Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;27:139-148

Accepted: December 12, 2010

Regulation of Serotonin Transport in Human Platelets by Tyrosine Kinase Syk

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Key Words
Human platelets • Serotonin transport • Syk tyrosine kinase

Abstract
Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter involved in the regulation of numerous neuro-physiological processes. The circulating level of 5-HT is regulated by the membrane transporter SERT present both in the presynaptic nerve terminals and blood platelets. 5-HT transport is a process tightly regulated by a variety of factors including protein phosphorylation. Aim of this study was to ascertain if also the SERT Tyr-phosphorylation mediated by Syk-kinase concurs to the regulation of SERT activity. Indeed we found that 5-HT uptake decreased upon platelet exposure to piceatannol or Syk-inhibitor II, two structurally unrelated inhibitors of the tyrosine-kinase Syk. Tyr-phosphorylation of anti-SERT-immuno-stained proteins in membrane extracts and in anti-SERT-immuno-precipitates, decreased upon platelet treatment with piceatannol, in parallel with a reduction of Syk-activity. Syk was immuno-revealed in the anti-SERT immuno-precipitates, which displayed a piceatannol-sensitive kinase activity towards SERT itself and the Syk-substrate α-sinuclein. Syk inhibitors also caused a decrease of the monensin-induced 5-HT-efflux from platelets and of imipramine binding to them. It is concluded that, in addition to the phosphorylation of SERT mediated by various other kinases, also that catalyzed by Syk might play an important role in the 5-HT transport, likely favoring the transporter conformation exposing the neurotransmitter binding sites.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter that plays an important role in the regulation of a variety of neuro-physiological processes and neuro-pathological disorders including cellular signaling, mood, anger, depression, and hypertension [1-5]. Serotonin exerts its effects through a variety of membrane receptors present both in the central and peripheral nervous systems, and in several non-neuronal tissues. Serotonin is synthesized in the central nervous

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system (CNS) and in the gut enterochromaffin cells. Both 5-HT transport from blood stream into the platelets, and the reuptake, following its release, at the synaptic level is mediated by a specific transporter SERT that displays similar structural and functional properties [6-8]. Serotonin is transported from the platelet cytosol into the dense granules by the vesicular monoamine transporter VMAT2, that is inhibited by reserpine [9].

SERT-mediated transport is a tightly regulated process, that depends on the glycosylated and phosphorylated state of the transporter, which exerts a significant impact on the duration and concentration of monoamines present in the synaptic cleft [10-13].

SERT is constituted by 12-transmembrane domains (TM) with five loops and the NH_2- and COOH-termini in the cytoplasm, [14-16], and a large extracellular loop situated between the transmembrane domains TM3 and TM4 and containing glycosylation sites [2, 17].

It is well known that serotonin is released from platelets mainly through exocytosis of the dense granules, a process associated to the cellular activation. However 5-HT efflux from platelets, as well as from SERT-expressing cells, can be also obtained by alkalinisation of the dense granules with permeable amine or inhibitors of vacuolar H^+-ATPase; by amphetamine-like substances [18, 19], or by manipulating the cytosolic concentration of Na^+, K^+ and Ca^{2+} [20-23]. The present study is part of a research project directed to explore further possible factors that regulate the platelet 5-HT transport. Our results demonstrate that in reserpine-treated platelets both serotonin uptake, and its release induced by the Na^+ ionophore monensin, correlate with Syk-mediated tyrosine phosphorylation of SERT. Syk tyrosine kinase is a non receptor protein kinase mostly expressed in hematopoietic cells and involved in a variety of cellular functions, i.e. signaling, tumor progression, autoimmune response and inflammation [24-26]. Syk has been implicated in both platelet outside-in and inside-out signaling cascades, including collagen- and thrombin-induced activation, dense granule secretion and activated integrin αIIbβ3-dependent functions [27-31]. Syk activity is correlated to its phosphorylation at specific tyrosine residues [24, 32].

Materials and Methods

Reagents

Apyrase, prostacyclin, and reserpine, were purchased from Sigma Chemicals (St. Louis, MO); [^{14}C]serotonin and [^{3}H]imipramine, were from GE Healthcare (Buckinghamshire, U.K.), and [γ^{32}P]ATP from PerkinElmer Radiochemicals (San Jose, CA); whereas fura 2/AM, ionomycin, SU6656, (trans-3,3',4,5'-tetra-hydroxy-stilbene), PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazol-[3,4-d]pyrimidine, piceatannol, and Syk-inhibitor II were from Calbiochem (Darmstadt, Germany). The protease inhibitor cocktail was from Roche Diagnostic (Mannheim, Germany); goat serotonin transporter antibody ST(C-20):sc-1458, anti-Syk and protein G Plus-Agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-phospho-Syk (Y525/526) was from Cell Signaling Technology (Danvers, MA), anti-SERT antibody AB1594P from Chemicon International Inc. (Temecula, CA), and mouse anti-phosphotyrosine monoclonal antibody PY20 from ICN Biotechnology (Irvine, CA). All other reagents were of analytical grade.

