Serotonin (5-HT) Transport in Human Platelets is Modulated by Src-Catalysed Tyr-Phosphorylation of the Plasma Membrane Transporter SERT

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Key Words

Serotonin (5-HT) • SERT • Platelets • Src-kinase • Tyrosine phosphorylation

Abstract

Background/Aim: platelets possess tightly regulated systems for serotonin (5-HT) transport. This study analysed whether the 5-HT transport mediated by the plasma-membrane transporter SERT is regulated by its Tyr-phosphorylation. **Methods:** 5-HT transport was determined by filtration techniques, while immunoblotting procedures were adopted for detecting the Tyr-phosphorylation of SERT in human platelet fractions. **Results:** 5-HT accumulation in platelets pre-treated with reserpine, which prevents the neurotransmitter transport into the dense granules, decreased upon cellular exposure to PP2 and SU6656, two structurally unrelated inhibitors of Src-kinases. By contrast, the protein Tyr-phosphatase inhibitor pervanadate increased the 5-HT accumulation. Anti-SERT immunostaining of the platelet fractions showed a major band displaying an apparent molecular mass of 50 kDa, indicating that, during the analytical procedure, SERT underwent proteolysis, which was counteracted by addition of 4 M urea in the cellular

disrupting medium. The Tyr-phosphorylation degree of SERT immunoprecipitated from membrane extracts decreased by platelet treatment with SU6656 or PP2, and enhanced upon pervanadate treatment. The anti-SERT immunoprecipitates displayed anti-Src immunostaining and in vitro kinase activity towards a Src-specific peptide-substrate. Platelet treatment with PP2 or SU6656 also caused a decrease in the imipramine binding to platelets. It was concluded that the Src-mediated SERT Tyr-phosphorylation regulates the 5-HT transport by affecting the neurotransmitter binding sites.

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Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter which regulates a variety of vascular, smooth muscle, and platelet functions [1-4]. Serotonin is mainly formed in the enterochromaffin cells of the gut and transported in circulation by platelets which take up it by means of transport systems displaying properties similar to those of neurones [2-6]. A plasma membrane transporter (SERT), which is inhibited by the antidepressant imipramine, transports 5-HT from extra-cellular to cytosolic

compartment [7]. A second carrier, i.e. vesicular monoamine transporter (VMAT), which is specifically inhibited by reserpine, transports 5-HT from the cytosol into the dense granules [7].

Serotonin is released from platelets by exocytosis of the dense granules that accompanies the platelet activation [8]. Efflux of 5-HT from platelets also occurs upon enhancement of intra-granular pH obtained by addition of membrane permeable amines or inhibitors of the vacuolar ATPase [9, 10]. Under these conditions a 5-HT efflux takes place from the dense granules to the cytosol and from the cytosol to the extra-cellular compartment. Serotonin efflux from platelet cytosol is also induced by increasing the cytosolic concentration of Ca^{2+} or Na^+ [11-13]. It has been postulated that this process occurs through SERT, which is a hydrophobic polypeptide with 12 membrane-spanning segments containing multiple consensus sites for various protein kinases in the cytosolic domains [3, 14]. This suggests that the transporter activity might be modulated by its phosphorylation. In fact, it has been reported that activators of cGMP-dependent protein kinases [15] and protein kinase C (PKC) [13, 16-21], and the tyrosine kinase inhibitors genistein and 2,5-hydroxycinnamate [22] cause a decrease of the 5-HT transport in human platelets and other cellular types expressing SERT.

The present study shows that the 5-HT accumulation in human platelets correlates with the degree of Src-mediated Tyr-phosphorylation of SERT.

Materials and Methods

Materials

Apyrase, prostacyclin, reserpine, 4-nitrophenyl phosphate (pNPP), and catalase were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.); [^{14}C]serotonin and [^3H]imipramine were from Amersham Pharmacia Biotech (Little Chalfont, U.K.); whereas fura 2/AM, ionomycin, SU6656, (*trans*-3,3',4,5'-tetrahydroxy-stilbene), PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazol-[3,4-*d*]pyrimidine), PP3 (4-amino-7-phenylpyrazol-[3,4-*d*]pyrimidine), and sodium orthovanadate were from Calbiochem (Darmstadt, Germany). Benzophurane isophthalate acetoxymethyl ester (SBFI/AM) was from Molecular Probes (Leiden, The Netherlands), the protease inhibitor cocktail from Roche Diagnostic (Mannheim, Germany), the goat serotonin transporter antibody ST(C-20): sc-1458 and anti-Src antibody Src(N-16):sc-19 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.), the mouse anti-phosphotyrosine monoclonal antibody PY20 from ICN Biotechnology (Irvine, CA, U.S.A.), and protein G PLUS-Agarose from D.B.A. Italia, (Milano, Italy). Pervanadate was freshly prepared by mixing 20 mM sodium orthovanadate with an equal volume of 40 mM H_2O_2 , after 15

