Presence and Characterization of the Serotonin Transporter in Human Resting Lymphocytes

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Although evidence exists of the presence of a serotonin (5-HT) reuptake system in lymphocytes, no information is available on the pharmacological characterization of this structure. Our study aimed to investigate this matter, therefore, by means of the binding of [^{3}H]-paroxetine ([^{3}H]PAR), a selective 5-HT reuptake inhibitor (SSRI), which is considered the ligand of choice for binding studies. Lymphocytes were obtained from a pool of 20 healthy subjects who volunteered for the study. The results showed the presence of a specific and saturable [^{3}H]PAR binding to lymphocyte membranes, with a Hill number close to unity indicative of the presence of one site only. The most potent drugs inhibiting [^{3}H]PAR binding were SSRIs (paroxetine, fluoxetine, citalopram) followed by clomipramine, imipramine, and 5-HT, whereas haloperidol, mazindol, and nomifensine had a negligible effect. These findings suggest that [^{3}H]-PAR in human resting lymphocytes specifically labels the 5-HT transporter.

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The serotonin (5-HT) transporter complex represents one of the main targets of the action of tricyclic antidepressants and selective 5-HT reuptake inhibitors (SSRIs). The transporter subserves the function of removing the neurotransmitter once released in the synaptic cleft and returning it to presynaptic terminals, where it is metabolized by monoaminoxidases or stored in secretory vesicles. Structurally, it belongs to the class of carrier proteins, characterized by 12 hydrophobic membrane-spanning domains, showing a certain degree of similarity with the GABA and the dopamine transporter (Amara and Kuhar 1993; Blakely et al. 1994; Lesch et al. 1994; Merickel et al. 1997).

The most quoted biochemical model of the 5-HT transporter is that of a supramolecular complex with different binding sites, such as those for [^{3}H]-imipramine ([^{3}H]IMI), the ligand that has been used to label it for some years (Briley et al. 1979; Ieni et al. 1985; Meyerson et al. 1987). However, pharmacological studies in this field have shown a heterogeneity of [^{3}H]IMI binding sites when desipramine is used to define the "specific" binding (Hrdina 1989): desipramine-defined IMI binding appears to consist of two subpopulations, with only that of a proteic nature, 5-HT–sensitive and Na-dependent being present in serotonergic neurons and related to the 5-HT transporter (Marcusson et al. 1985). [^{3}H]-Paroxetine ([^{3}H]PAR), a SSRI, has subsequently seemed to label the 5-HT transporter protein more specifically (Mellerup et al. 1983; Marcusson et al. 1988), and its use in clinical studies is currently replacing that of [^{3}H]IMI (D'Haenen et al. 1988; Lawrence et al. 1993; Faludi et al. 1994; Maguire et al. 1995; Marazziti et al. 1996).

The 5-HT transporter has been found and cloned in different tissues, including human brain and blood platelets (Marcusson and Ross 1990; Humphrey et al. 1991; Ramamoorthy et al. 1993a, 1993b; Qian et al. 1995; Padbury et al. 1997), and it appears identical in the two structures, as confirmed by sequence homologies through cloning studies (Blakely et al. 1991; Lesch et al. 1993a,b).

Although evidence exists of the presence of a 5-HT reuptake mechanism in circulating lymphocytes (Faraj et al. 1994), no information is available on the pharmacological characterization of this structure.

Our study aimed, therefore, to investigate the presence of the 5-HT transporter in human resting lymphocytes by means of the specific binding of [3H]PAR and to characterize it pharmacologically.

MATERIALS AND METHODS

Materials

[3H]PAR (specific activity: 20.2 Ci/mmol) was purchased from New England Nuclear (NEN, Milan, Italy); 5-HT was obtained from Research Biochemicals Inc. (RBI, Natuck, MA, USA). Imipramine and clomipramine were a gift from Ciba-Geigy AG (Basel, Switzerland); fluoxetine from Eli-Lilly Co. (Indianapolis, IN, USA); paroxetine from SmithKline Beecham Pharmaceuticals (Betchworth, UK) and citalopram from H. Lundbeck A/S (Copenhagen, Denmark); mazindol, nomifensine and haloperidol were purchased from Sigma (Milan, Italy). All other reagents were standard laboratory reagents of analytical grade.

