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Histamine release, an undesired effect of thrombin inhibitors with basic character, is mediated through direct activation of G_i proteins

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Abstract

The common structural feature of LK direct thrombin inhibitors is a strong basic group attached to the azaphenylalanine scaffold, which is important for the appropriate interaction at the thrombin active site. Our previous results have shown that this basic group could be responsible for a reduction of tracheal air flow and a fall of mean arterial pressure in anaesthetized rats, an undesired effect of direct thrombin inhibitors which correlated with their ability to release histamine from mast cells.

In the present study, we investigated the mechanism of LK direct thrombin inhibitors-induced histamine release from rat peritoneal mast cells. We demonstrated that thrombin inhibitors with basic character (LK-732, LK-639 and LK-6063) provoked release of histamine from mast cells, while less basic analogs (LK-658, LK-633 and LK-6062) had no effect. Histamine released by LK-732 and LK-639 was suppressed by removal of sialic acid residues by neuraminidase and by pertussis toxin, an inhibitor of G_i protein activity. Additional demonstration that G proteins are the targets of LK-732 and LK-639 was provided by the increase of GTPγS binding rate to G proteins in rat brain cortical membranes. Our results indicate that basic direct thrombin inhibitors LK-732 and LK-639 provoke release of histamine from mast cells by direct activation of G_i proteins through the similar biochemical pathway as basic secretagogues.

Keywords: Thrombin inhibitor; Mast cell; Histamine release; G protein; Basic secretagogue; Azaphenylalanine scaffold

1. Introduction

Thrombin plays a central role in thrombosis, which is one of the leading causes of cardiovascular disease and morbidity in developed societies. Existing treatments for thrombotic disorders involving heparin, low molecular weight heparin, hirudin or warfarin have numerous limitations, such as thrombocytopenia, lack of oral bioavailability and unpredictable anticoagulant response (Agnelli and Sonaglia, 2002; Ammar et al., 1997; Hirsh and Weitz, 1999). The discovery and development of direct, noncovalent and orally bioavailable thrombin inhibitors are therefore an important goal for many pharmaceutical laboratories (Boos et al., 2005; Linkins and Weitz, 2005; Nutescu et al., 2006; Pfau, 2003; Serebruany et al., 2006). Thrombin inhibitors built on the azaphenylalanine scaffold, which bind directly to thrombin and block the enzyme's interactions with its substrates, represent a new group of direct thrombin inhibitors with promising in vitro anticoagulant (Obreza et al., 2004; Zega et al., 2004) and in vivo antithrombotic effects (Peternel et al., 2005). Their common structural feature responsible for the appropriate interaction at the thrombin active site is a strong basic group attached to the azaphenylalanine scaffold (Obreza et al., 2004; Zega et al., 2004).

Our previous studies have shown that direct thrombin inhibitors built on the azaphenylalanine scaffold with a basic character are able to release histamine from rat peritoneal mast cells

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(Peternel et al., 2006). In addition, in vivo studies have demonstrated that these agents induce reduction of tracheal air flow and a fall of mean arterial pressure after i.v. administration in anaesthetized rats. In contrast, less basic inhibitors did not induce release of histamine from mast cells and did not affect tracheal air flow and mean arterial pressure (Peternel et al., 2006).

Basic secretagogue-elicited mast cell activation is beside antigenic pathway that occurs in response to antigens the main signaling pathway of serosal mast cell activation (Metcalfe et al., 1997). From the original observation that mastoparan, a wasp venom peptide, activates mast cells through direct interaction with G proteins, basic secretagogues (compound 48/80, cationic peptides) are believed to act through receptor-independent manner by direct activation of G_i protein (Aridor et al., 1990; Ferry et al., 2002; Higashijima et al., 1988; Mousli et al., 1990a,b,c).

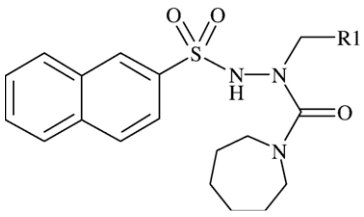
The aim of the present study was to investigate the mechanism whereby LK direct thrombin inhibitors built on the azaphenylalanine scaffold with basic character induce release of histamine from rat peritoneal mast cells.

