were centrifuged (1,500 rpm, 5 min, 4°C) and washed twice with saline solution (1,000 rpm, 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

6.2 μl of a freshly prepared concentrated solution of each drug 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 rpm 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 rpm, 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 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. 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 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 Grynkiewicz et al. [1985] and the calibration of fluorescence values versus pH as per Thomas et al. [1979]. 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 ± SEM.

RESULTS

In this work, we checked the release of histamine, intracellular Ca^{2+} and pH changes in HMC-$1^{560, 816}$ in the presence of stimuli.

We have previously described that alkalinisation induces histamine release in HMC-1^{560} in a dose-dependent manner [Pernas-Sueiras et al., 2005]. We showed that intracellular pH increases after NH_4Cl addition without changing cytosolic Ca^{2+} concentrations. Now we treated HMC-$1^{560, 816}$ cells in the same way and could observe a dose-dependent activation of exocytosis (Fig. 1A). Histamine release increased with increasing NH_4Cl concentrations. Further, we observed intracellular pH and Ca^{2+} after adding different concentrations of NH_4Cl. As Figure 1B shows, pH increases immediately after drug addition, where at the highest concentration (100 mM) induced the highest alkalinisation (pH 8.2) and the lowest (20 mM) caused a more slightly increase (pH 7.9). After an initial peak, cytosolic pH

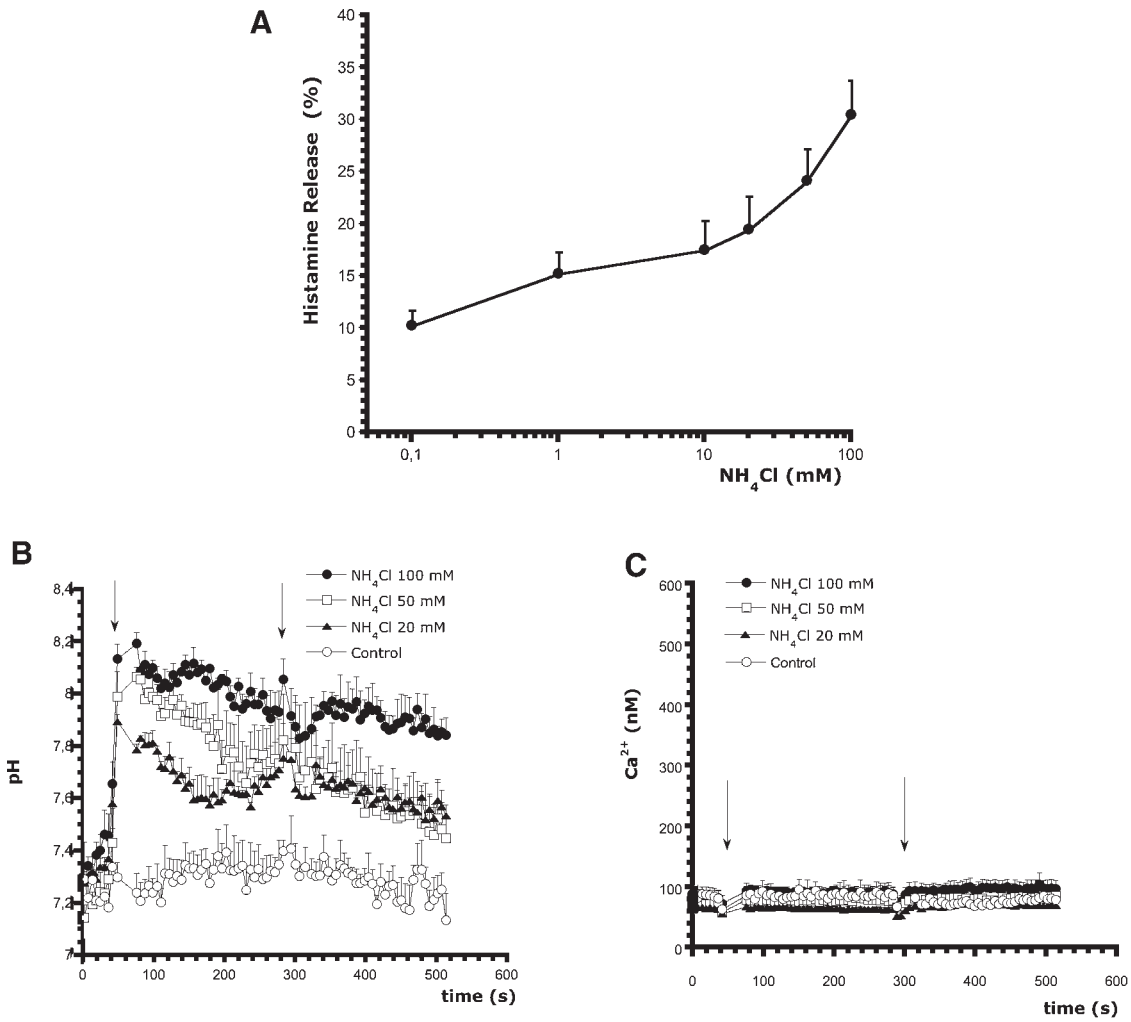

Fig. 1. Effect of NH_4Cl in HMC-$1^{560, 816}$ cells. **A**: Dose–response of histamine released in HMC-$1^{560, 816}$ cells in presence of NH_4Cl. Different concentrations of NH_4Cl were added and histamine release was measured after 10 min of incubation in saline solution. **B**: Variation of cytosolic Ca^{2+} levels in the presence of 20, 50, 100 mM NH_4Cl. The first arrow indicates the addition of NH_4Cl and second that Ca^{2+} (1 mM) was added. **C**: Intracellular pH profile in cells subject to the protocol described in (B). Mean ± SEM of four experiments.

slowly decreased. The experiments were initiated in Ca^{2+} free medium and later on Ca^{2+} was restored to the extracellular medium. Figure 1C shows that Ca^{2+} addition did not affect the declining pH. None of the NH_4Cl concentrations modified intracellular Ca^{2+} levels. During the whole experiment, cytosolic Ca^{2+} concentration remained constant at a baseline.