Preparation of platelet suspensions

Blood samples were collected from healthy volunteers, who had taken no medication during the preceding 2 weeks, with their informed consent and in accordance with the Helsinki declaration. Blood was immediately mixed with one-tenth volume of citric-anticoagulant ACD (75 mM sodium citrate, 40 mM citric acid, 130 mM dextrose, pH 6.0), supplemented with 20 mU/ml apyrase and 0.8 μg/ml prostacyclin, and centrifuged for 20 min at 200 *g*. In order to eliminate the possible residual contaminating red blood cells the supernatant platelet-rich plasma (PRP) was supplemented with further 5% ACD and centrifuged again for 5 min at 200 *g*. The new PRP was further centrifuged for 20 min at 750 *g*, and the spun down platelets were re-suspended, unless otherwise indicated, at $2x10^8$ cells/ml of basal buffer consisting of (mM): 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose, and 15 Tris/HEPES, (pH 7.4) [33].

Serotonin transport in human platelets

Serotonin uptake by platelets was determined as described elsewhere [33]; briefly: 0.2 μM [^{14}C]5-HT was generally added to platelet suspensions ($2x10^8$ cells/ml), the incubations were stopped by mixing the samples with equal volumes of ice-cold glutaraldehyde solution (3% vol/vol). Samples were filtered through Millipore HAWD 013 filters, which were washed twice with the glutaraldehyde solution and counted in a scintillation beta counter. Experiments were carried out in triplicate and the net uptake was calculated as difference between the amount of [^{14}C]5-HT incorporated by platelets in the absence and presence of the SERT inhibitor imipramine [9]. Serotonin efflux was monitored with the same procedure adopted for uptake using platelets pre-loaded with [^{14}C]5-HT [23].

Determination of cytosolic Ca^{2+} and Na^+ concentration

Cytosolic [Ca^{2+}] and [Na^+] were determined by means of the fluorescent probe fura 2/AM and benzophurane isophtalate (SBFI) respectively, as previously reported [23]. Probe-loaded platelets were resuspended in the basal medium ($2x10^8$ cells/ml) and treated according to the experimental protocol. Fluorescence was measured in a thermostated, magnetically stirred quartz cuvette, using the excitation wavelengths of 340 and 345/385 nm and the emission wavelengths of 505 and 490 nm and for Ca^+ and Na^+ determination respectively.

Detection of Tyr-phosphorylated SERT in platelet membrane extracts and immuno-precipitates

Platelets were suspended in the basal medium at a count of $1.5x10^9$ cells/ml, and aliquots (1-2 ml) of the suspension were incubated according to the experimental protocol. In the first set of experiments the reactions were stopped by addition of one-tenth volume of lysis buffer consisting of (mM concentration): 500 NaCl, 100 KCl, 20 EDTA, 10 EGTA, 10 sodium vanadate, 50 sodium p-nitrophenyl-phosphate (pNPP), 200 Tris-HCl, pH 7.4, Triton-X100 (1% vol/vol), and supplemented with the protease inhibitor cocktail Complete (1 tablet/ml). All the following steps were performed at 4°C. After 15 min incubation the lysates were centrifuged at 100,000 *g* for 40 min. The pelletted membranes were resuspended in 100 µl of the lysis buffer diluted ten fold and supplemented with Triton-X100 (0.6 % vol/vol), SDS (0.4 % vol/vol), and sodium deoxycholate (0.4 % wt/vol) kept for 1 h under stirring and then ultra-centrifuged for 1 h at 100,000 *g*. Aliquots of the extracted protein solution (20-40 µg proteins, determined according to Bradford [34], were subjected to SDS/PAGE (10% gels), the separated proteins were immediately electrophoretically transferred to nitrocellulose membranes, treated with anti-phosphotyrosine (PY20 from ICN Biotechnology), or anti-SERT antibody, (either ST(C-20):sc-1458, from Santa Cruz Biotechnology, or AB1594P from Chemicon International), followed by secondary peroxidase-conjugate antibody and detected by the enhanced chemi-luminescence technique (ECL, Amersham Pharmacia Biotech, S. Francisco, CA) [35].

SERT immuno-precipitates (IPs) were obtained from the membrane extracts (120-160 µg protein), diluted with three volumes of buffer devoid of detergents, and pre-cleared by treatment with the protein G Plus-Agarose (30 µl/ml incubated for 60 min, followed by 10 min centrifugation at 3000 *g*). Anti-SERT antibody (5 µg/ml) was then added to the suspensions and incubated (2-3 h) under mild mixing. IPs obtained after 10 min centrifugation at 3000 *g*, were washed three times and solubilised in Laemmli buffer, subjected to SDS-PAGE and immuno-blotted with either anti-SERT or anti-P-Tyr antibodies. Occasionally the anti-phosphotyrosine immuno-blots were stripped and reprobed with anti-SERT antibody.