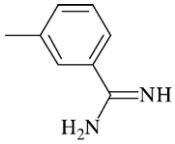
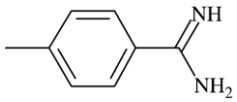
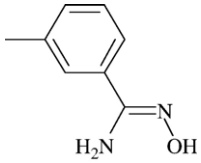
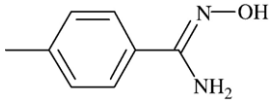
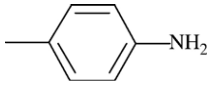
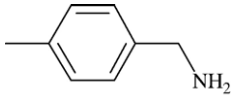
2. Materials and methods

2.1. Materials

Bovine albumin, D-(+)-glucose, mastoparan, compound 48/80, type V neuraminidase from *Clostridium perfringens*, Triton X-100 and pertussis toxin were supplied by Sigma (St. Louis, USA). HEPES (2-[4-(2-hydroxyethyl]-1-piperazinyl) ethanesulfonic acid) was purchased from Merck (Darmstadt, Germany), Percoll® was from Pharmacia Biotech AB (Uppsala, Sweden) and guanosine 5′-[γ-^{35}S]triphosphate from Amersham (Uppsala, Sweden). LK direct thrombin inhibitors (Table 1) were synthesized at the Faculty of Pharmacy (University of Ljubljana, Slovenia) according to published procedures (Obreza et al., 2004; Zega et al., 2004).

Table 1
Structures and pK_b values of direct thrombin inhibitors built on azaphenylalanine scaffold



Compound	R1	pK_b
LK-732		2.10
LK-639		1.85
LK-658		10.70
LK-633		10.89
LK-6062		9.56
LK-6063		4.64

2.2. Animals

Study was carried out using male Wistar rats (260–380 g, Experimental Medical Centre, Medical Faculty, University of Ljubljana, Slovenia). Rats were housed four to five per cage. The room temperature was kept at 23 ± 1 °C. Food and water were available ad libitum. All animals received care in compliance with the European Convention on Animal Care. The protocol received the approval of the Veterinary Administration of the Republic of Slovenia with regard to the care and use of laboratory animals.

2.3. Mast cells isolation and purification

Rats were anaesthetized with ether, decapitated and exsanguinated. 10 ml of buffered-salt solution was injected into the peritoneal cavity and then the abdomen was gently massaged for 1.5 min. Mixed rat peritoneal cells were suspended in buffer-salt solution with the following composition (mM): NaCl 134.0, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.0, Tris–HCl 12.5, glucose 1 mg/ml, bovine albumin 1 mg/ml, pH 7.4. The cell suspension was then centrifuged at 220×*g* for 10 min and supernatant discarded. For preparation of purified mast cells, the cells were transferred to a HEPES-buffered (32 mmol/l) Percoll solution (1017 g/ml). A gradient of Percoll was created by centrifugation at 21,000×*g* for 30 min at 4 °C. After centrifugation, mast cells were located in the lower third of the gradient with a purity of more than 98%. Percoll was removed by washing the mast cell fraction in buffer-salt solution (Knudsen et al., 1993; Stempelj and Ferjan, 2005).

2.4. Mast cells treatment

Mast cells, suspended in buffer-salt solution, were aliquoted in plastic tubes to attain final volume 0.5 ml, each containing approximately 2×10^5 mast cells. The cells were pre-incubated with pertussis toxin (1–300 ng/ml) for 2 h or with neuraminidase

(2 U/ml) for 1 h at 37 °C and then stimulated with direct thrombin inhibitors (3–500 μM) or with compound 48/80 (1 μg/ml) for further 15 min. When cytotoxic effect of compounds was assessed, mast cells were pre-incubated for 30 min at 4 °C and then stimulated at this temperature with LK direct thrombin inhibitors (200 μM), compound 48/80 (1 μg/ml) or with Triton X-100 (0.1%) for 30 min. The secretion of histamine was stopped by immersing the tubes in ice-cold bath and by the addition of ice-cold buffer-salt solution. After centrifugation of mast cells, the supernatants were collected and the cell pellets resuspended in 0.9% NaCl and allowed to stand in a boiling water bath for 3 min to release residual histamine (Knudsen et al., 1993). Appropriate controls to determine spontaneous histamine release in the absence of stimuli were prepared for each experiment. When direct thrombin inhibitors were dissolved in dimethyl sulfoxide (DMSO), the final concentration of this solvent was less than 0.5%. Control experiments confirmed that DMSO at these concentrations (<0.5%) did not affect the release of histamine.

