Partial purification and characterization of the sodium-ion-coupled 5-hydroxytryptamine transporter of rat cerebral cortex

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A procedure for the extensive purification of the Na⁺-coupled 5-hydroxytryptamine transporter of rat cerebral cortex has been developed. The 5-hydroxytryptamine transporter was solubilized with the non-ionic detergent digitonin, and the detergent extracts were subjected to sequential affinity chromatography on a citalopram-based agarose support and wheat-germ-agglutinin-Sepharose. 5-Hydroxytryptamine transporters in the affinity-purified preparation were identified by using the selective 5-hydroxytryptamine-uptake inhibitor [³H]paroxetine, and were shown to display a similar pharmacological profile to those present in particulate preparations. An overall transporter purification of around 2000-fold was achieved with a 9% recovery. SDS/PAGE of affinity-chromatographed material starting from detergent extracts incubated in the presence or absence of 1 mM-citalopram indicated that a polypeptide of *M*_r 73000 corresponded to the 5-hydroxytryptamine-transporter protein.

INTRODUCTION

Na⁺-coupled 5-hydroxytryptamine (5HT)-transport systems play an important physiological role in 5HT homeostasis [1]. For example, the plasma-membrane 5HT-transport system present in platelets and mast cells permits these cells to concentrate this biogenic amine. Also, the 5HT transporter at serotonergic nerve terminals functions to terminate 5HT neurotransmission and to maintain low synaptic-cleft levels of this neurotransmitter. With the suggestion that enhancement of neurotransmission at serotonergic synapses by inhibition of 5HT uptake would provide antidepressant activity [2], the 5HT transporter has become an important drug-research target. Selective inhibitors of the 5HT-transport system include fluoxetine [3], sertraline [4], citalopram [5] and litoxetine [6].

In addition to uptake studies with [³H]5HT [7], radiolabelled selective inhibitors of 5HT uptake, such as [³H]paroxetine [8–11] and [³H]citalopram [12], have been used as probes to characterize the Na⁺-coupled 5HT transporter. For example, analyses of [³H]paroxetine binding in competition inhibition, kinetic dissociation and chemical modification experiments indicate that the selective non-tricyclic 5HT-uptake inhibitors, as well as the tricyclic compound imipramine, bind to mutually exclusive overlapping domains at the substrate-recognition site of the 5HT transporter [11].

Nonetheless, despite the physiological and clinical importance of the 5HT transporter and its well-studied characterization using membrane preparations, the molecular properties of this transport system remain to be elucidated. In this context, studies aimed at determining the primary and secondary structure of this protein will be of prime importance. To this end, we have previously reported the solubilization of the 5HT transporter of rat cerebral cortex and shown that the solubilized macromolecule retains its substrate- and inhibitor-binding sites [13]. We now report an extensive purification by an affinity-chromatographic protocol of this detergent-solubilized 5HT-transporter preparation.

England Nuclear, Boston, MA, U.S.A. Digitonin was purchased from Biosynth A.G., Staad, Switzerland, Affi-Gel 10 from Bio-Rad, Paris, France, wheat-germ agglutinin (WGA)-Sepharose 6MB from Pharmacia, St. Quentin, France, and Pierce Excellulose GF-5 desalting columns from Pierce. Fluoxetine hydrochloride, sertraline hydrochloride, citalopram oxalate, imipramine hydrochloride, litoxetine benzoate, 5-(aminomethyl)-1-(4-fluorophenyl)-*N,N*-dimethyl-1,3-dihydroisobenzofuran-1-propanamine dimaleate was provided by the Chemistry Department of Synthélabo Recherche, Paris, France. *N*-Acetylglucosamine, 5HT creatinine sulphate, phenylmethanesulphonyl fluoride (PMSF), benzamidine hydrochloride, EDTA, benzethonium hydrochloride, dithiothreitol and aprotinin were purchased from Sigma, Paris, France. The TSK-G4000 SW and TSK-G3000 SW steric-exclusion columns (size 7.5 mm × 600 mm) were obtained from LKB-Pharmacia, St. Quentin, France, and the Centricon-30 filters were from Amicon, Epernon, France. Other chemicals and reagents were of analytical grade.