Subjects

Twenty healthy young subjects (eight female and 12 male; aged between 18 and 30 years; mean age ± SD: 25 ± 3) were included. All subjects were drug-free, in good physical health, as documented by a general check-up and by normal blood and urine tests, and with no familial or past personal history of psychiatric or neurological disorders, as assessed by a psychiatric interview. All gave their informed consent to participation in this study, which was approved by the Ethics Committee of Pisa University.

Lymphocyte Preparation

Venous blood (50 ml) was drawn between 8:00 and 9:00 A.M. from fasting subjects by antecubital venipuncture, collected in heparinized (10 UI/ml blood) plastic tubes and processed immediately. Lymphocytes were isolated according to our modification of the Boyum method (1968). Blood was centrifuged for 15 minutes at 300 g to

obtain a pellet containing red cells, platelets, granulocytes, lymphocytes (P1), and platelet-rich plasma (PRP). Platelets were precipitated from PRP by centrifugation at 30,000 g for 10 minutes to obtain platelet-free plasma. P1 was diluted 1:1 with Emagel (Behring AG, Marburg) and centrifuged for 30 minutes at 550 g at room temperature over a density gradient of Lymphoprep (Nycomed, Oslo, Norway). Lymphocytes were harvested and washed in phosphate-buffered saline (PBS) (150 mmol/L NaCl, 2.5 mmol/L KH2PO4, 11.27 mmol/L NaHPO4, pH 7.4) at 300 g for 15 minutes. The resulting pellet was diluted with 1 ml PBS, stratified on 7 ml of platelet-free plasma and centrifuged at 60 g for 15 min. Lymphocytes were washed twice in platelet-free plasma to obtain a sample completely separated from platelets. Washed lymphocytes were resuspended in PBS and counted with a hemocytometer. The purity of lymphocyte preparations was controlled by means of an optical microscope (Ortholux, LEITZ) in a Burker chamber. Lymphocytes were colored according to the May-Grunwald methods, and pictures were taken (Figure 1) showing no contamination with other blood elements.

Lymphocyte Membrane Preparation

The fresh lymphocyte pellet was resuspended in ice-cold 5 mmol/L Tris HCl buffer, pH 7.4, containing protease inhibitors (200 μg/ml bacitracine, 160 μg/ml benzamidine, 20 μg/ml soybean trypsin inhibitor), lysed using a Polytron homogenizer and centrifuged at 50,000 g for 10 minutes at 4°C. The ensuing pellet was resuspended in ice-cold 50 mmol/L Tris HCl buffer, pH 7.4, containing protease inhibitors and centrifuged at 50,000 g for 15 minutes at 4°C. The pellet was then washed with 8 ml buffer (50 mmol/L Tris HCl, 120 mmol/L NaCl, 5 mmol/L KCl, pH 7.4) and stored at −80°C until the assay, which was performed within 2 weeks.