2.5. Histamine release assay

Histamine was assayed spectrofluorometrically both in pellets (residual histamine) and in supernatants (histamine released) by using a modified method of Shore (Shore et al., 1959) in a spectrofluorometer Shimadzu RF-1501 at excitation wavelength 360 nm and emission wavelength 450 nm. The release of histamine was calculated as a percentage of the total histamine content of the sample. All values were corrected for the spontaneous histamine release (7.1±3.2%).

2.6. Membrane preparation and the rate of GTPγS binding

Rat brain cortical membranes were prepared according to the protocol of McKenzie (1992). Until used, they were kept frozen in the concentration of 1–2 mg protein/ml as determined by the method of Lowry et al. (1951). The initial rate of binding of [^{35}S]GTPγS to G proteins in the membranes was followed as described by McKenzie (1992), with minor modifications (Bavec et al., 1999). Briefly, the membranes (final protein concentration in the assay mixture was kept around 0.05 mg/ml) were incubated for 3 min in the absence and presence of LK direct thrombin inhibitors (200 μM) or mastoparan (100 μM) with 0.5 nM [^{35}S]GTPγS at 25 °C in TE-buffer (10 mM Tris–HCl+0.1 mM EDTA), pH 7.5. The unbound [^{35}S]GTPγS was removed by rapid filtration of the reaction mixture through Millipore GF/C glass-fiber filters under vacuum. The remaining radioactivity in filters was determined in the LKB 1214 Rackbeta liquid scintillation counter. Blank values were determined by replacing the membranes with buffer.

2.7. Calculation of ionization constants

Apparent negative logarithm base ionization constant (pK_b values) for dissociation centre of the investigated direct thrombin inhibitors were calculated using ACD/pK_a software (ACD Labs, Toronto, Canada). The calculations were performed for a temperature of 25 °C and zero ionic strength in aqueous solution. The accuracy of the calculated values is within ±0.5 p$K_{a/b}$ units.

2.8. Statistical analysis

Results are expressed as the mean±S.E.M. One-way analysis of variance (ANOVA) with Dunnett's post-test was used to assess the difference between control and treated groups. If just two groups were compared, two-tailed *t*-test was used. $P<0.05$ was considered to be significant. Statistical analysis was performed using Graph Pad Prism software 4.02 (San Diego, USA).

3. Results

3.1. Effect of direct thrombin inhibitors on histamine release

Mast cells were treated with increasing concentrations (3–300 μM) of LK direct thrombin inhibitors with (LK-732, LK-639, LK-6063) or without (LK-658, LK-639, LK-6062) basic character (Table 1). A significant amount of histamine was released by basic thrombin inhibitors LK-732, LK-639 and LK-6063 at concentrations above 30 μM.

At the concentration 200 μM, the responses to LK-732, LK-639 and LK-6063 were 63.7±2.3%, 67.3±3.6% and 85.0±0.8%, respectively (Fig. 1). In contrast, histamine was not released by LK-658 (pK_b=10.70) and LK-633 (pK_b=10.89), less basic analogs of LK-732 (pK_b=2.10) and LK-639 (pK_b=1.85), respectively. Compounds LK-658 and LK-633 have attached amidoxime group to the aromatic ring instead of amidine group which is responsible for the basic character of LK-732 and LK-639 (Table 1) (Obreza et al., 2004; Zega et al., 2004). In addition, absence of a basic character in structurally different thrombin inhibitor LK-6062 (pK_b=9.56) also resulted in abolishment of histamine release.