Methods

Coupling of the reduced citalopram derivative [5-(aminomethyl)-1-(4-fluorophenyl)-*N,N*-(dimethyl)-1,3-dihydroisobenzofuran-1-propanamine dimaleate] to an agarose support. Triethylamine (2 mmol) was added to the reduced citalopram derivative dimaleate salt (0.5 mmol) dissolved in 20 ml of dimethyl sulphoxide to liberate the free base. Affi-Gel 10 (25 ml) was washed with 6 × 2 vol. of dimethyl sulphoxide. The washed resin was then added to the liberated base solution and the mixture gently shaken for 24–48 h at room temperature in the dark. Subsequently, ethanolamine (2.4 mmol) was added and the incubation continued for 4 h. The gel was then extensively washed with dimethyl sulphoxide, distilled water and 50 mM-Tris/HCl, pH 7.4, containing 120 mM-NaCl and 5 mM-KCl (buffer A). The gel was stored in the dark in buffer A containing 0.02% NaN₃.

Membrane preparation. Crude membrane preparations of rat cerebral cortex were prepared as described previously [8].

Solubilization of the 5HT transporter. The 5HT transporter was solubilized from the membrane fractions with the non-ionic detergent digitonin as described previously [13]. Briefly, membranes were gently stirred for 1 h at 4 °C in buffer A containing 1% (w/v) digitonin, 2.23 mM-EDTA, 0.16 mM-benzethonium

EXPERIMENTAL

Materials

[³H]Paroxetine (925 GBq/mmol) was obtained from New

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; WGA, wheat-germ agglutinin; 5HT, 5-hydroxytryptamine.

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chloride, 1 mM-benzamidine hydrochloride, 100 μ M-PMSF and 16 m-units of aprotinin/ml at a protein concentration of 1.5–2.5 mg/ml. The mixture was then centrifuged at 48000 g for 1 h at 4 °C, the supernatant filtered through a 0.22 μ m-pore-size filter and the filtrate used as the source of solubilized 5HT transporter.

Affinity chromatography on citalopram-based agarose. In a routine experiment, 165–200 mg of solubilized membrane protein, corresponding to 100–150 pmol of [3 H]paroxetine-binding sites, were passed through a column containing 4 ml of citalopram-based-agarose resin at 4 °C at a maximum flow rate of 10 ml/h. The affinity resin was washed at 4 °C with at least 10 vol. of 10 mM-Tris/HCl buffer, pH 7.4, containing 250 mM-NaCl, 0.05 % (w/v) digitonin, 100 μ M-PMSF and 2.23 mM-EDTA (buffer B). The resin was further washed at 30 °C with 3 vol. of 10 mM-Tris/HCl, pH 7.4, containing 150 mM-KCl, 0.05 % digitonin, 100 μ M-PMSF and 1 mM-dithiothreitol (buffer C), followed by buffer C + 1 mM-litoxetine.

Affinity chromatography on WGA-Sepharese. The litoxetine eluate from the citalopram-based-agarose column was incubated with 1 ml of WGA-Sepharese for at least 5 h at 4 °C on a roller shaker. The WGA-Sepharese was then transferred to a column and washed at 4 °C with 10–15 vol. of buffer C, followed by buffer C containing 450 mM-*N*-acetylglucosamine.

Protein determination. Protein was determined by the method of Bradford [14]. The protein dye reagent and the pure BSA used as the standard protein were obtained from Bio-Rad (Paris).