The Ca^{2+} ionophore ionomycin is another reported stimulus for histamine release in HMC-1^{560}. The stimulation is based on a dose-dependent intracellular Ca^{2+}-increase combined with pH-increase. In HMC-$1^{560, 816}$ we found as well a release of histamine depending on the dose of ionomycin that was added (Fig. 2A). At the point where ionomycin was added (Fig. 2B), intracellular Ca^{2+} concentration increased rapidly due to the release of the cation from intracellular reservoirs. Subsequent, cytosolic Ca^{2+} dropped down fast to the baseline concentration. The following addition of Ca^{2+} (1 mM) to the medium induced a second intracellular Ca^{2+} increase, due to the influx of the ion from extracellular. Figure 2B shows that intracellular Ca^{2+} reached higher levels with higher concentrations of ionomycin, both in the depletion of intracellular reservoirs (first peak), and in a similar trend when Ca^{2+} enters from the extracellular medium (second peak). Observing pH levels, Figure 2C, in the same experiments, pH baseline moved around 7.2 and increased after adding Ca^{2+}.

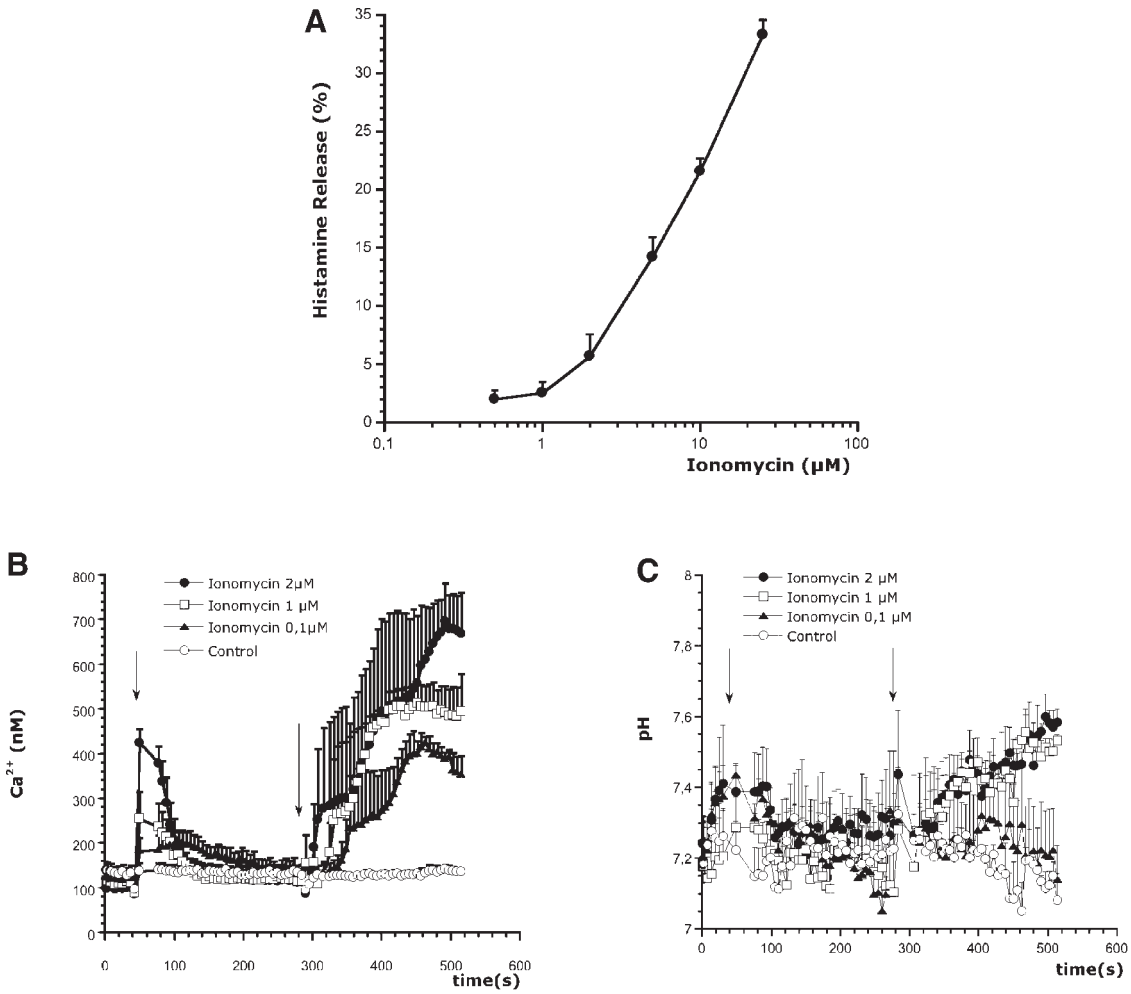

Fig. 2. Effect of ionomycin in HMC-$1^{560, 816}$ cells. **A**: Dose–response of histamine released in HMC-$1^{560, 816}$ cells in presence of ionomycin. Different concentrations of ionomycin were added and histamine release was measured after 10 min of incubation in saline solution. **B**: Variation of cytosolic Ca^{2+} levels in the presence of 0.1, 1, 2 μM ionomycin. The first arrow indicates the addition of NH_4Cl and second that Ca^{2+} (1 mM) was added. **C**: Intracellular pH profile in cells subject to the protocol described in (B). Mean ± SEM of four experiments.