3.2. Cytotoxic effect of direct thrombin inhibitors on histamine release

In order to determine if histamine released by direct thrombin inhibitors was evoked by a cytotoxic action, we performed the experiments at 4 °C. Histamine release induced at 37 °C by

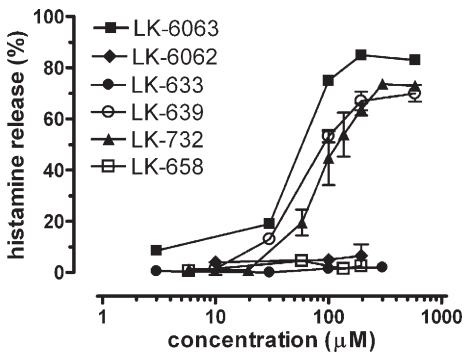


Fig. 1. Effect of direct thrombin inhibitors built on the azaphenylalanine scaffold on histamine release from isolated rat peritoneal mast cells. Results are given as mean±S.E.M. of four experiments.

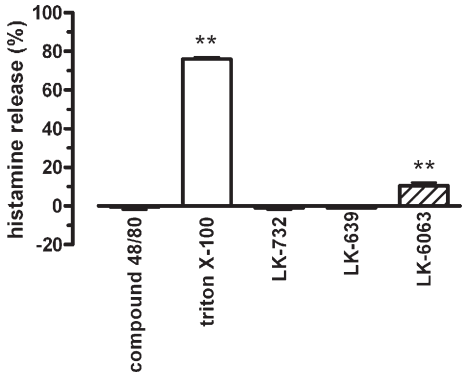


Fig. 2. Effect of low temperature on LK direct thrombin inhibitors-induced histamine release from isolated rat peritoneal mast cells. Cell were pre-incubated for 30 min at 4 °C before the addition of LK-732 (200 μM), LK-639 (200 μM), LK-6063 (200 μM), compound 48/80 (1 μg/ml) or Triton X-100 (0.1%) to the medium for 30 min. Results are given as mean±S.E.M. of four experiments. ANOVA, $^{**}P<0.01$.

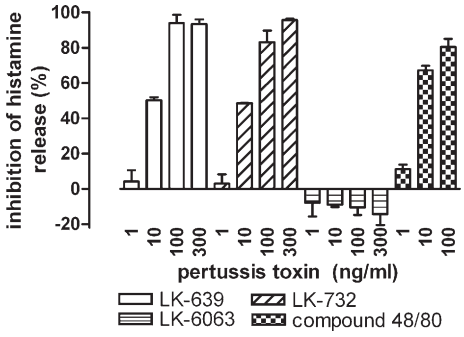


Fig. 3. Effect of pertussis toxin on histamine release induced by LK direct thrombin inhibitors built on the azaphenylalanine scaffold. Cells were pre-incubated with pertussis toxin for 2 h at 37 °C before the addition of LK-732 (200 μM), LK-639 (200 μM), LK-6063 (200 μM) or compound 48/80 (1 μg/ml) to the medium for 15 min. Results are given as mean±S.E.M. of four experiments.

LK-732 (63.7±2.3%, 200 μM) and LK-639 (67.3±3.6%, 200 μM) was completely abolished when mast cells were stimulated at 4 °C, whereas LK-6063 at the concentration of 200 μM still induced a significant 10.5±1.7% histamine release (Fig. 2). Treatment of mast cells at 4 °C by compound 48/80 (1 μg/ml), a well-known activator of G_i proteins in mast cells (Mousli et al., 1990a,b), also abolished histamine secretion, but did not prevent cytolytic effect of Triton X-100 (0.1%). These results suggest that histamine release induced by LK-732 and LK-639 was not due to non-specific cytotoxicity, whereas secretion induced by 200 μM LK-6063 was partially induced by its cytotoxic effect.