min incubation at room temperature the excess of hydrogen peroxide was removed by addition of 1000 units of catalase. The synthetic peptide cdc2(6-20), which reproduces the sequence 6-20 of cdc2 protein, was kindly provided by Prof. O. Marin (University of Padova, Italy).

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 $\mu\text{U}/\text{ml}$ apyrase and 0.8 $\mu\text{g}/\text{ml}$ prostacyclin, and centrifuged for 20 min at 200 g. The supernatant platelet-rich plasma (PRP) was supplemented with further 5% ACD and re-centrifuged for 10 min at 200 g. The new PRP was further centrifuged for 20 min at 750 g. The spun down platelets were re-suspended, unless otherwise indicated, in the basal buffer consisting of (mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10 glucose, and 10 Tris/Hepes, (pH 7.4), at a concentration of 2×10^8 cells/ml. Where indicated acetylsalicylic acid treatment was performed by addition (0.1 mM final concentration) of a freshly prepared solution in 0.1 M NaHCO_3 , to platelet-rich plasma, followed by 20 min incubation at 36°C.

Serotonin transport in human platelets

Serotonin uptake by platelets was determined as described elsewhere [13]; briefly: 0.5 μM [^{14}C]5-HT was added to platelet suspensions, the incubations were stopped by mixing the samples with equal volumes of ice-cold glutaraldehyde solution (3% v/v) and filtration through Millipore HAWD 013 filters, which were washed 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 [7]. Serotonin efflux was monitored with the same procedure adopted for uptake using platelets pre-loaded with [^{14}C]5-HT [13].

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

Cytosolic [Ca^{2+}] was determined by means of the fluorescent probe fura 2 as previously reported [13], whereas the cytosolic [Na^+] was determined with the fluorescent indicator benzophurane isophthalate (SBFI) essentially as described in [23]. SBFI/AM solution (5 mM in dimethyl sulfoxide) was mixed with an equal volume of the non-ionic detergent Pluronic F-127 (20% w/v), added to platelet suspensions (final concentration 4 μM) and incubated for 60 min at 37°C. Platelet suspensions, supplemented with prostaglandin PGI_2 (0.1 $\mu\text{g}/\text{ml}$), were centrifuged (700 g for 20 min), platelets were re-suspended in the basal medium (2×10^8 cells/ml) and treated according to the experimental protocol. Fluorescence was measured in a thermostated, magnetically stirred quartz cuvette, using the dual excitation wavelengths of 345 and 385 nm and the emission wavelength of 490 nm. Calibration was performed with known Na^+ concentrations and the ionophore monensin (6 μM).

Detection of tyrosine phosphorylation of protein membrane extracts and SERT immunoprecipitates