Detection of Syk activity displayed by anti-SERT- and anti-Syk-IPs

Anti-SERT immuno-complexes were obtained from membrane extracts as above described, while anti-Syk IPs were derived from cell lysates. The phosphorylation activity displayed by anti-SERT- and anti-Syk-IPs was measured by suspending the immuno-complexes in 30 µl of phosphorylation medium containing 50 mM Tris/HCl, (pH 7.5), 6 mM $MnCl_2$, 5 mM $MgCl_2$, 10 µM [γ^{32}P]ATP (specific activity 3000 dpm/pmol), 100 µM sodium orthovanadate. Following 5 min incubation at 30°C, samples were subjected to SDS/PAGE (9% gel) and the ^{32}P-incorporated in the protein bands was evaluated using the Cyclone Plus Storage Phosphor System (PerkinElmer, Waltham, MA), which created a digitized image file that was analyzed by the optiQuantTM Image Analysis software, and the radioactivity incorporated in the protein was expressed in Digital Light Units (DLU). In parallel experiments the phosphorylation of the Syk-substrate α-synuclein by SERT-IPs was performed by adding 0.5 µM α-synuclein to the phosphorylation medium. Samples were subjected to 15% SDS/PAGE, stained by Coomassie-brilliant blue, and the ^{32}P-radioactivity incorporated in the protein was determined as above described.

Analysis of Sky autophosphorylation

Anti-Syk IPs, obtained from platelet lysates, were subjected to SDS/PAGE (9 % gel), blotted and immuno-stained with the site-specific anti-phospho-Syk(Tyr525/526) antibodies. Blots were then stripped and reprobed with anti-Syk antibodies.

Measurement of imipramine binding

Imipramine binding to platelets was essentially determined as previously reported [35]: platelet suspensions ($2x10^8$ cells/ml), prepared as above described, were incubated at 37°C with 5 nM [^{3}H]imipramine (21 µCi/mmol) in the presence (unspecific binding) or absence of 200 nM fluoxetine. At prefixed times the platelets were filtered on Millipore apparatus through Whatman GF/C filters and washed three times with 5 ml of the basal buffer supplemented with 0.5 mg/ml bovine serum albumin. The filters were then counted in a scintillation beta counter.

Measurement of ATP secretion and acridine orange accumulation in human platelets

ATP release was monitored by means of luciferin/luciferase, whereas acridine orange accumulation was fluorimetrically measured as previously described [33].

Statistical analysis

Analysis of statistical significance of the differences was performed by the non parametric Mann-Whitney U-test.

Results

Platelet pre-incubation with either piceatannol or Syk-inhibitor II, two structurally unrelated, specific inhibitors of the tyrosine kinase Syk [36, 37], brought about a decrease of the serotonin (5-HT) transport in human platelets (Figs. 1A and 1B). The inhibitor concentrations (35 µM piceatannol and 10 µM Syk-inhibitor II) were chosen on the basis of preliminary experiments showing that these were the lowest inhibitor concentrations that gave an almost maximal inhibition on the 5-HT transport (not shown).

In order to understand the action mechanism of the Syk inhibitors we first of all verified whether they induced by themselves either exocytosis of the dense granules, monitored as ATP secretion, or decrease of cellular accumulation of acridine orange, a basic fluorescent probe that, similarly to 5-HT, accumulates into the dense granules driven by the acid intra-granular pH [38]. The negative

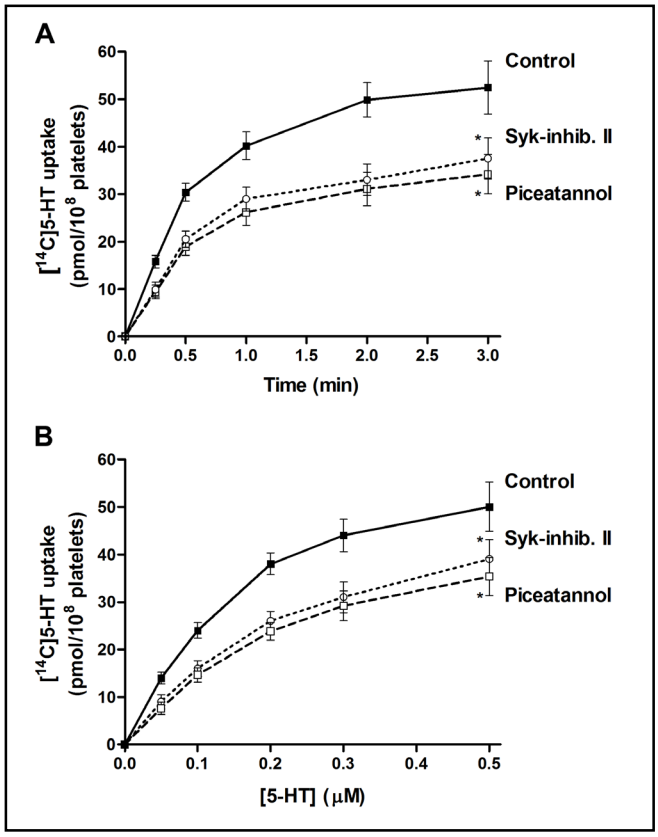

Fig. 1. Effect of the Syk-kinase inhibitors piceatannol and Syk-inhibitor II on serotonin ([^{14}C]5-HT) uptake in human platelets. (A) Time-course of the [^{14}C]5-HT (0.2 μM) accumulation. (B) Concentration dependent serotonin uptake (1 min); 35 μM piceatannol or 10 μM Syk-inhibitor II or vehicle (control) were pre-incubated for 30 min prior to addition of [^{14}C]5-HT. (A and B): values are means of 6 experiments performed in triplicate, with ± S.D. indicated by vertical bars; * statistically significance of differences p< 0.01 *vs* control.