3.3. Effect of pertussis toxin on direct thrombin inhibitors-induced histamine release

To test whether LK thrombin inhibitors induce histamine release from mast cells through activation of G_i proteins, a mechanism that was established for basic secretagogues (Nakamura and Ui, 1985; Saito et al., 1987), we pre-treated mast cells with pertussis toxin, an inhibitor of G_i protein. We demonstrated that pertussis toxin (1–300 ng/ml) almost completely inhibited histamine release induced by 200 μM of LK-732 and LK-639 (Fig. 3). The inhibition induced by 300 ng/ml of pertussis toxin was 95.6±0.6% for LK-732 and 93.4±2.5% for LK-639. Similarly, compound 48/80 (1 μg/ml)-induced histamine release was reduced by pre-treatment of mast cells with pertussis toxin. In contrast, pertussis toxin at concentrations up to 300 ng/ml failed to inhibit histamine release induced by 200 μM of LK-6063, suggesting that LK-6063 induces histamine release through mechanism distinct from that established for basic secretagogues.

3.4. Effect of neuraminidase on direct thrombin inhibitors-induced histamine release

In order to determine whether sialic acid residues on mast cell surface are the initial binding site for basic thrombin inhibitors, we studied the effect of neuraminidase, which removes sialic acid residues from the plasma membrane (Mousli et al., 1989), on basic thrombin inhibitors-induced histamine release. Pre-treatment of mast cells for 1 h with neuraminidase (2 U/ml) inhibited histamine release induced by LK-732 (200 μM) and LK-639 (200 μM) as well as by compound 48/80 (1 μg/ml) (Fig. 4). The inhibition was 66.5±4.2%, 60.3±2.3% and 74.0±4.0% for LK-732, LK-639 and compound 48/80, respectively. In contrast, pre-treatment with neuraminidase had no effect on histamine secretion induced by 200 μM of LK-6063. These results suggest that thrombin inhibitors LK-732 and LK-639, but not LK-6063, interact with sialic acid residues on mast cell surface and then cross the membrane and activate G_i proteins.

3.5. Effect of direct thrombin inhibitors on rate of GTPγS binding

The activation of histamine secretion from mast cells by LK-732 and LK-639 appeared to be similar to that of synthetic polyamine compound 48/80 and to various polycationic

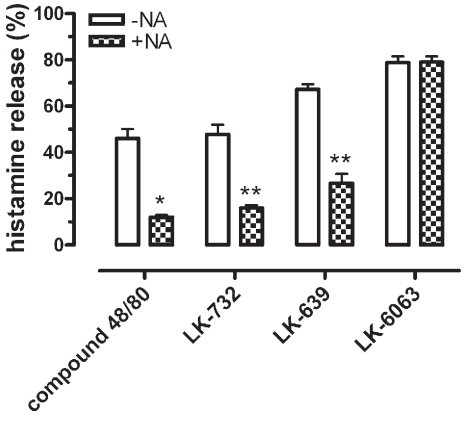


Fig. 4. Effect of neuraminidase on histamine release induced by LK direct thrombin inhibitors built on the azaphenylalanine scaffold. Cells were pre-incubated in the presence (+NA) or absence (−NA) of neuraminidase (2 U/ml) for 1 h at 37 °C before the addition of LK-732 (200 μM), LK-639 (200 μM), LK-6063 (200 μM) or compound 48/80 (1 μg/ml) to the medium for 15 min. Results are given as mean±S.E.M. of four experiments. *t*-test, $^{*}P<0.05$, $^{**}P<0.01$.

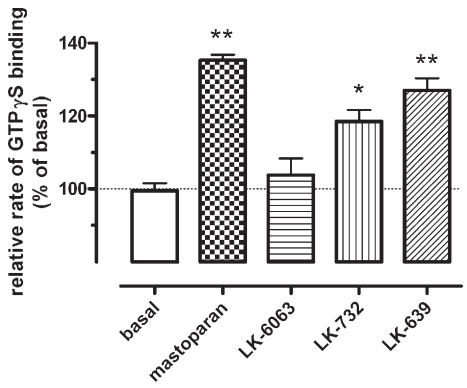


Fig. 5. Effect of LK direct thrombin inhibitors built on the azaphenylalanine scaffold on the rate of [^{35}S]GTPγS binding to G proteins from rat brain cortical membranes. Effects of LK-732 (200 μM), LK-639 (200 μM) and LK-6063 (200 μM) on the GTPγS binding were compared with the effect of mastoparan (100 μM). Basal rate of GTPγS binding (100%, dashed line) was 37 fmol/min/mg protein. Results are given as mean±S.E.M. of four experiments. ANOVA, *P<0.05, **P<0.01.