Platelets suspended in the basal medium (6 ml containing 1.5×10^9 cells/ml) were incubated according to the experimental protocol. In the first set of experiments the reactions were stopped by addition of lysis buffer consisting of (final concentration, mM): 100 NaCl, 2 EDTA, 1 EGTA, 1 sodium vanadate, 10 KCl, 5 sodium p-nitrophenyl-phosphate (pNPP), 20 Tris-HCl, pH 7.4, Triton-X100 (0.1% v/v), and supplemented with the protease inhibitor complete (1 tablet/ml). All the following steps were performed at 4°C. The lysates were centrifuged at 60,000 g for 30 min. The pelleted membranes were re-suspended in 300 µl of the above medium supplemented with sodium deoxycholate (0.4 % w/v), Triton-X100 (0.4 % v/v) and SDS (0.8 % v/v), kept for 2 h under stirring and then ultra-centrifuged for 1 h at 200,000 g. An aliquot of the extracted protein solution was subjected to SDS/PAGE (10% gels), the separated proteins were immediately electrophoretically transferred to nitrocellulose membranes, treated with anti-phosphotyrosine or anti-SERT antibody, followed by secondary peroxidase-conjugate antibody and detected by the enhanced chemi-luminescence technique (ECL, Amersham Pharmacia Biotech). Since the immunostaining with a specific antibody revealed that SERT underwent a proteolytic process during the analytical procedures, we sought for the conditions in which the SERT proteolysis was prevented. The procedure chosen for the detection and immunoprecipitation of SERT consisted in the platelet lysis in a medium containing 4 M urea and 2 % (w/v) SDS, ultra-centrifugation of the lysates for 1 h at 200,000 g, re-suspension of the pelleted membranes in a medium containing 1% (v/v) NP40, 0.25% (w/v) deoxycholate (DOC), antiprotease cocktail (1 tablet/ml together with 1 mM NaF and 1 mM pNPP), and 1 mM sodium orthovanadate. The membrane extracts were mixed with two volumes of the same buffer devoid of detergents and used for the immunoprecipitation of SERT which was obtained, after the preclearing of the suspension with the protein G Plus-Agarose (60 min incubation with 30 µl/ml at 4°C followed by 10 min centrifugation at 3,000 g) by incubating the anti-SERT antibody under mild mixing (5 µg/ml, for 3 h at 4°C). The immunoprecipitates (IPs), obtained after 10 min centrifugation at 3,000 g, were washed three times and solubilised in Laemmli buffer, subjected to SDS-PAGE and immunoblotted with either anti-SERT or anti-P-Tyr antibodies. Occasionally the anti-phosphotyrosine immunoblots were stripped and re-probed with anti-SERT antibody.

Determination of the Src kinase activity

Src-kinase activity displayed by anti-SERT immunocomplexes, obtained as above described, was measured in 50 µl of phosphorylation medium containing 50 mM Tris/HCl, pH 7.5, 6 mM $MnCl_2$, 5 mM $MgCl_2$, 10 µM $[\gamma^{33}P]$ ATP (specific activity 1000 dpm/pmol), 100 µM sodium orthovanadate and 200 µM cdc2(6-20), a Src-specific peptide-substrate. Following 5 min incubation at 30°C, samples were subjected to SDS/PAGE (15% gels) and the peptide phosphorylation was evaluated using an Instant Imager (Meriden, CT).

Measurement of imipramine binding

Platelet suspensions, prepared as above described, were incubated with 5 nM $[^3H]$ 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 and washed three times with 5 ml of the basal Tyrode 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 [13].

Statistical analysis

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

Results

Serotonin (5-HT) uptake by human platelets decreased upon cellular treatment with protein Tyr-kinase inhibitors including the non-specific inhibitor genistein [24], and two rather specific inhibitors of the Src Tyr-kinases, SU6656 and PP2 [25-29], (Fig. 1A). SU6656 is considered a highly selective inhibitor of the Src-kinases, while PP2 has been reported to inhibit also other cellular protein kinases as the PDGF receptor kinase, mitogen-activated protein kinase, protein kinase B (Akt) and Abelson tyrosine kinase (Abl) [26-28]. We obtained similar results with both compounds, which are structurally unrelated, and then they indicate that the decreased 5-HT transport was likely ascribable to the inhibition of the Src-kinases [29]. By contrast 5-HT accumulation increased upon platelet pre-treatment with the protein tyrosine phosphatase inhibitor pervanadate [30], and was not significantly modified by PP3, an inactive analogue of PP2 (Fig. 1A). None of the used Tyr-kinase inhibitors induced: i) exocytosis of the dense granules, (monitored as ATP secretion); ii) decrease of cellular accumulation of acridine orange, (a basic fluorescent probe which, similarly to 5-HT, accumulates into the dense granules driven by the acid intra-granular pH, [13]), or iii) cytosolic $[Ca^{2+}]$ increase, (an event that causes a reduction of the 5-HT transport [13, 31]), (not shown).