We examined also the influence of STI571 after long-time incubation in HMC-$1^{560, 816}$ cells. Up to now, it is reported that this drug does not have effects in this cell line.

First, we tested the viability of the cells in the presence of different concentrations of STI571. As Figure 3 shows, in contrast to HMC-1^{560}, HMC-$1^{560, 816}$ cells did not die when they were exposed to the TyrK inhibitor in concentrations between 0.1 nM and 10 μM.

Furthermore, we incubated HMC-$1^{560, 816}$ cells with 25 nM during 24 h in culture medium and performed afterwards histamine assays. We selected this concentration from previous experiences in HMC-1^{560} cells. Surprisingly, spontaneous histamine release was significantly decreased in STI571 24 h pre-incubated HMC-$1^{560, 816}$ cells (Fig. 4). This result gave us reason to investigate more the behaviour of these cells concerning to long-time incubation with STI571 and release of histamine. We stimulated them with NH_4Cl (50 mM) after 24-h pre-incubation with STI571 (25 nM). As Figure 5A shows, pre-treated cells released more histamine than control cells after alkalinisation. In contrast, HMC-$1^{560, 816}$ cells did not change their histamine response when they were pre-incubated with STI571 and further stimulated with ionomycin (2 μM), compared to ionomycin treated cells (Fig. 5B).

In HMC-1^{560}, the effect of NH_4Cl was due to a higher increase of pH in cells pre-incubated with the TyrK inhibitor after alkalinisation. To investigate this in HMC-$1^{560, 816}$, intracellular was measured after 24 h STI571 (25 nM) pre-treatment. In Figure 6A, it is shown that pH_i reached higher values in STI571 pre-incubated HMC-$1^{560, 816}$ cells, whereas intracellular Ca^{2+} did not change at any moment of the experiments (Fig. 6B).

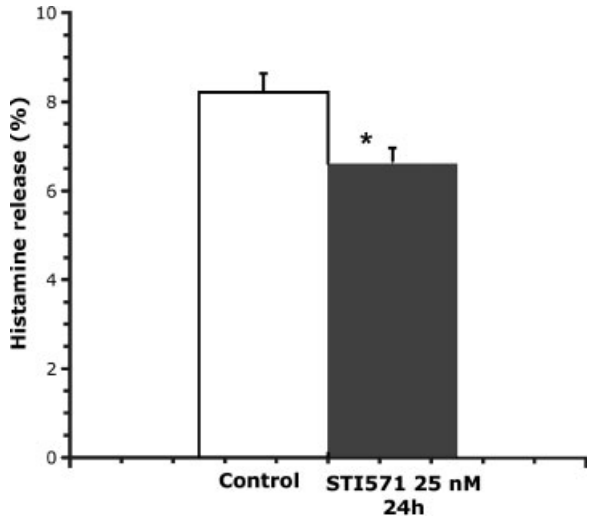

Fig. 4. Effect of STI571 on HMC-$1^{560, 816}$ spontaneous histamine release after 24 h of incubation. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. Histamine release was checked after 10 min of incubation in saline solution at 37°C. Mean ± SEM of four experiments. (*) Significant differences between control and drug pre-incubated cells.

From previous studies, we know that PKC stimulation by the phorbol ester PMA increases the release of histamine after NH_4Cl stimulation in STI571 pre-incubated HMC-1^{560} cells (manuscript in press). As Figure 7A shows, STI571 increased significantly alkalinisation-induced histamine release after treatment with PMA (100 ng/ml) in HMC-$1^{560, 816}$. However, we could not observe any differences neither in cytosolic Ca^{2+}, nor in pH levels comparing STI571 pre-incubated cells with control cells

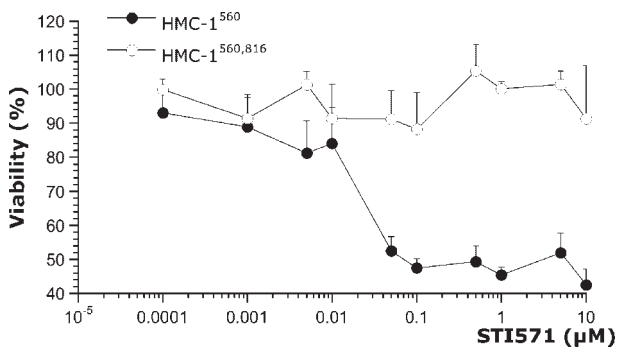

Fig. 3. HMC-1^{560} and HMC-$1^{560, 816}$ viabilities after 24 h incubation with STI571. Different concentrations of STI571 were added in culture medium and cells were incubated for 24 h at 37°C. Cell viability was checked by MTT test. Control cells with non-STI571 treatment, were used as 100% viability and ethanol treatment as 100% dead, in each experiment. Mean ± SEM of four experiments.