peptides including mastoparan that act directly on G proteins (Mousli et al., 1990a,b,c). Therefore, we tested the effect of thrombin inhibitors that provoke release of histamine on the rate of GTPγS binding to G proteins in rat brain cortical membranes. As shown in Fig. 5, the rate of GTPγS binding to G proteins was enhanced by mastoparan (100 μM), which is in accordance with previous findings (Shin et al., 1994), as well as by LK-732 (200 μM) and LK-639 (200 μM). On the other hand, LK-6063 (200 μM) did not have significant effect on the rate of GTPγS binding to G proteins.

4. Discussion

We have previously reported that basic LK direct thrombin inhibitors built on the azaphenylalanine scaffold cause reduction of tracheal air flow and a fall of mean arterial pressure in anaesthetized rats. This undesired effect of LK direct thrombin inhibitors correlated with their ability to release histamine from rat peritoneal mast cells and was diminished by reducing of their basicity (Peternel et al., 2006). In the present study, we demonstrated that LK compounds with basic character release histamine from mast cells by direct activation of G_i protein, with a mechanism similar to that reported for basic peptides and various basic amines such as compound 48/80 (Mousli et al., 1990a,b,c).

Our results demonstrated that direct thrombin inhibitors LK-732, LK-639 and LK-6063, which all have attached basic group to the aromatic ring (Table 1), provoked release of histamine from rat peritoneal mast cells in a concentration-dependent manner. In contrast, less basic direct thrombin inhibitors LK-658, LK-633 and LK-6062 at the same concentrations did not elicit histamine release. These results support our previous findings indicated that histamine release induced by direct thrombin inhibitors built on the azaphenylalanine scaffold could be diminished by reducing the basicity of terminally located basic groups of the compound (Peternel et al., 2006). The concentrations of direct thrombin inhibitors that provoked release of histamine from rat peritoneal mast cells were higher in comparison to that reported for their antithrombotic effect (Peternel et al., 2005, 2006). Moreover, our previous results demonstrated a weak correlation between the concentration of LK direct thrombin inhibitors required for the inhibition of the enzymatic activity of thrombin and their histamine release activity (Peternel et al., 2006). Therefore, the effect of direct thrombin inhibitors built on the azaphenylalanine scaffold on histamine release could be avoided without the loss of their desired interaction at the thrombin active site. In addition, we found that although all LK direct thrombin inhibitors with basic character released histamine from mast cells at the similar concentration range, only secretion induced by LK-732 and LK-639 was temperature-dependent and was completely blocked at low temperature (4 °C). In contrast, LK-6063 still induced significant histamine release at 4 °C indicating its action also through cytotoxic mechanism.

Sensitivity to pertussis toxin, an inhibitor of G_i protein activity, and to neuraminidase, which hydrolyzes the sialic acid residues from the plasma membrane, is the most used criteria to characterize basic secretagogue-induced serosal mast cell activation (Ferry et al., 2002). We found that pre-treatment of mast cells with pertussis toxin blocked release of histamine induced by LK-732, LK-639 and compound 48/80, but did not influence the release induced by LK-6063. Moreover, treatment of mast cells by neuraminidase suppressed release of histamine induced by LK-732, LK-639 and compound 48/80, whereas respond to LK-6063 remained unchanged. These results demonstrate that direct thrombin inhibitors LK-732 and LK-639 interact with sialic acid residue on mast cell membrane, followed by the activation of pertussis toxin-sensitive G_i protein leading to histamine release. Additional demonstration that G proteins are direct target of LK-732 and LK-639 was provided by determination of GTPγS binding rate in rat brain cortical membranes. LK-732 and LK-639 enhanced the rate of GTPγS binding to G proteins, whereas LK-6063 was without significant effect.