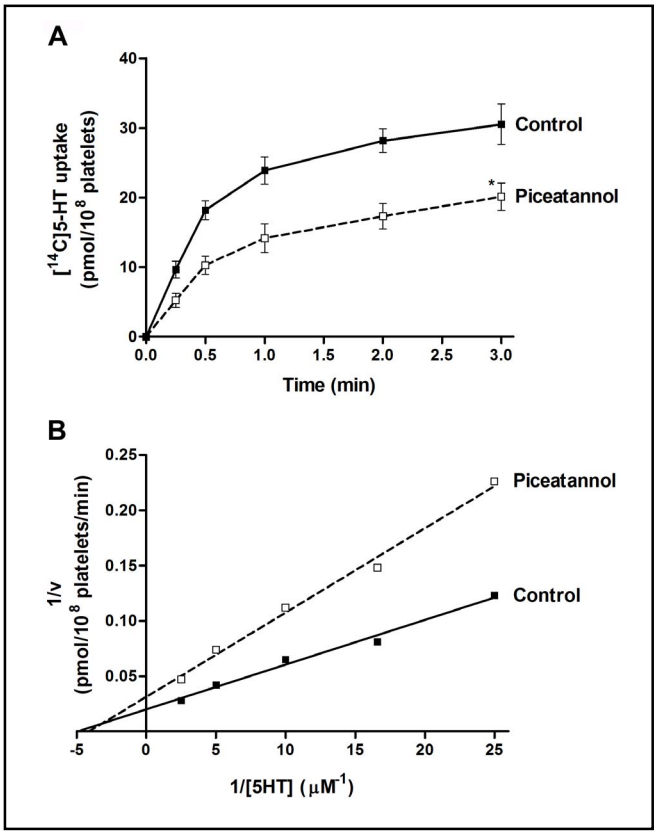

Fig. 2. Serotonin uptake in reserpine pre-treated platelets. (A) Time course of [^{14}C]5-HT (0.2 μM) uptake by platelets preincubated with reserpine (20 μM, 10 min), in the absence or presence of 35 μM piceatannol or vehicle (control) (30 min). (B) double reciprocal plots of [^{14}C]5-HT transport (at 1 min) versus its concentration. Values are means of six experiments performed in triplicate; *statistically significant difference p< 0.01 *vs* control.

results obtained (not shown) indicated that the tyrosine-kinase inhibitors did not affect the "driving force" of 5-HT accumulation into the dense granules. We then analyzed whether piceatannol reduced the 5-HT transport also in platelets pre-treated with reserpine, an inhibitor of the serotonin carrier of the dense granule membranes VMAT2, thus limiting the serotonin accumulation to the cytosolic compartment [39]. The amount of serotonin taken up by reserpine-treated platelets was, as expected, less than that taken up by untreated platelets, and the inhibitory effect displayed also in these conditions by piceatannol on the 5-HT transport indicated that the kinase inhibitor acted at the level of the plasmatic membrane SERT (Fig. 2A). The double reciprocal plots of the 5-HT amount taken up versus 5-HT concentration demonstrate that, in the experimental conditions adopted, piceatannol decreased by about 40% the V_{max} of the serotonin transport (from 54 to 35 pmol/10^{8}platelets/min), without significantly modifying its affinity towards the platelet transporter. In fact the calculated *Km* resulted to be 0.22 μM and 0.24 μM in the absence and presence of piceatannol respectively, indicating that the kinase inhibitor displayed an inhibitory effect of a non-competitive type (Fig. 2B).

These results prompted us to examine whether the above effects displayed by the Tyr-kinase-inhibitors on 5-HT transport were associated to changes in the Tyr-phosphorylation of SERT. To this purpose the immuno-detection of SERT in platelet membrane extracts was first of all performed. Membrane extracts, obtained as