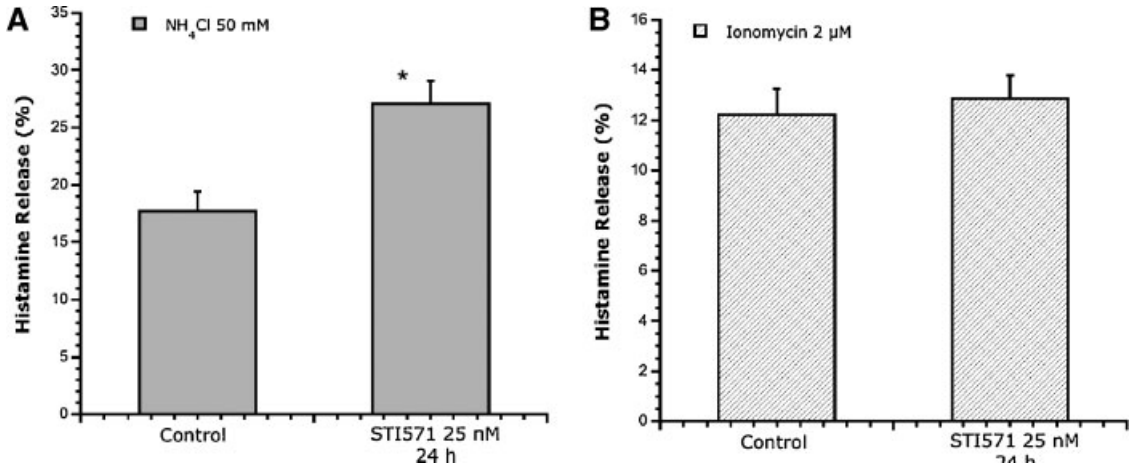

Fig. 5. Effect of STI571 on alkalinisation- and ionomycin-induced histamine release in HMC-$1^{560, 816}$ cells. STI571 (25 nM) 24 h pre-incubated cells were stimulated with NH_4Cl (50 mM) (**A**) or ionomycin (2 μM) (**B**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments. (*) Significant differences between control and drug pre-incubated cells.

(data not shown). There was no effect when STI571 pre-treated cells were stimulated with ionomycin (2 μM) in combination with PMA (100 ng/ ml) (Fig. 7B).

When PKC was inhibited by GF109203X (500 nM), 24-h pre-incubation with STI571 did not modify histamine release neither in cells stimulated by NH_4Cl (50 mM) (Fig. 8A), nor by ionomycin (0.1 μM) (Fig. 8B). HMC-$1^{560, 816}$ cells with PKC inhibited by Gö6976 (100 nM) showed the same behaviour (Fig. 8C,D). In contrast, as Figure 9A shows, when PKC was inhibited by rottlerin (10 μM) and subsequently NH_4Cl (50 mM) stimulation took place, surprisingly STI571 pre-treated cells enhanced histamine release. That meant that when TyrK and PKC were inhibited the effect on histamine release was potentiated. We could not observe any difference comparing STI571 and control cells, when they were treated with rottlerin (10 μM) and afterwards stimulated with ionomycin (Fig. 9B). In continuation, we performed rottlerin experiments in the fluorescent microscope, shown in Figure 10. HMC-$1^{560, 816}$ cells were pre-incubated with STI571 (25 nM) during 24 h like in histamine assay. We began the experiments in Ca^{2+}-free medium. After obtaining a baseline, we added NH_4Cl (50 mM). As

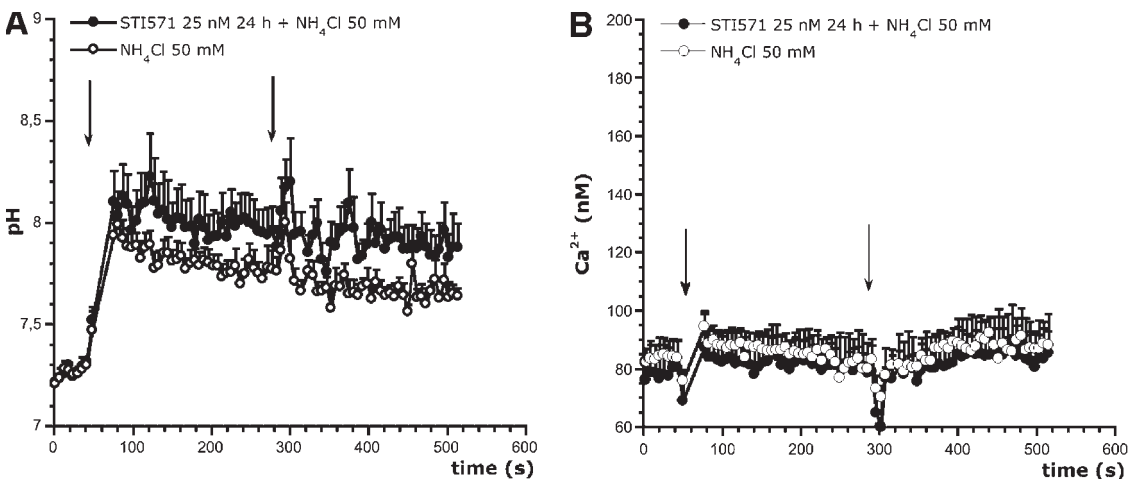

Fig. 6. Effect of STI571 on intracellular Ca^{2+} and pH levels in HMC-$1^{560, 816}$ cells. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. Cytosolic pH (**A**) and Ca^{2+} (**B**) changes were observed. The first arrow indicates the addition of NH_4Cl (50 mM) and second that Ca^{2+} (1 mM) was added. Mean ± SEM of four experiments.

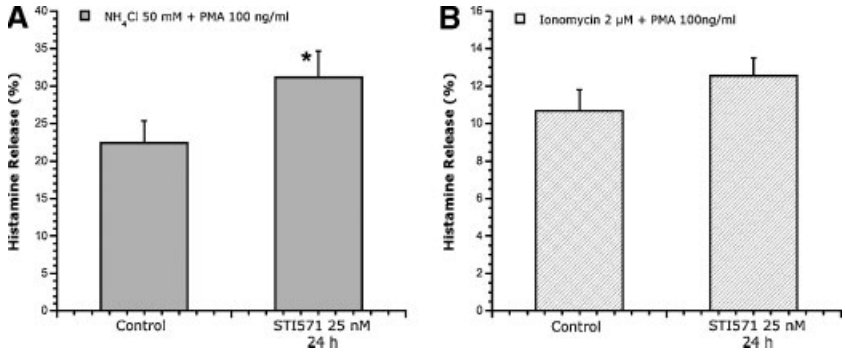

Fig. 7. Effect of PKC activation and STI571 on alkalinisation- and ionomycin-induced histamine release in HMC-1[560, 816]. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with PMA (100 ng/ml) 10 min and afterwards stimulated with NH_4Cl (50 mM) (**A**) or ionomycin (2 µM) (**B**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments. (*) Significant differences between control and drug pre-incubated cells.