Taken together, our findings suggest that basic direct thrombin inhibitors LK-732 and LK-639 provoke release of histamine from mast cells by direct activation of G_i proteins through the similar biochemical pathway as basic secretagogues. On the other hand, LK-6063-induced histamine release is partially mediated through a cytotoxic action. In addition, we have shown that less basic direct thrombin inhibitors (LK-658, LK-633 and LK-6062) have no histamine release activity. The mechanism of action of basic secretagogues described in rat peritoneal mast cells has also been demonstrated in human skin mast cells (Church et al., 1991; Columbo et al., 1996; Emadi-Khiav et al., 1995; Mousli et al., 1994). Therefore, we assume that the reactivity of human mast cells to LK direct thrombin inhibitors may be similar to that with rat peritoneal mast cells.

From our study, we can conclude that histamine release induced by basic LK direct thrombin inhibitors as well as by other drugs with basic character may be an important undesired effect that should be taken into consideration in the development of new drugs. Moreover, we suggest that studies determining histamine release activity of drugs with basic character could be included in the high throughput toxicological screening assays.

Acknowledgement

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References

Agnelli, G., Sonaglia, F., 2002. Perspectives on antithrombotic agents: from unfractionated heparin to new antithrombotics. Haematologica 87, 757–770.

Ammar, H.O., Ghorab, M., el-Nahhas, S.A., Makram, T.S., 1997. Improvement of the biological performance of oral anticoagulant drugs: 1. Warfarin. Pharmazie 52, 627–631.

Aridor, M., Traub, L.M., Sagi-Eisenberg, R., 1990. Exocytosis in mast cells by basic secretagogues: evidence for direct activation of GTP-binding proteins. J. Cell Biol. 111, 909–917.

Bavec, A., Jureus, A., Cigic, B., Langel, Ü., Zorko, M., 1999. Peptitergent PD1 affects the GTPase activity of rat brain cortical membranes. Peptides 20, 177–184.

Boos, C.J., Hinton, A., Lip, G.Y., 2005. Ximelagatran: a clinical perspective. Eur. J. Intern. Med. 16, 267–278.

Church, M.K., Okayama, Y., el-Lati, S., 1991. Mediator secretion from human skin mast cells provoked by immunological and non-immunological stimulation. Skin. Pharmacol. 4 (Suppl 1), 15–24.

Columbo, M., Horowitz, E.M., Kagey-Sobotka, A., Lichtenstein, L.M., 1996. Substance P activates the release of histamine from human skin mast cells through a pertussis toxin-sensitive and protein kinase C-dependent mechanism. Clin. Immunol. Immunopathol. 81, 68–73.

Emadi-Khiav, B., Mousli, M., Bronner, C., Landry, Y., 1995. Human and rat cutaneous mast cells: involvement of a G protein in the response to peptidergic stimuli. Eur. J. Pharmacol. 272, 97–102.

Ferry, X., Brehin, S., Kamel, R., Landry, Y., 2002. G protein-dependent activation of mast cell by peptides and basic secretagogues. Peptides 23, 1507–1515.

Higashijima, T., Uzu, S., Nakajima, T., Ross, E.M., 1988. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). J. Biol. Chem. 263, 6491–6494.

Hirsh, J., Weitz, J.I., 1999. New antithrombotic agents. Lancet 353, 1431–1436.

Knudsen, T., Ferjan, I., Johansen, T., 1993. Effect of ouabain, digoxin and digitoxigenin on potassium uptake and histamine release from rat peritoneal mast cells. FEBS Lett. 321, 127–131.

Linkins, L.A., Weitz, J.I., 2005. Pharmacology and clinical potential of direct thrombin inhibitors. Curr. Pharm. Des. 11, 3877–3884.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

McKenzie, F.R., 1992. Basic techniques to study G-protein function. In: Milligan, G. (Ed.), Signal transduction. A practical approach. Oxford University Press, Oxford.

Metcalfe, D.D., Baram, D., Mekori, Y.A., 1997. Mast cells. Physiol. Rev. 77, 1033–1079.

Mousli, M., Bronner, C., Bueb, J.L., Tschirhart, E., Gies, J.P., Landry, Y., 1989. Activation of rat peritoneal mast cells by substance P and mastoparan. J. Pharmacol. Exp. Ther. 250, 329–335.