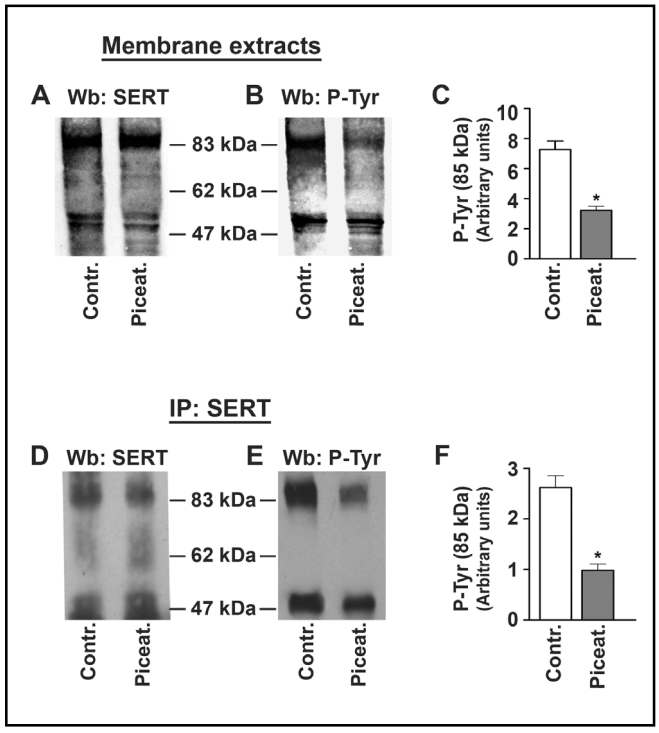

Fig. 3. Immuno-detection of SERT and Tyr-phosphorylated proteins in platelet membrane extracts and anti-SERT immuno-precipitates (IPs). Membrane extracts obtained from platelets pre-treated for 30 min with: vehicle (control) or 35 μM piceatannol (piceat.) were subjected to SDS/PAGE, blotted and immuno-stained with anti-SERT (A) and anti-P-Tyr (B) antibodies. Densitometric determination of the Tyr-phosphorylation of the 85 kDa band (C). Anti-SERT IPs obtained from platelet membrane extracts were blotted and immuno-stained with anti-SERT (D) and anti-P-Tyr (E) antibodies as the membrane fractions. The degree of Tyr phosphorylation of the 85 kDa was densitometrically determined by the Gel-Pro Analyzer version 3.1, designed by Media Cybernetics Inc., (Bethesda, MD), and normalized for the amount of immuno-precipitated SERT (F). Blots are representative of four experiments performed with different platelet specimens. Statistically significant differences *$p < 0.005$ *vs* control.

described in the "Materials and methods" section, were subjected to SDS-PAGE, transblotted and immuno-stained with anti-SERT and anti-P-Tyr antibodies. A major band displaying an apparent molecular mass of about 85 kDa, together with few minor protein bands of lower molecular mass, emerged in the nitrocellulose membranes exposed to anti-SERT antibody (Fig. 3A). Since the predicted molecular mass of SERT is 72 kDa [7], the finding of an abundant anti-SERT protein band of about 85 kDa suggested that the transporter was likely present in its glycosylated form [17, 40]. On the other hand the anti-SERT bands displaying molecular masses lower than the expected 72 kDa indicated that the serotonin transporter likely underwent a proteolytic degradation during the experimental procedure, even in the presence of a protease inhibitor cocktail [41].

Even if the accurate analysis of the results was rather difficult it appeared that platelet pre-treatment with the Syk-inhibitor piceatannol reduced the Tyr-phosphorylation of the protein bands corresponding to SERT (Fig. 3B). The densitometric analysis of anti-P-Tyr immuno-stained 85 kDa band confirmed this conclusion (Fig. 3C).

We then analysed the degree of Tyr-phosphorylation of the anti-SERT-immuno-precipitates obtained from membrane extracts. The anti-SERT bands of IPs obtained from platelets pre-treated with piceatannol resulted less Tyr-phosphorylated than those obtained from vehicle-treated platelets (Figs. 3D, 3E and 3F). Similar results were also obtained in reserpine-treated platelets (not shown).

The co-immuno-precipitation of specific enzymes with their substrate-antibodies is a widely used technical approach [42]. So in order to confirm that SERT was a potential substrate of Syk we first of all verified if a piceatannol-sensitive Tyr-kinase coimmuno-precipitated with the anti-SERT antibody. To this purpose the anti-SERT-IPs were incubated in a phosphorylating medium containing [γ^{32}P]ATP, and the radioactive phosphate incorporated in the protein band corresponding to SERT, was measured (Figs. 4A-a, 4A-b). In the adopted experimental conditions SERT resulted indeed phosphorylated, and its phosphorylation was reduced in the presence of piceatannol. Moreover the presence of Syk in the anti-SERT-IPs, was revealed by both their anti-Syk immuno-staining (Fig. 4A-c), and the piceatannol-sensitive capability of SERT-IPs to *in vitro* phosphorylate the Syk-substrate α-synuclein [43], (Fig. 4A-d).

We then analysed the activation state of Syk in control and piceatannol-treated platelets. Since Syk activation is associated with its autophosphorylation at Tyr525/526, located in the activation loop [24, 32], we determined the phosphorylation level of these two specific residues. The kinase was immuno-precipitated from cellular lysates and immuno-stained with anti-phospho-Syk (Y525/526) antibodies. As expected, the extent of Syk autophosphorylation was reduced in cells treated with the inhibitor in comparison to that of control platelets, while the amount of total Syk was similar (Fig. 4B-a). Interestingly, the inhibitory effect of piceatannol persisted also in the Syk-immuno-complexes obtained from