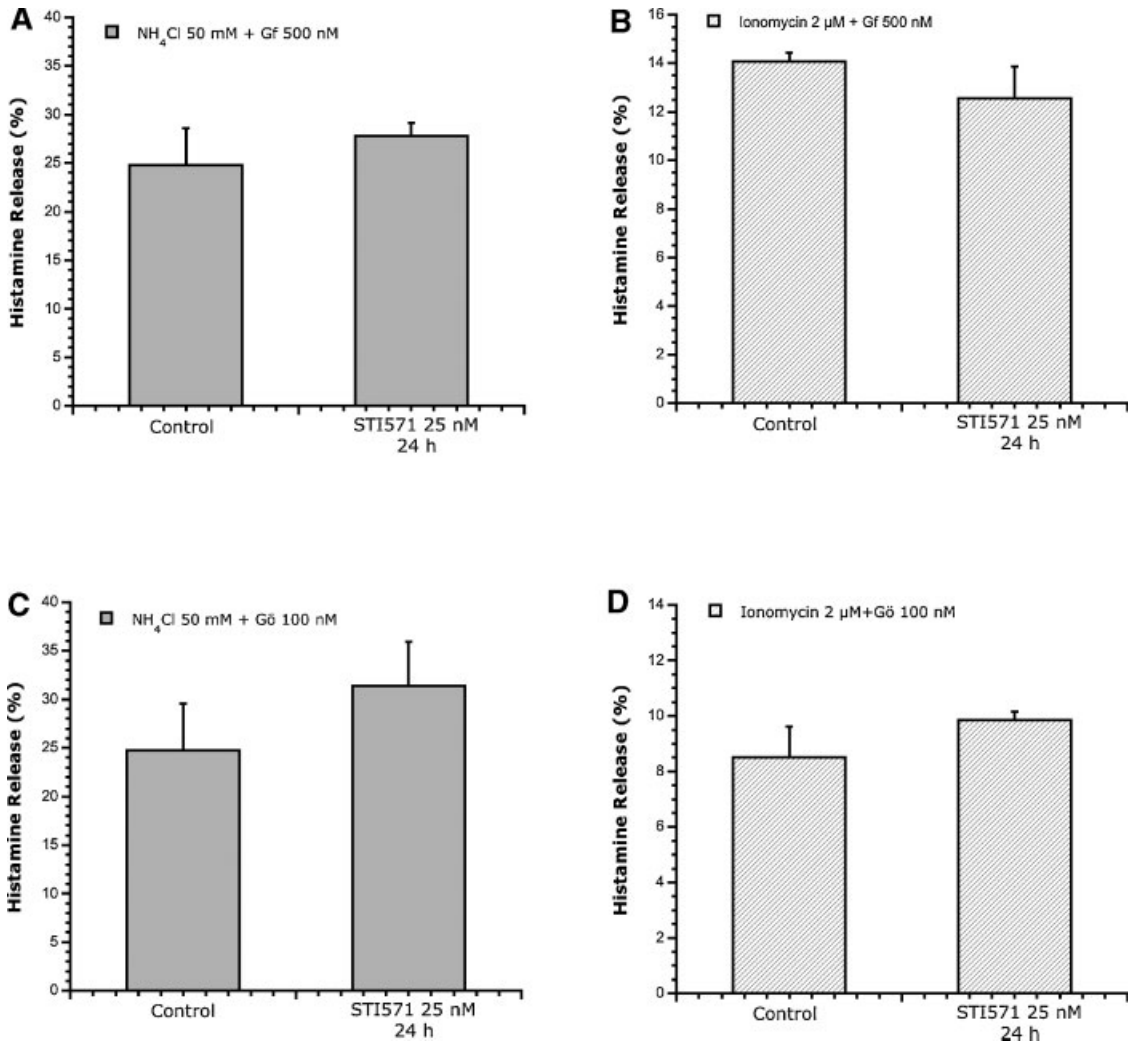

Fig. 8. Effect of PKC inhibition and STI571 on alkalinisation- or ionomycin-induced histamine release in HMC-1[560, 816]. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with Gö6976 (100 nM) and GF109203X (500 nM) 10 min and afterwards stimulated with NH_4Cl (50 mM) (**A**,**C**) or with ionomycin (2 µM) (**B**,**D**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments.