Gel electrophoresis. Samples were concentrated to 80 μ l by using Amicon Centricon 30 filters and 40 μ l of SDS sample buffer [composition: 10 % SDS, 10 % (v/v) glycerol, 0.012 M-Tris/HCl, pH 6.8, and 5 % (v/v) β -mercaptoethanol] was added. The samples were then stored at –80 °C until electrophoresis. Subsequently, the samples were analysed on SDS/10%-polyacrylamide gels by the procedure of Laemmli [15]. After electrophoresis, the gels were fixed and silver-stained with the Bio-Rad silver-staining kit.

[3 H]Paroxetine binding. The binding of [3 H]paroxetine to membrane and digitonin-solubilized preparations was performed as described previously [13]. In the case of affinity-chromatography eluates, fractions were subjected to gel-exclusion chromatography on Pierce Excellulose GF-5 columns before performing the binding assays.

RESULTS

Purification of the 5HT transporter by affinity chromatography

In preliminary experiments, the behaviour of digitonin-solubilized 5HT-transporter preparations of rat cerebral cortex was examined upon chromatography on an agarose support to which a reduced citalopram derivative had been covalently coupled (Fig. 1). [3 H]Paroxetine-binding assays used to assess 5HT transporter activity revealed that this support adsorbed 65 % of the solubilized transporter applied. In contrast, an agarose support to which ethanolamine had been covalently coupled did not retain the 5HT transporter, as 100 % of the applied [3 H]paroxetine binding sites were recovered in the pass-through fraction from this column. Subsequently, this citalopram-based-agarose support was used as the affinity support to purify the 5HT transporter from digitonin extracts of rat cerebral-cortex membranes containing 100–150 pmol of [3 H]paroxetine-binding activity. At this scale the affinity-chromatography profile showed that most (typically 65–80 %) of the 5HT-transporter activity was absorbed by the support, whereas the bulk of the protein was unretarded (Fig. 2). The support was washed with high-ionic-strength buffer, and the

5HT transporter was eluted in the presence of 1 mM-litoxetine. The litoxetine eluate was then adsorbed on WGA-Sepharese, and, after extensive washing of this support, [3 H]paroxetine-binding sites were eluted with 450 mM-*N*-acetylglucosamine.

The data from a typical purification experiment are shown in Table 1. The recovery of [3 H]paroxetine-binding sites after solubilization of rat cerebral-cortical membrane preparations with 1 % (w/v) digitonin in the presence of a proteinase-inhibitor

Fig. 1. Chemical structure of the citalopram-based-agarose support

This affinity-chromatography support (c) was prepared by coupling the reduced citalopram derivative (a) to an *N*-hydroxysuccinimide-activated agarose gel as described in the Experimental section. Structure (b) is citalopram.

Fig. 2. Citalopram-based-agarose chromatography of digitonin-solubilized rat cerebral-cortex 5HT-transporter activity

Solubilized 5HT transporter (120 pmol of [3 H]paroxetine-binding sites), prepared as described in the Experimental section, was applied to a column containing 4 ml of the citalopram-based-agarose at a flow rate of approx. 10 ml/h. The column was then washed with buffers B and C, and the 5HT transporter was eluted with buffer C containing 1 mM-litoxetine as described in the Experimental section. Fraction volumes were 4 ml. The binding of [3 H]paroxetine to samples containing litoxetine was determined after subjecting samples to gel-exclusion chromatography on Pierce Excellulose GF-5 columns.

Table 1. Affinity-chromatography purification of the 5HT transporter of rat cerebral cortex

Typically, 100–150 pmol of solubilized 5HT transporter was extracted with 1 % digitonin from 80 ml of crude membranes prepared from 8 g of rat cerebral cortex. The solubilized extracts underwent successive affinity-chromatography steps on a citalopram-based-agarose resin and WGA-Sepharese as described in the Experimental section.