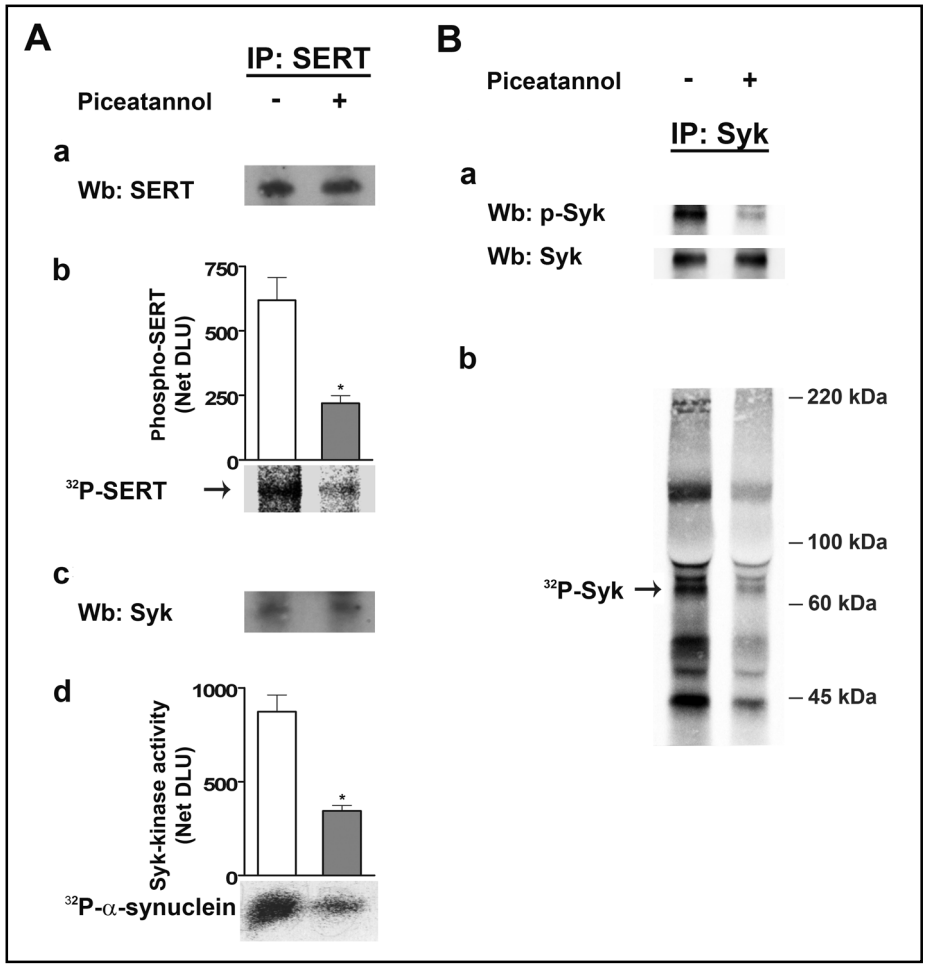

Fig. 4. Co-immuno-precipitation of Syk with SERT and their phosphorylation. (A) Anti-SERT IPs, obtained from platelet membrane extracts, were incubated with [γ^{32}P]ATP, in a phosphorylating medium, as detailed in Materials and methods, in the absence or presence of 10 μM piceatannol. To better highlight the SERT band, the samples were solubilised in Laemmli buffer devoid of β-mercaptoethanol, subjected to SDS/PAGE and immuno-stained with anti-SERT and anti-Syk antibodies (A-a and A-c, respectively). ^{32}P-radioactivity incorporated in the SERT-band was determined by Cyclone Plus Storage Phosphor System and the optiQuantTM Image Analysis software (A-b). In parallel experiments, the phosphorylation of the Syk-substrate α-synuclein by SERT-IPs was performed as described in Materials and methods (A-d). The radioactivity incorporated in the proteins is expressed in Digital Light Units (DLU) (± S.E.M.). (B) Anti-Syk IPs obtained from the lysates of control and piceatannol-treated platelets were immuno-stained with the site-specific phospho-Syk antibodies anti-pTyr525/526. Blots were then stripped and reprobed with anti-Syk antibodies (B-a). In parallel experiments anti-Syk IPs were incubated in a phosphorylating medium containing [γ^{32}P]ATP and the ^{32}P-radioactivity incorporated in the protein-bands was analysed as detailed in Materials and methods (B-b). Figures are representative or means of three separate experiments. *p< 0.001 *vs* control.

piceatannol-treated platelets. In fact their kinase activity, measured *in vitro* by incubating the Syk-immuno-complexes in a phosphorylating medium containing [γ^{32}P]ATP, was strongly reduced by piceatannol pre-treatment not only towards the Syk-itself but also on various other proteins co-immuno-precipitated with Syk (Fig. 4B-b).

It has been reported that imipramine inhibits the serotonin uptake by binding to platelet membrane SERT, and its binding has been correlated with the magnitude of 5-HT transport [35, 44, 45]. We therefore analysed the effect of the kinase-inhibitors piceatannol and Syk-inhibitor II on [^{3}H]imipramine binding to platelets and found that the kinase inhibitors decreased significantly (about 40%) this process (Fig. 5).

We have then investigated whether the Syk-kinase inhibitors affected the efflux of [^{14}C]5-HT from reserpine-treated platelets preloaded with radioactive serotonin.