In order to assess whether the modulators of the protein Tyr-phosphorylation affected the serotonin entry from the extra-cellular compartment into the cytoplasm

Fig. 1. Effect of modulators of protein Tyr-phosphorylation on serotonin ($[^{14}\text{C}]5\text{-HT}$) uptake in human platelets. Suspensions of control (A), or reserpine pre-treated platelets ($10\text{ }\mu\text{M}$ for 10 min) (B), were pre-incubated for 30 min with: vehicle (control), $100\text{ }\mu\text{M}$ genistein, $5\text{ }\mu\text{M}$ 6656, $5\text{ }\mu\text{M}$ PP2, $5\text{ }\mu\text{M}$ PP3, or $50\text{ }\mu\text{M}$ pervanadate, prior to addition of $0.5\text{ }\mu\text{M}$ $[^{14}\text{C}]5\text{-HT}$ and further incubation for the indicated time. Values are means of 4 experiments performed in triplicate with S.D. indicated by vertical bars. Statistical significance of differences: * $p < 0.01$, ** $p < 0.005$.

or from the latter into the dense granules, we analysed the 5-HT uptake in platelets pre-treated with reserpine, which inhibits the serotonin carrier of the dense granule membranes VMAT, and thus limits the serotonin accumulation into the cytosolic compartment [7]. All the tested protein kinase inhibitors caused a decrease of the serotonin uptake, while the protein phosphatase inhibitor pervanadate enhanced the 5-HT accumulation, also in reserpine-treated platelets (Fig. 1B), indicating that the compounds acted at the level of the plasmatic membrane SERT.

Fig. 2. Monensin-induced efflux of 5-HT from reserpine-treated platelets. Reserpine-treated platelets were loaded with $[^{14}\text{C}]5\text{-HT}$ and then incubated for 30 min with: only vehicle (control), $5\text{ }\mu\text{M}$ SU6656, $5\text{ }\mu\text{M}$ PP2, or $50\text{ }\mu\text{M}$ pervanadate. Monensin ($0.5\text{ }\mu\text{M}$) was added at the arrow. Values are means of 3 experiments performed in triplicate with S.E. indicated by vertical bars.

We then investigated whether the modulators of Tyr-phosphorylation affected the efflux of pre-loaded $[^{14}\text{C}]5\text{-HT}$ from reserpine-treated platelets, efflux which was induced by increasing the cytosolic $[\text{Na}^+]_i$ with the Na^+/H^+ exchanger monensin [11-13]. Neither the monensin-induced $[\text{Na}^+]_i$ increase (not shown), nor the efflux of labelled serotonin were affected by platelet pre-treatment with the inhibitors of either Src-kinases (PP2 and SU6656) or Tyr-phosphatases (pervanadate) (Fig. 2).

It is well known that imipramine inhibits the serotonin uptake by binding to platelet membranes, and its cellular binding has been correlated with the magnitude of 5-HT transport [7, 32, 33]. We therefore analysed whether the above inhibitors modified the $[^3\text{H}]$ imipramine binding, and found that while in control conditions $3.3 \pm 0.5\text{ pmol}$ of imipramine bound to 1×10^8 platelets after 2 min incubation (mean $\pm$ S.D., $n=5$), in the presence of either PP2 or SU6656 ($5\text{ }\mu\text{M}$, 30 min) the imipramine bound to platelets decreased by 43 ± 8 and $37 \pm 9\%$, respectively.

Even if the preincubation with pervanadate did not significantly affect the amount of bound imipramine (not shown), we thought worthy to examine whether the above effects displayed by the Tyr-phosphorylation modulators

Fig. 3. Immunodetection of SERT and Tyr-phosphorylated proteins in human platelet membrane fraction. Anti-SERT and anti-P-Tyr immunostaining of proteins extracted from membranes of platelets pre-treated for 30 min with: vehicle (control), 5 μ M PP2, 5 μ M SU6656 or 50 μ M pervanadate. Blots are representative of four experiments performed with different platelet specimen.

on 5-HT transport were associated to with 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 were obtained, as described in the Methods section, by lysis of platelets with low detergent containing solution (0.1% v/v Triton-X100), centrifugation of lysates, treatment of the pelleted fractions with high detergent solution (0.4% w/v sodium deoxycholate, 0.4% v/v Triton-X100 and 0.8% v/v SDS), and ultracentrifugation of the suspensions. The membrane extracts were subjected to SDS/PAGE and immuno-stained with anti-SERT and anti-P-Tyr antibodies as described in the Materials and Methods section. A major band displaying an apparent molecular mass of about 50 kDa together with two minor protein bands of about 78 and 100 kDa emerged in the electrophoretic gel exposed to anti-SERT antibody (Fig. 3). Even if the high number of Tyr-phospho-proteins hindered the analysis of results, it appeared that platelet pre-treatment with the Src-inhibitors PP2 and SU6656 reduced the Tyr-phosphorylation of the protein bands corresponding to SERT, while this protein (together with a number of other proteins) appeared more phosphorylated in the cells pre-incubated with the Tyr-phosphatase inhibitor pervanadate (Fig. 3).