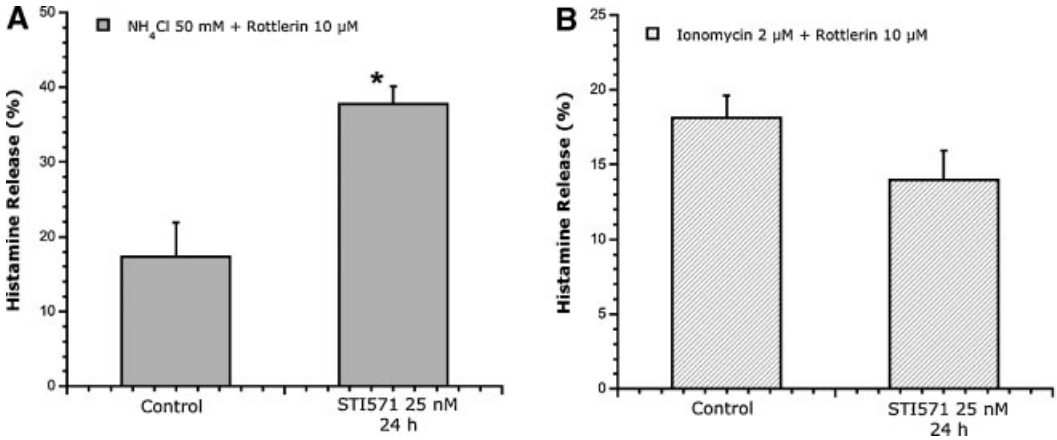

Fig. 9. Effect of rottlerin and STI571 on alkalinisation- or ionomycin-induced histamine release in HMC-$1^{560, 816}$. Cells pre-incubated with STI571 (25 nM) during 24 h were pre-treated with rottlerin (10 μM) 10 min and afterwards stimulated with NH_4Cl (50 mM) (**A**) or with ionomycin (2 μM) (**B**) during 10 min in saline solution at 37°C and histamine release was measured. Mean ± SEM of four experiments.

Figure 10 shows, pH increased significantly more in STI571 pre-treated cells at the point of NH_4Cl addition than in control cells. After adding Ca^{2+} (1 mM) to the medium, pH seemed to elevate lightly again.

DISCUSSION

It is described in previous reports that NH_4Cl induces alkalinisation and no Ca^{2+} increase in rat mast cells [Alfonso et al., 2000]. In these cells, it was suggested for the first time that pH increase by itself could be an enough signal to induce histamine release. Studies in HMC-1 have clearly demonstrated that they also show this behaviour after alkalinisation. Exocytotic process takes place without affecting intracellular Ca^{2+} concentrations [Pernas-Sueiras et al., 2005, 2006a,b]. In those studies, we used HMC-1^{560} cells that carry a mutation in the proto-oncogene c-kit that leads to a Glycin → Valin amino acid exchange in position 560 of the receptor TyrK KIT.

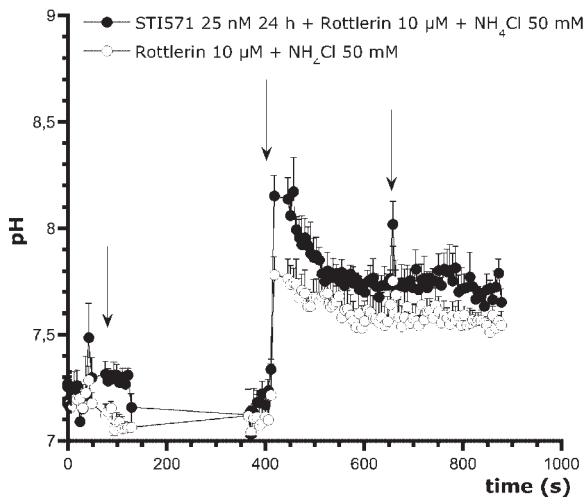

Fig. 10. Effect of rottlerin and STI571 on alkalinisation-modulated intracellular pH levels in HMC-$1^{560, 816}$ cells. Cells were pre-incubated for 24 h in the presence of STI571 (25 nM) in culture medium. Cytosolic pH changes were observed. The first arrow indicates the addition of rottlerin (10 μM), the second of NH_4Cl (50 mM) and the third that Ca^{2+} (1 mM) was added. Mean ± SEM of three experiments.

In the present work, the HMC-$1^{560, 816}$ subline with one amino acid change more in position 816 was used to check if its behaviour is similar regarding release of histamine after stimulation. This is important since there are already differences reported between both sublines [Sundstrom et al., 2003; Gleixner et al., 2005].

Results shown confirmed the same dose-dependent histamine release in presence of NH_4Cl in HMC-$1^{560, 816}$ like in cells with one mutation. We also demonstrated that intracellular alkalinisation took place without changing Ca^{2+} concentrations. However, pH increased much more in HMC-$1^{560, 816}$ than in HMC-1^{560} after alkalinisation. When HMC-$1^{560, 816}$ cells were treated with 50 mM of NH_4Cl, pH increased about 0.8 units; in contrast, treating HMC-1^{560} with the same concentration, pH rose only about 0.3 units higher, whereas histamine release was almost the same. We suggest that pH control has a larger importance in HMC-$1^{560, 816}$ than in HMC-1^{560},

but more studies have to be addressed in this context.

The Ca^{2+} ionophore ionomycin is another stimulus reported to induce exocytosis in HMC-1^{560} [Pernas-Sueiras et al., 2005] and other mast cells [Teofoli et al., 1999]. In HMC-1^{560} the stimulation is due to an increase of intracellular Ca^{2+} concentration in combination with an alkalinisation in presence of extracellular Ca^{2+}. We could demonstrate that histamine release can be stimulated with ionomycin in a dose-dependent manner, also in HMC-$1^{560, 816}$. We saw that HMC-$1^{560, 816}$ cells did not reach a plateau at concentrations up to 10 μM of ionomycin as HMC-1^{560}, comparing both histamine release profiles [Pernas-Sueiras et al., 2005]. Other differences were shown in intracellular Ca^{2+} observations. After ionomycin addition, cytosolic Ca^{2+} concentration increased due to the release of the cation from intracellular reservoirs. This raise was more steeply but reached lower values in HMC-$1^{560, 816}$ than in HMC-1^{560}. When Ca^{2+} entered from the extracellular medium, intracellular Ca^{2+} concentration increased less and more slowly in cells with two mutations. The presence of the second mutation modifies the regulatory mechanisms for calcium.