Preparation	³ H]Paroxetine binding (pmol/mg of protein)*	Total no. of ³ H]paroxetine binding sites (pmol)*		Yield (%)		Purification (fold)	
		Applied	Recovered	Stepwise	Overall	Stepwise	Overall
Crude membranes	0.53		159	100	100	1	1
Digitonin extract	0.62	159	130	82	82	1.2	1.2
Reduced citalopram derivative-agarose eluate	205†	113	14	12	10	331	386
WGA-Sepharese eluate	1100†	11	10	91	9	5.4	2070

* As measured in binding assays with 0.5 nM-³H]paroxetine.

† ³H]Paroxetine binding in eluates measured after samples had been subjected to gel-exclusion chromatography.

Fig. 3. SDS/PAGE of 5HT-transporter preparation after the final WGA-Sepharese step

5HT-transporter activity was solubilized from rat cerebral-cortical membranes as described in the Experimental section. The solubilized extracts were then divided into two equal batches. One batch was incubated for 1 h at 4 °C with 1 mM-citalopram. Both preparations then underwent parallel affinity-chromatography purifications on citalopram-based-agarose resin and WGA-Sepharese as described in the Experimental section, and equivalent samples of the final *N*-acetylglucosamine eluates were prepared for SDS/PAGE. The samples were electrophoresed on an SDS/10%-polyacrylamide gel, and the gel was subsequently fixed and silver-stained. The gel was calibrated with the following *M_r* markers: β -galactosidase (*M_r* 116000), phosphorylase *b* (*M_r* 97400), BSA (*M_r* 66000), ovalbumin (*M_r* 45000) and carbonic anhydrase (*M_r* 29000). Similar SDS/PAGE patterns were obtained by performing the above experimental protocol three times.

cocktail was 82 %. Affinity chromatography on the citalopram-based-agarose support gave a 331-fold purification with a yield of 12 %. The WGA-Sepharese chromatography resulted in 5.4-fold purification. Total purification of the 5HT transporter was about 2000-fold, with a final purity of 1.1 nmol of ³H]paroxetine-binding sites/mg of protein.

Table 2. Profile of ³H]paroxetine binding to various 5HT-transporter preparations

³H]Paroxetine binding to the membrane-bound, digitonin-solubilized and affinity-purified 5HT-transporter preparations was performed as described in the Experimental section. The competitive inhibition experiments were carried out with 0.2 nM-³H]paroxetine, with 6–8 concentrations of each inhibitor. *K_i* values were calculated from *IC*₅₀ values by using the Cheng-Prusoff correction [*K_i* = *IC*₅₀/(1 + *c*/*K_d*)], where *c* is the concentration of ³H]paroxetine and *K_d* the equilibrium dissociation constant]. The values shown represent means of 2–3 independent experiments: ND, not determined.

Agents	<i>K_d</i> or <i>K_i</i> of 5HT-transporter preparation (nM)		
	Membrane-bound	Solubilized	Affinity-purified
³ H]Paroxetine	0.15*	0.14*	0.15
Citalopram	1*	2.8*	7
Sertraline	1.26	ND	0.84
Litoxetine	3	ND	8
Imipramine	41*	57.8*	64
5HT	698*	466*	2500

* Values taken from [13].

SDS/PAGE of the affinity-purified 5HT transporter

This revealed a number of polypeptide bands upon protein staining (Fig. 3, lane A). In order to ascertain which of these polypeptide(s) corresponded to the 5HT transporter, a parallel purification was performed in which citalopram (1 mM) was added to solubilized receptor preparation before adsorption on to the citalopram-based agarose. Under these latter conditions no ³H]paroxetine-binding activity was subsequently eluted, and a major polypeptide band (*M_r* 73000) was found to be absent upon analysis by SDS/PAGE (Fig. 3, lane B). This indicates that the polypeptide of *M_r* 73000 is probably the 5HT-transporter protein.