As the predicted molecular mass of SERT is about 72 kDa [14], the finding of an abundant anti-SERT protein

Fig. 4. Immunodetection of SERT in membrane extracts prepared in the presence of urea. Anti-SERT immunostained proteins of membrane extracts and membrane anti-SERT immunoprecipitates (IPs) obtained in the absence or presence of 4 M urea. The anti-SERT IPs were also probed with anti-P-Tyr antibody. Other technical details are reported in the Materials and Methods section. Blots are representative of four separate experiments.

band of 50 kDa indicated that the serotonin transporter likely underwent a proteolytic degradation during the preparation procedure even if this was performed in the presence of a protease inhibitor cocktail. The proteolytic process was notably counteracted by the addition of high concentration of urea in the cellular disrupting medium. In fact, under these conditions, a major anti-SERT protein band corresponding to about 85 kDa was found in the membrane extract (Fig. 4). Experiments were then performed in order to optimize the experimental conditions for extracting and immunoprecipitating non-proteolysed SERT. Eventually the chosen procedure (described in detail in the Materials and Methods section), consisted in the platelet lysis in a medium containing 4 M urea and 2% (w/v) SDS. The anti-SERT immunoprecipitation was performed in the extract solutions diluted with two volumes of medium devoid of detergents. The immunoprecipitates (IPs) were solubilised in Laemmli buffer, subjected to SDS-PAGE and immunoblotted with either anti-SERT or anti-P-Tyr antibodies. Representative immunoblots of anti-SERT IPs from membrane extracts are reported in Figure 4, which shows two relatively abundant Tyr-phosphorylated protein bands of about 50 and 85 kDa together with a weakly phosphorylated band of about 140 kDa.

The 85 kDa band, which was chosen as paradigmatic SERT band, resulted less Tyr-phosphorylated in the

Fig. 5. Detection of Tyr-phosphorylation and Src-kinase activity of anti-SERT IPs from platelets treated with inhibitors of the Src-kinases or protein Tyr-phosphatases. Membrane extracts obtained from platelets, pre-treated as in Fig. 1, were immunoprecipitated with anti-SERT antibody. IPs were blotted and immunostained with anti-P-Tyr, anti-SERT, or anti-Src antibodies (A). The anti-SERT IP, separately obtained from control platelets, was divided in four equivalent fractions and assayed for the kinase activity towards the Src-specific peptide substrate cdc-2(6-20). The enzyme activity was determined in the absence or presence of the Tyr-phosphorylation modulators, added in the assay medium at the above reported concentration, and incubated for 10 min before starting the reaction by ATP addition (B). Blots are representative of 5 separate experiments, while enzyme activity values are means $\pm$ S.D. of 4 experiments. * $p < 0.001$.

IPs obtained from platelets pre-treated with PP2 or SU6656, and more phosphorylated in the IPs from pervanadate pre-treated platelets (Fig. 5A). Similar behaviour was accordingly observed also in the 50 kDa and 140 kDa protein bands, (not shown).

The coimmunoprecipitation of enzyme-substrate complexes is an event frequently observed [34-36], so in order to confirm that the Tyr-phosphorylation of SERT was mediated by the Src-kinase(s), we sought whether Src, which is the most expressed member of this family in platelets, coimmunoprecipitated with the transporter in anti-SERT IPs. Figure 5 shows that Src-kinase was

indeed present in these IPs, as revealed both by the anti-Src immunoblotting (Fig. 5A), and by the finding that anti-SERT IP complexes eluates were able to phosphorylate *in vitro* a substrate specific for the Src-kinases (Fig. 5B, control lane). As expected, the Src activity displayed by anti-SERT IPs obtained from control platelets was drastically reduced upon addition of either PP2 or SU6656 in the assay medium, while was almost unaffected upon addition of pervanadate (Fig. 5B).