The efflux was induced by increasing the cytosolic [Na^+] by addition of the Na^+/H^+ exchanger monensin [20, 21, 23]. Under the experimental conditions adopted, both Syk-kinase inhibitors piceatannol and Syk-inhibitor II, caused a significant decrease of 5-HT efflux (Fig. 6) without modifying the cytosolic [Na^+] (not shown).

Discussion

Platelets are not capable of synthesizing serotonin but they can accumulate the neurotransmitter from blood.

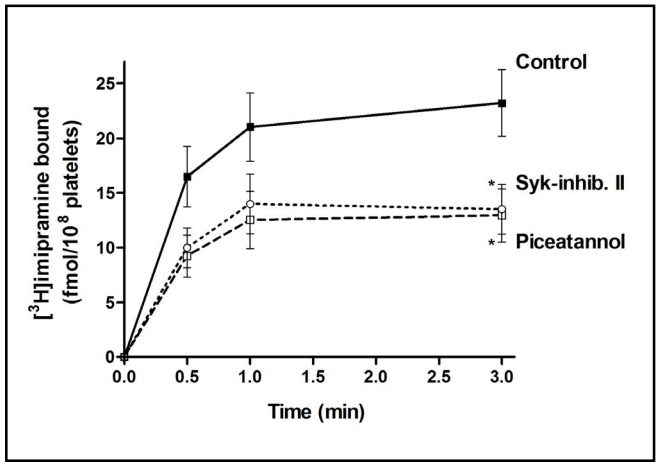

Fig. 5. Imipramine binding to platelets. Time-course of imipramine (5 nM) binding to human platelets preincubated for 30 min with vehicle (control), or 35 μM piceatannol or 10 μM Syk-inhibitor II. Data are means of four determinations with ± S.D. indicated by vertical bars; *p< 0.005 *vs* control.

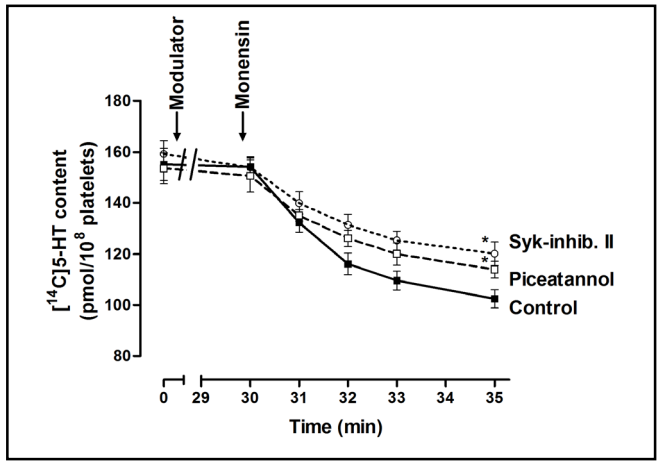

Fig. 6. Efflux of 5-HT from reserpine-treated platelets. Platelets loaded with [^{14}C]5-HT were then incubated for 30 min with: only vehicle (control), or 35 μM piceatannol or 10 μM Syk-inhibitor II. Monensin (0.5 μM) was added at the arrow. Values are means of four experiments performed in triplicate with S.D. indicated by vertical bars; *p< 0.01 *vs* controls.

Platelet 5-HT transporters display characteristics similar to those of neurones, therefore these particular cells constitute a suitable model for the study of serotonin transport [6-8, 40]. The plasma membrane SERT contains in the cytosolic domains multiple consensus sites for various protein kinases [7], and its PKC-dependent phosphorylation was shown to regulate the transport efficiency of amines, including 5-HT, in synaptosomes, human platelets, and in a variety of cells expressing SERT [10-12, 33, 46-48]. It has been also reported that the degree of SERT-phosphorylation can be increased by inhibition of the phosphatase PP2A, or activation of PKA, cyclic GMP-dependent protein kinases (PKG), and p38 mitogen-activated protein kinase (MAPK), and that these phosphorylative processes induce a rapid increase in SERT expression and/or function [13, 49, 50].

In the present study we show that the 5-HT uptake by human platelets also depends on the degree of SERT Tyr-phosphorylation mediated by the Syk-kinase. This conclusion is first of all based on the decreased serotonin uptake found in reserpine treated platelets pre-incubated with the selective Syk-kinase inhibitors piceatannol and Syk-inhibitor II, two structurally unrelated compounds [36, 37]. Accordingly the protein bands corresponding to anti-SERT-immuno-stained bands in membrane extracts and anti-SERT immuno-precipitates resulted less Tyr-phosphorylated when obtained from platelets pre-treated with piceatannol. Furthermore the presence of the Syk-kinase in the anti-SERT IPs, as revealed by both its immuno-staining and the piceatannol-sensitive *in vitro* kinase activity displayed by anti-SERT IPs towards the SERT itself and the Syk-substrate α-synuclein, is consistent with the conclusion that Syk is involved in the SERT Tyr-phosphorylation. In this context it is interesting to mention that it has been recently reported that α-synuclein interacts with SERT forming a heteromeric complex [51].