[3H]PAR Binding Assay

On the day of assay, pellets were resuspended and homogenized in an assay buffer of 50 mmol/L Tris, 120 mmol/L NaCl, 5 mmol/L KCl (pH 7.4) (B1), yielding a final protein concentration of about 50 to 100 μg/tube. Proteins were measured according to the Peterson method (1977). The [3H]PAR binding was carried out according to the method of Marazziti et al. (1996). Membranes were incubated in a final volume of 2.0 ml B1 buffer. To test the saturability of [3H]PAR specific binding sites, the lymphocyte membranes were incubated with eight increasing concentrations of [3H]PAR, ranging from 0.01 to 1.5 nmol/L. Nonspecific binding was accounted for by incubating similar samples with 10 μmol/L fluoxetine. After 60 minutes at 22°C, the incubation was halted by the addition of 5 ml of ice-cold B1 buffer.
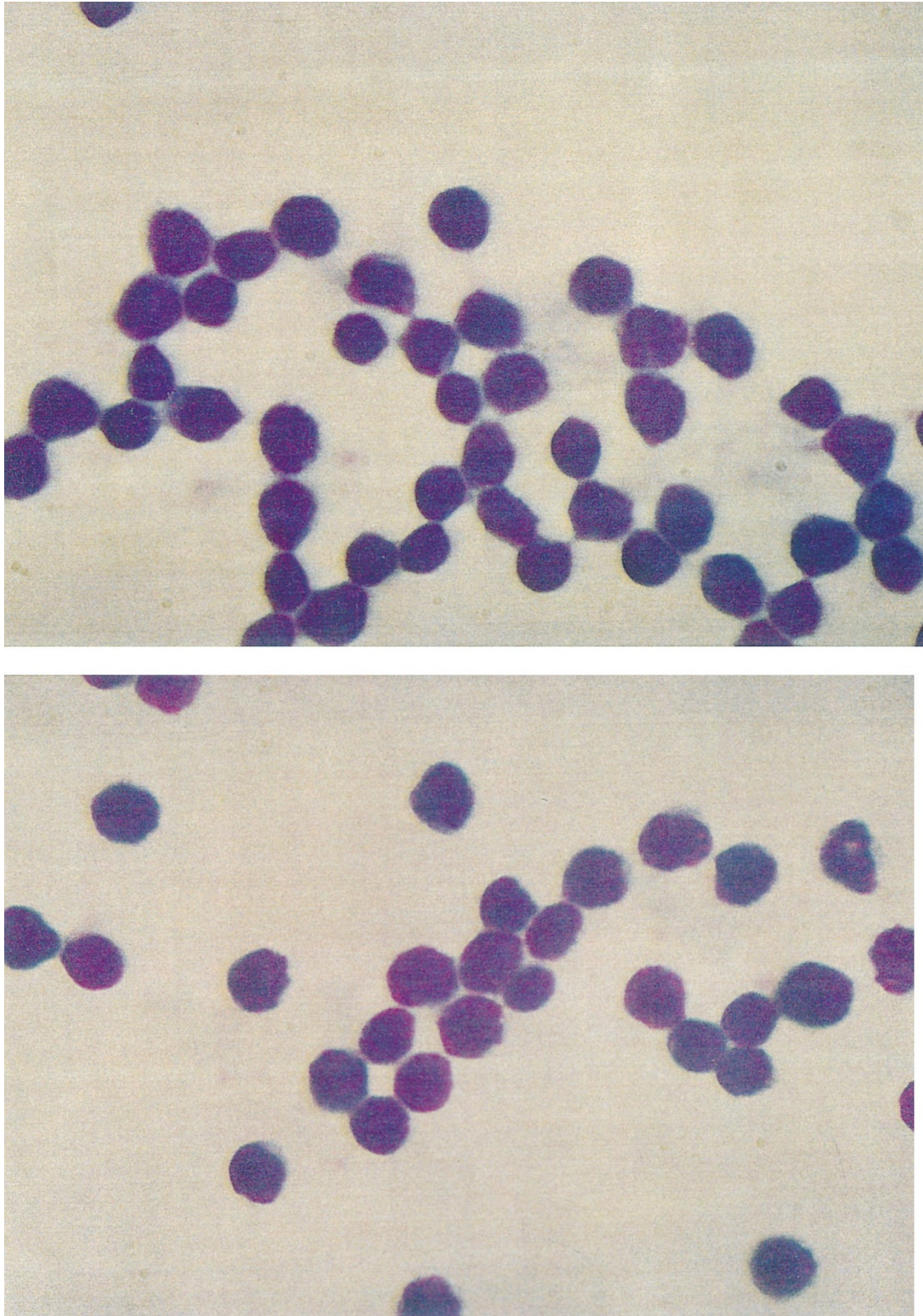
Figure 1. Images of isolated lymphocytes not contaminated by other blood cells.

Samples were rapidly filtered under vacuum through Whatman GF/C filters previously soaked in polyethylene-immine solution (0.3%) and washed three times with 5 ml of assay buffer. Filters were then placed in vials with 4 ml of Ready Protein scintillation cocktail (Beckman), and radioactivity was measured by means of a beta-counter (Packard 1600 Tricarb).

Sodium Dependence

To assess the sodium dependence, we analyzed also the [3H]PAR binding with a single PAR concentration and varying sodium concentrations. Lymphocyte membranes were preincubated at 22°C for 15 minutes in 50 mmol/L Tris HCl buffer, then [3H]PAR (0.15 nmol/L) and NaCl at concentrations ranging from 100 μmol/L to 500 mmol/L were added.