Discussion

Blood platelets possess 5-HT transporters displaying characteristics similar to those of neurones, therefore they constitute a suitable model for the study of the transport of this neurotransmitter [3-5, 14]. The SERT-mediated 5-HT uptake is a tightly regulated process. SERT contains multiple consensus sites for various protein kinases in the cytosolic domains [14], and its PKC-dependent phosphorylation was previously shown to regulate the 5-HT transport in a variety of cells expressing SERT as well as in human and mouse platelets [13, 16-21, 37, 38]. It was also found that serotonin accumulation in platelets decreases in the presence of the tyrosine kinase inhibitors genistein and 2,5-hydroxycinnamate [22].

The present results show that 5-HT uptake by reserpine-treated platelets depends on the degree of Tyr-phosphorylation of SERT mediated by Src-kinase. This conclusion was first of all based on the decreased serotonin uptake found in platelets pre-treated with the Src-kinase inhibitors SU6656 and PP2, which are two structurally unrelated compounds and their combined use has been proposed for identifying the specific physiological role(s) of the Src-kinases [29]. The higher inhibition displayed by genistein on 5-HT transport, in comparison to that exerted by SU6656 and PP2, is probably due to the inhibitory action of this compound on a wide range of Tyr-kinases which likely are also involved in the Tyr-phosphorylation of SERT.

The decreased SERT Tyr-phosphorylation is accompanied by a diminution of platelet imipramine binding. A linear relationship between imipramine binding and 5-HT uptake was found in platelets [32, 33], and a competition between imipramine and 5-HT for the same SERT sites was proposed [7, 39]. Therefore the observed changes in the imipramine binding suggest that the Tyr-phosphorylation of SERT is involved in the regulation of the 5-HT transport by modifying the structure of the transporter and thus the number or affinity of the serotonin binding

sites. It might be worth mentioning that imipramine binding has been proposed as a diagnostic parameter for panic disorders, depression and Parkinson's disease [40-42].

We have previously hypothesized that the monensin-elicited serotonin efflux from platelets might occur through the outward directed SERT [31], however at variance with the inhibitory effect exhibited on the serotonin uptake, the Src-kinase inhibitors did not affect the monensin-induced efflux of the neurotransmitter. This different effect of the Tyr-phosphorylation modulators between 5-HT uptake and efflux is likely ascribable to the asymmetric structure of SERT which has been shown to regulate the inward and outward directed transport with different mechanisms [13, 14]. This conclusion is consistent with the previously reported different inhibitory action of imipramine on the accumulation and efflux of serotonin in human platelets [13].

The present results also exclude that the reduced 5-HT accumulation observed in platelets exposed to Src-kinase inhibitors could depend on the increase in Ca^{2+} or Na^{+} cytosolic concentration, which we have previously demonstrated to inhibit the serotonin uptake [13].

It has been recently demonstrated that SERT undergoes a deep proteolytic degradation during the extraction from cells, and that its degradation depends very much on the preparation conditions [43]. Accordingly, we have found that the usually employed anti-protease cocktails only slightly prevented the SERT proteolysis, which was considerably counteracted by high urea concentration. Indeed the anti-SERT immunoblots obtained in these conditions revealed two major bands displaying apparent molecular masses of about 85 kDa and 140 kDa, which are values higher than that predicted of 72 kDa. In agreement with the Qian group observations [44], we think that this discrepancy is likely due to the glycosylation and

aggregation of the transporter, respectively.

The coimmunoprecipitation of the Src-kinase with the transporter, as revealed by both the immunological response and the *in vitro* kinase activity of anti-SERT IPs towards a Src-specific peptide substrate, indicated that Src is involved in the SERT Tyr-phosphorylation. This conclusion is also supported by the finding that the *in vitro* kinase activity displayed by anti-SERT IPs was drastically inhibited by SU6656 and PP2 added to the incubation medium (Fig. 5).

In conclusion the present results indicate that the Src-mediated Tyr-phosphorylation of SERT may contribute to the modulation of its transporter efficiency and might represent a new potential target in the therapeutic strategies of the neurologic disorders linked to anomalous organic distribution of serotonin.

Abbreviations

5-HT (5-hydroxytryptamine, serotonin); SERT (serotonin transporter of plasma membrane); VMAT (vesicular monoamine transporter); IP (immunoprecipitate); PRP (platelet rich plasma); pNPP (p-nitrophenylphosphate).

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