The SERT-immuno-staining performed on the membrane extracts and anti-SERT IPs revealed a major band displaying an apparent molecular mass (Mr) of about 85 kDa and few minor bands of lower Mr. The major band displaying a Mr higher than that predicted of 72 kDa is likely due to glycosylation of the transporter [2, 17]. Whereas the protein bands displaying Mr lower than 72 kDa most likely originated from a proteolytic process occurring during the SERT isolation procedure [41]. In fact we have found that SERT fragmentation increased by prolonging the precipitation time (from 2 h to overnight), while it decreased in the presence of high urea concentration (4 M) in the cellular disrupting medium (not shown).

The decreased SERT Tyr-phosphorylation in Syk-kinase-inhibitor treated platelets is associated to a diminution of platelet imipramine binding. It has been previously found a linear relationship between this process and 5-HT uptake [44, 45], and a competition between imipramine and 5-HT for the same SERT sites was proposed [39, 52]. Therefore the decrease of both

imipramine binding and serotonin transport, caused by Syk-inhibitors, is likely due to the fact that the Syk-mediated Tyr-phosphorylation is required for making SERT to assume an appropriate conformation exposing the external serotonin binding sites. In fact it has been demonstrated that SERT transports 5-HT into platelets, in symport with Na^+ and Cl^- and in exchange with a K^+ ion, by means of a mechanism based on the alternate access model in which various conformational changes are required for the exposition of substrate-binding sites. In this model the transporter functions by alternately exposing the substrate-binding sites to cytoplasmatic and extracellular faces of the plasma membrane [39, 53]. Experiments are in progress to determine the specific aminoacid target(s) of Syk and its (their) localization in the SERT primary structure. Interestingly in this context it has been recently demonstrated that calmodulin kinase II acts at the C terminus level of dopamine transporter [54], while the N terminus conformation is crucial for amphetamine-induced monoamine efflux [55].

It was previously found that serotonin accumulation in platelets was reduced by their treatment with broad-spectrum Tyr-kinase inhibitors [56], and that a decreased 5-HT transport was parallel to a reduced Src-dependent SERT-phosphorylation [35]. Here we found an inhibitory effect on 5-HT transport displayed by Syk-inhibitors, suggesting that SERT contains different Tyr-residue targets for different Tyr-kinases that likely concur to the regulation of SERT conformation and activity.

As mentioned in the Introduction, serotonin efflux from platelets can be obtained by manipulating the cytosolic ionic concentration [20, 57], in particular that of Na^+ and Ca^{2+} [23]. Since the Syk-inhibitors used neither modified the $[Na^+]_c$ and $[Ca^{2+}]_c$ (not shown), nor induced *per sè* an efflux of serotonin, we could exclude that the reduced 5-HT accumulation observed in platelets exposed to kinase inhibitors was consequent to a 5-HT efflux induced by them. Indeed, at variance with previous findings obtained with Src-inhibitors [35], we have now found that the serotonin efflux induced by the Na^+ ionophore monensin from reserpine-treated platelets diminished in platelets pre-treated with piceatannol or Syk-inhibitor II. The different behaviour of the two classes of inhibitors is likely dependent on the fact that the Tyr-phosphorylation catalysed by Syk, but not by Src, is required for SERT achievement of a conformation favouring the serotonin getting out from platelets. It should be noted that conflicting results have been reported on the efflux pathways of serotonin from the platelet cytosol into the extracellular compartment, mainly based on the different inhibitory effect shown by imipramine and fluoxetine on this process [18, 21, 23]. Interestingly, it has been recently reported that not only the inward but also the outward transport of serotonin is enhanced in depressed patients suffering seasonal affective disorders [58].

It is well known that the SERT-mediated 5-HT transport is a process regulated by a variety of factors, and that its magnitude, together with the function of 5-HT receptors, is associated to depression [4, 5, 11, 58, 59], so SERT constitutes a major therapeutic target in anti-depressive therapies [60]. A bulk of literature indicate that Syk plays a crucial role in the phosphorylation of multiple proteins regulating a variety of important cellular fiunctions [24-31]. Even if it can not be ruled out that the reduced 5-HT transport induced by Syk-inhibitors may be due to the decrease in Tyr-phosphorylation of other molecules than SERT, the present results suggest that Syk contributes to the modulation of SERT activity through its Tyr-phosphorylation and concurs to the regulation of the organic distribution of serotonin. Therefore Syk might constitute a new potential device in the therapeutic strategies for pathologies arising from anomalous organic distribution of the neurotransmitter.

Abbreviations

5-HT (serotonin); SERT (serotonin transporter of plasma membrane); VMAT (vesicular monoamine transporter); Syk (spleen tyrosine kinase); PRP (platelet rich plasma); IP (immuno-precipitate).

Acknowledgements

The authors like to acknowledge the financial support by the University of Padova (Italy).

Note: Part of these results has been presented at the Int. Congr. "Biogenic Amines: Biochemical, Physiological and Clinical Aspects"; Alberè di Tenna (TN) Italy. May, 2008.

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