Specificity of [3H]PAR Binding

Lymphocyte membranes were incubated, under the same conditions described above, with a constant concentration of [3H]PAR (0.4 nmol/L) and eight concentrations, ranging from 0.5 nmol/L to 10 μmol/L, of imipramine and clomipramine (tricyclic antidepressants), paroxetine, fluoxetine, and citalopram (SSRIs), mazindol and nomifensine (compounds with dopaminergic activity), haloperidol (a typical neuroleptic), and 5-HT.

Data Analysis

The equilibrium-saturation binding parameters, i.e., the maximum binding capacity (Bmax, fmol/mg protein) and the dissociation constant, (K_d, nmol/L), as well as the drug competition binding data were analyzed by means of the iterative curve-fitting computer programs EBDA and LIGAND (McPherson 1985). The potency of drugs in displacing [3H]PAR was expressed as Ki values, calculated by means of the formula Ki = IC50/(1 + C/Kd) (Cheng and Prusoff 1973), where IC50 represents the concentration of the drug inhibiting 50% of the specific radioligand binding and C is the radioligand concentration.

RESULTS

Under our experimental conditions described above, the specific [3H]PAR binding represented approximately 75% of the total binding it was saturable, and of high affinity (K_d, mean ± SEM = 0.17 ± 0.02 nmol/L). The saturation was reached at a concentration of 1 nmol/L of [3H]PAR (Figure 2). The maximum binding capacity (mean ± SEM) was 220 ± 26 fmol/mg protein (Table 1).
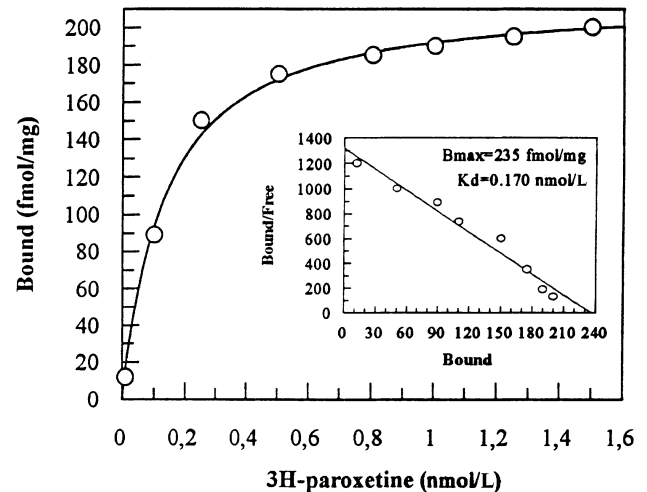

Figure 2. Saturation curve and Scatchard analysis of [3H]PAR binding to lymphocyte membranes.

The Scatchard analysis of the equilibrium saturation binding data resulted in a straight line, consistent with the presence of a homogenous population of binding sites (Figure 2).

With regard to sodium dependence, the results reported in Figure 3 showed that the binding increased significantly with increasing sodium concentrations until the plateau, reached at 110 mmol/L, and that the EC 50 was 70.

Displacement experiments showed that the tested compounds possessed different degrees of inhibitory potency, the most effective being paroxetine, followed by fluoxetine, citalopram, clomipramine, imipramine, 5-HT, mazindol, nomifensine, and haloperidol (Table 2).

Under our experimental conditions, the displacing curves of the different compounds were all monophasic, with the Hill number close to unity.

DISCUSSION

The results of our study showed the presence of a high-affinity (in nmol/L order), specific and saturable [3H]PAR binding in membranes obtained from human lymphocytes in resting conditions. The Scatchard analysis and the Hill coefficient close to unity are indicative

Table 1. [3H]PAR Binding Parameters (Bmax and K_d) in Human Lymphocyte Membranes

	Age	Bmax	K_d
12 M, 8 F	25 ± 3	220 ± 26	0.17 ± 0.02

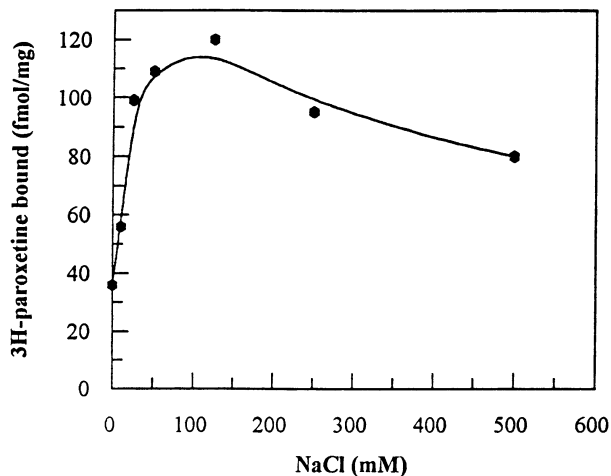M = male; F = female; Age = years (mean ± SD); Bmax = fmol/mg protein (mean ± SEM); K_d = nmol/L (mean ± SEM).