Mousli, M., Bronner, C., Bockaert, J., Rouot, B., Landry, Y., 1990a. Interaction of substance P, compound 48/80 and mastoparan with the alpha-subunit C-terminus of G protein. Immunol. Lett. 25, 355–357.

Mousli, M., Bronner, C., Landry, Y., Bockaert, J., Rouot, B., 1990b. Direct activation of GTP-binding regulatory proteins (G-proteins) by substance P and compound 48/80. FEBS Lett. 59, 260–262.

Mousli, M., Bueb, J.L., Bronner, C., Rouot, B., Landry, Y., 1990c. G protein activation: a receptor-independent mode of action for cationic amphiphilic neuropeptides and venom peptides. Trends Pharmacol. Sci. 11, 358–362.

Mousli, M., Hugli, T.E., Landry, Y., Bronner, C., 1994. Peptidergic pathway in human skin and rat peritoneal mast cell activation. Immunopharmacology 27, 1–11.

Nakamura, T., Ui, M., 1985. Simultaneous inhibitions of inositol phospholipid breakdown, arachidonic acid release, and histamine secretion in mast cells by islet-activating protein, pertussis toxin. A possible involvement of the toxin-specific substrate in the Ca2+-mobilizing receptor-mediated biosignaling system. J. Biol. Chem. 260, 3584–3593.

Nutescu, E.A., Shapiro, N.L., Chevalier, A., 2006. New anticoagulant agents: direct thrombin inhibitors. Clin. Geriatr. Med. 22, 33–56.

Obreza, A., Stegnar, M., Trampus-Bakija, A., Prezelj, A., Urleb, U., 2004. Synthesis and in vitro evaluation of new azaphenylalanine derivatives as serine protease inhibitors. Pharmazie 59, 739–743.

Peternel, L., Stegnar, M., Drevensek, G., Budihna, M.V., Bozic, M., Zega, A., Stalc, A., Cerne, M., Urleb, U., 2005. Antithrombotic potential of new direct thrombin inhibitors built on the azaphenylalanine scaffold in two rat venous thrombosis models. Thromb. Haemost. 93, 437–442.

Peternel, L., Stempelj, M., Cerne, M., Zega, A., Obreza, A., Oblak, M., Drevensek, G., Budihna, M.V., Stanovnik, L., Urleb, U., 2006. Direct thrombin inhibitors built on the azaphenylalanine scaffold provoke degranulation of mast cells. Thromb. Haemost. 95, 294–300.

Pfau, R., 2003. Structure-based design of thrombin inhibitors. Curr. Opin. Drug. Discov. Devel. 6, 437–450.

Saito, H., Okajima, F., Molski, T.F., Sha'afi, R.I., Ui, M., Ishizaka, T., 1987. Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and-independent histamine release from mast cells and basophils. J. Immunol. 138, 3927–3934.

Serebruany, M.V., Malinin, A.I., Serebruany, V.L., 2006. Argatroban, a direct thrombin inhibitor for heparin-induced thrombocytopaenia: present and future perspectives. Expert. Opin. Pharmacother. 7, 81–89.

Shin, Y., Moni, R.W., Lueders, J.E., Daly, J.W., 1994. Effects of the amphiphilic peptides mastoparan and adenoregulin on receptor binding, G proteins, phosphoinositide breakdown, cyclic AMP generation, and calcium influx. Cell. Mol. Neurobiol. 14, 133–157.

Shore, P.A., Burkhalter, A., Cohn Jr., V.H., 1959. A method for the fluorometric assay of histamine in tissues. J. Pharmacol. Exp. Ther. 127, 182–186.

Stempelj, M., Ferjan, I., 2005. Signaling pathway in nerve growth factor induced histamine release from rat mast cells. Inflamm. Res. 54, 344–349.

Zega, A., Mlinsek, G., Solmajer, T., Trampus-Bakija, A., Stegnar, M., Urleb, U., 2004. Thrombin inhibitors built on an azaphenylalanine scaffold. Bioorg. Med. Chem. Lett. 14, 1563–1567.