Differences in the two sublines apart from the mutations have been already described. HMC-$1^{560, 816}$ has a higher proliferation rate in culture, whereas the cells are smaller and more homogenous. HMC-1^{560} cells are heterogeneous in size and showed homotypic aggregation. HMC-$1^{560, 816}$ has a normal diploid DNA profile but HMC-1^{560} indicate an increased DNA content [Sundstrom et al., 2003]. However, one of the most interesting differences is the response of the two cell lines in presence of KIT TyrK inhibitor STI571. Whereas STI571 induces apoptosis and inhibits proliferation in HMC-1^{560} cells, it does not affect HMC-$1^{560, 816}$ cells due to the conformation change in the kinase of KIT, that avoids drug binding [Heinrich et al., 2000; Ma et al., 2002; Akin et al., 2003; Roskoski, 2003].

Long-time incubation of HMC-1^{560} cells with STI571 modifies histamine release, spontaneous release decreases and alkalinisation-induced histamine release increased, caused by a pH_i increase to higher values after NH_4Cl addition (manuscript in press). STI571 could inhibit other Tyr kinases, and in this study, we showed that STI571 affected histamine release in HMC-$1^{560, 816}$ similarly after long-time incubation. This suggests that STI571 might have other binding sites. STI571 is reported to bind the ATP binding site of the KIT kinase in HMC-1^{560} [Roskoski, 2003]. The drug is not able to ligate the same site in HMC-$1^{560, 816}$, because of the conformation change in the KIT kinase caused by the amino acid exchange in position 816 [Ma et al., 2002; Sundstrom et al., 2003]. If the drug would selectively inhibit KIT TyrK, we could not observe similar effects in HMC-1^{560} and HMC-$1^{560, 816}$ cells. We have suggested that inhibition takes place in other Tyr kinases, since we found that lavendustin A and genistein, two unselective TyrK, were also able to reduce spontaneous histamine release after long-time incubation HMC-1^{560} (manuscript in press). The two drugs induced also an increase in alkalinisation-induced exocytosis. This might be through Src Tyr kinases, since it is reported that their inhibition by genistein avoids intracellular pH increase by inhibiting an anion Cl^-/HCO_3^- exchanger in cardiomyocytes [Puceat et al., 1998].

Our cell viability test and studies of other pharmacological groups demonstrate clearly that KIT TyrK inhibition takes place in HMC-1^{560} but not in HMC-$1^{560, 816}$ [Heinrich et al., 2000; Akin et al., 2003]. Therefore proliferation and histamine release might have completely unconnected signalling pathways. This agrees with other studies that have reported that Ras-MAPK-ERK and PI3K pathways do not seem to be essential for exocytosis in this cell line [Kempna et al., 2004]. These pathways are directly connected with KIT and essential for proliferation [Linnekin, 1999]. In contrast, regarding histamine release, pH seems to play a mayor role in HMC-1, as it was reported in rat mast cells [Vilarino et al., 1999; Alfonso et al., 2000]. Modulation of pH can be due to the activity of anion Cl^-/HCO_3^- [Puceat et al., 1998] or cation H^+/Na^+ [Alfonso et al., 1994, 1998; Friis and Johansen, 1996] exchanger.

It has been described that STI571 act very selective on KIT TyrK [Heinrich et al., 2000]. The selectivity of the compound should be studied in other enzymes and ion exchangers, since pH is influenced after long-term drug incubation. It should also be bear in mind that HMC-$1^{560, 816}$ are more sensitive to extracellular pH changes.

Our results show that STI571 does not modify ionomycin-induced histamine release in cells

with one or two mutations. It seems that the pathway that is modified by STI571 is not linked to that of ionomycin and probably not to intracellular Ca^{2+} concentrations. Until now, we could never observe that cytosolic Ca^{2+} has a different profile in STI571 pre-incubated cells.

Finally, we checked the influence of PKC modulation on STI571 effects in HMC-$1^{560, 816}$ since we had earlier reported that activation of PKC by PMA increased histamine response after alkalinisation in STI571 pre-treated HMC-1^{560}cells (manuscript in press). We found the same behaviour of HMC-$1^{560, 816}$ cells.

Surprisingly, STI571 pre-incubated HMC-$1^{560, 816}$ cells with rottlerin inhibited PKC, released more histamine after alkalinisation. This effect might be linked to the fact that pH rose to higher values in STI571 cells. Since the TyrK inhibitor was discovered by testing of compounds for inhibition of PKC [Shah et al., 2006]. Maybe this potentiated effect is due to a common binding site of the two inhibitors that modify pH regulation in HMC-$1^{560, 816}$. Nevertheless, as far as we know, it has not been reported that PKC inhibition induced histamine release in mast cells. In contrast, we could not observe an effect of PKC inhibition in HMC-1^{560}. The difference could be due to the higher pH sensitivity of HMC-$1^{560, 816}$.

More studies should be made in HMC-$1^{560, 816}$ concerning to activating processes and the influence of the mutations in this context especially in pH modulation. Much work is already done in HMC-1^{560} and this is a good starting point to compare the two sublines in the future. This is important because both lines appear in different diseases and drug targeting could be more efficient with a better knowledge of intracellular signalling.

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REFERENCES

Akin C, Metcalfe DD. 2004. Systemic mastocytosis. Annu Rev Med 55:419–432.

Akin C, Brockow K, D'Ambrosio C, Kirshenbaum AS, Ma Y, Longley BJ, Metcalfe DD. 2003. Effects of tyrosine kinase inhibitor STI571 on human mast cells bearing wild-type or mutated c-kit. Exp Hematol 31:686–692.

Alfonso A, Botana MA, Vieytes MR, Botana LM. 1994. Functional characterization of the Na(+)-H+ exchanger in rat mast cells: Crosstalks between different kinase pathways. Eur J Pharmacol 267:289–296.