Figure 3. Effect of increasing concentrations of sodium on [³H]PAR binding to lymphocyte membranes. Each point represents the mean ± SEM of three separate experiments each performed in duplicate.

that [³H]PAR identifies one site only, as already reported in other tissues (Mellerup et al. 1983; Marcusson et al. 1988).

Our findings clearly indicate that [³H]PAR labels the 5-HT transporter protein with the same affinity as that observed in the central nervous system and platelets (Mellerup et al. 1983; Bäckstrom et al. 1989; Rotondo et al. 1996). The results of experiments on sodium dependence showed that [³H]PAR binding required optimal sodium concentration, similar to that found in platelets and brain sections (Mann and Hrdina 1992): this finding also supports the presence of 5-HT transporter in lymphocyte membranes. Although lymphocytes were reported to carry the specific reuptake system for 5-HT (Faraj et al. 1994), the present data may be considered the first demonstration of this structure in human lymphocytes by means of binding techniques.

Further support of the notion that [³H]PAR was labeling the 5-HT transporter derives from the results of

the pharmacological displacement studies. The most active compound was paroxetine, followed by other SSRIs, fluoxetine, and citalopram in that order. Clomipramine, a nonselective SRI was more active than imipramine, but they were both powerful compounds, whereas 5-HT had a lower effect and mazindol and nomifensine, potent inhibitors of the norepinephrine and the dopamine transporters, and haloperidol had no effect. The pharmacological profile overlapped with that obtained by our group in brain and platelet membranes (Rotondo et al. 1996): in fact, the different compounds showed the same rank of potency in all these tissues. The lack of activity of mazindol and nomifensine at the level of [³H]PAR binding shows that we were not labeling the dopamine transporter, which is present at a high concentration in lymphocytes (Faraj et al. 1991). The presence of the 5-HT transporter in human lymphocytes suggests its possible role in some immune functions, mediated by these cells and by 5-HT, whose role as an immunomodulator is currently emerging (Fillion et al. 1996). Although our method of purification of lymphocytes was accurate and we excluded the presence of other blood cells, we did not distinguish B from T lymphocytes: it is thus possible that different lymphocyte subpopulations under different conditions (not activated or activated) might reveal a heterogenous distribution of the 5-HT transporter: future studies should perhaps examine this question.

From a conceptual point of view, the presence of the 5-HT transporter in nucleate cells such as lymphocytes, might permit molecular genetic studies in patients, such as those affected by mood and anxiety disorders, where disturbances at the level of the platelet 5-HT transporter have been reported (Meltzer and Lowy 1987; Marazziti et al. 1996), although it is possible that the regulation of the 5-HT transporter may be different in nucleated and nonnucleated cells. Pursuing this line of research, recently Lesch et al. (1996) showed that a variant of the polymorphism in the 5-HT transporter reduces the expression of the transporter itself and the 5-HT uptake in lymphoblasts. However, the characteristics of the 5-HT transporter in cultured lymphoblasts may differ from those in resting lymphocytes.

Table 2. Ki (nmol/L) and Hill Number Values of Different Drugs Acting on [³H]PAR Binding to Lymphocyte Membranes

Drug	Ki (nmol/L)	Hill number (nH)
Paroxetine	0.15 ± 0.06	1.02 ± 0.015
Clomipramine	1.5 ± 0.10	0.96 ± 0.02
Fluoxetine	1.87 ± 0.09	0.99 ± 0.03
Citalopram	4.1 ± 0.3	0.96 ± 0.02
Imipramine	6 ± 1.6	0.97 ± 0.02
5-HT	630 ± 57.3	0.95 ± 0.03
Mazindol	2,000 ± 162	0.86 ± 0.04
Nomifensine	2,900 ± 280	0.91 ± 0.02
Haloperidol	3,000 ± 10	0.92 ± 0.02

Each value is the mean ± SEM of three independent determinations.

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