Alfonso A, Botana MA, Vieytes MR, Botana LM. 1998. Sodium, PMA and calcium play an important role on intracellular pH modulation in rat mast cells. Cell Physiol Biochem 8:314–327.

Alfonso A, Cabado AG, Vieytes MR, Botana LM. 2000. Calcium-pH crosstalks in rat mast cells: Cytosolic alkalinization, but not intracellular calcium release, is a sufficient signal for degranulation. Br J Pharmacol 130: 1809–1816.

Friis UG, Johansen T. 1996. Dual regulation of the Na+/H(+)-exchange in rat peritoneal mast cells: Role of protein kinase C and calcium on pHi regulation and histamine release. Br J Pharmacol 118:1327–1334.

Garcia-Montero AC, Jara-Acevedo M, Teodosio C, Sanchez ML, Nunez R, Prados A, Aldanondo I, Sanchez L, Dominguez M, Botana LM, Sanchez-Jimenez F, Sotlar K, Almeida J, Escribano L, Orfao A. 2006. KIT mutation in mast cells and other bone marrow hematopoietic cell lineages in systemic mast cell disorders: A prospective study of the Spanish Network on Mastocytosis (REMA) in a series of 113 patients. Blood 108:2366–2372.

Gleixner KV, Mayerhofer M, Aichberger KJ, Derdak S, Sonneck K, Bohm A, Gruze A, Samorapoompichit P, Manley PW, Fabbro D, Pickl WF, Sillaber C, Valent P. 2005. PKC412 inhibits in vitro growth of neoplastic human mast cells expressing the D816V-mutated variant of KIT: Comparison with AMN107, imatinib, and cladribine (2CdA) and evaluation of cooperative drug effects. Blood 107:752–759.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440–3450.

Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler A. 2000. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. Blood 96:925–932.

Kempna P, Reiter E, Arock M, Azzi A, Zingg JM. 2004. Inhibition of HMC-1 mast cell proliferation by vitamin E: Involvement of the protein kinase B pathway. J Biol Chem 279:50700–50709.

Linnekin D. 1999. Early signaling pathways activated by c-Kit in hematopoietic cells. Int J Biochem Cell Biol 31: 1053–1074.

Ma Y, Zeng S, Metcalfe DD, Akin C, Dimitrijevic S, Butterfield JH, McMahon G, Longley BJ. 2002. The c-KIT mutation causing human mastocytosis is resistant to STI571 and other KIT kinase inhibitors; kinases with enzymatic site mutations show different inhibitor sensitivity profiles than wild-type kinases and those with regulatory-type mutations. Blood 99:1741–1744.

Pernas-Sueiras O, Alfonso A, Vieytes MR, Botana LM. 2005. Mast cell exocytosis can be triggered by ammonium

chloride with just a cytosolic alkalinization and no calcium increase. J Cell Physiol 204:775–784.

Pernas-Sueiras O, Alfonso A, Vieytes MR, Botana LM. 2006a. PKC and cAMP positively modulate alkaline-induced exocytosis in the human mast cell line HMC-1. J Cell Biochem 99:1651–1663.

Pernas-Sueiras O, Alfonso A, Vieytes MR, Orfao A, Escribano L, Francisca SJ, Botana LM. 2006b. Calcium-pH crosstalks in the human mast cell line HMC-1: Intracellular alkalinization activates calcium extrusion through the plasma membrane Ca^{2+}-ATPase. J Cell Biochem 99:1397–1408.

Puceat M, Roche S, Vassort G. 1998. Src family tyrosine kinase regulates intracellular pH in cardiomyocytes. J Cell Biol 141:1637–1646.

Roskoski RJ. 2003. STI-571: An anticancer protein-tyrosine kinase inhibitor. Biochem Biophys Res Commun 309: 709–717.

Shah NP, Lee FY, Luo R, Jiang Y, Donker M, Akin C. 2006. Dasatinib (BMS-354825) inhibits KITD816V, an imatinib-resistant activating mutation that triggers neoplastic growth in most patients with systemic mastocytosis. Blood 108:286–291.

Shore PA. 1971. The chemical determination of histamine. Methods Biochem Anal (Suppl):89–97.

Sundstrom M, Vliagoftis H, Karlberg P, Butterfield JH, Nilsson K, Metcalfe DD, Nilsson G. 2003. Functional and phenotypic studies of two variants of a human mast cell line with a distinct set of mutations in the c-kit proto-oncogene. Immunology 108:89–97.

Teofoli P, Frezzolini A, Puddu P, De Pita O, Mauviel A, Lotti T. 1999. The role of proopiomelanocortin-derived peptides in skin fibroblast and mast cell functions. Ann NY Acad Sci 885:268–276.

Thomas JA, Buchsbaum RN, Zimniak A, Racker E. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 18:2210–2218.

Valent P, Horny HP, Escribano L, Longley BJ, Li CY, Schwartz LB, Marone G, Nunez R, Akin C, Sotlar K, Sperr WR, Wolff K, Brunning RD, Parwaresch RM, Austen KF, Lennert K, Metcalfe DD, Vardiman JW, Bennett JM. 2001. Diagnostic criteria and classification of mastocytosis: A consensus proposal. Leuk Res 25:603–625.

Vilarino N, Vieytes MR, Vieites JM, Botana LM. 1999. Modulatory effect of HCO3- on rat mast cell exocytosis: Cross-talks between bicarbonate and calcium. Biochem Biophys Res Commun 260:71–79.

Yavuz AS, Lipsky PE, Yavuz S, Metcalfe DD, Akin C. 2002. Evidence for the involvement of a hematopoietic progenitor cell in systemic mastocytosis from single-cell analysis of mutations in the c-kit gene. Blood 100:661–665.