Pharmacological profile of the affinity-purified 5HT transporter

The characteristics of ³H]paroxetine binding to the membrane-bound, solubilized and affinity-purified preparations are summarized in Table 2. Equilibrium saturation analysis of ³H]paroxetine binding to the affinity-purified 5HT-transporter preparation revealed the binding of this radioligand to a single class of high-affinity sites with a dissociation constant (*K_d*) similar to that displayed in the membrane-bound and solubilized

Fig. 4. High-performance steric-exclusion chromatography of affinity-purified 5HT-transporter activity

5HT-transporter-containing fractions after citalopram-based-agarose chromatography were concentrated to 200 μ l by Amicon Centricon-30 filtration. The concentrated 5HT transporter was chromatographed on one TSK-G4000 SW and one TSK-G3000 SW steric-exclusion column, linked in tandem. Mobile-phase conditions were 50 mM-Tris/HCl, pH 7.4, containing 120 mM-NaCl, 5 mM-KCl and 0.05 % digitonin at a flow rate of 0.5 ml/min. 5HT-transporter activity was located by [3 H]paroxetine-binding assays on the eluted fractions. The tandem-linked column was calibrated with the following protein standards: thyroglobulin (M_r 669000), aldolase (M_r 150000) and carbonic anhydrase (M_r 29000). The recovery of [3 H]paroxetine-binding sites was 45–55 % ($n = 3$ experiments).

preparations. In addition, in competitive-inhibition experiments 5HT and the selective 5HT-uptake inhibitors fluoxetine, litoxetine, sertraline and citalopram, and the tricyclic antidepressant drug imipramine, retained their potencies to inhibit [3 H]paroxetine binding to the affinity-purified preparation.

High-performance steric-exclusion chromatography

The litoxetine eluate obtained after the citalopram-based-agarose step was in some experiments subjected to high-performance steric-exclusion chromatography. Calibration of this chromatographic step with globular protein standards revealed that the 5HT transporter, assayed by [3 H]paroxetine binding, migrated as a digitonin-protein complex of M_r 260000 (Fig. 4).

DISCUSSION

In this paper we describe an affinity-chromatographic procedure to purify extensively the Na $^+$ -coupled 5HT transporter of rat cerebral cortex. The purification procedure involved sequential chromatography of digitonin-solubilized 5HT transporter on a citalopram-based-agarose support and WGA-Sephadex, which allowed purification within 55 h.

Affinity chromatography on the citalopram-based-agarose resin provided the principal purification step, with almost a 400-fold enrichment of [3 H]paroxetine-binding activity compared with that observed in the parent membrane preparation. This high degree of purification can be attributed to the following reasons. First, the citalopram-based-agarose resin exhibited a high capacity to adsorb digitonin-solubilized 5HT transporter (the resin removed 65–80 % of [3 H]paroxetine-binding sites from the applied solubilized preparations at a ratio of approx. 40 ml (50 mg) of detergent extracts/ml of resin). Interestingly, the reduced citalopram derivative used (Fig. 1a) for covalent coupling to Affi-Gel 10 retained a high affinity for the 5HT transporter ($K_i = 6$ nM for inhibition of [3 H]paroxetine binding to solubilized detergent extracts of rat cerebral cortex; D. Graham & S. Z. Langer, unpublished work). Presumably, further modification of this molecule by covalently coupling the amino group

through a long hydrophilic spacer to agarose did not have too dramatic an effect on its affinity for the 5HT transporter. A second reason for the effectiveness of this purification step might also be the use of a relatively high-ionic-strength (250 mM-NaCl) buffer to wash the support in order to remove non-specifically adsorbed proteins. Thirdly, the transporter was eluted in a Na $^+$ -free (KCl) buffer containing the selective 5HT-uptake inhibitor litoxetine. This elution was performed in a Na $^+$ -free buffer, as previous reports have indicated that the binding of radio-labelled 5HT-uptake inhibitors such as [3 H]imipramine [16], [3 H]paroxetine [16] and [3 H]citalopram [12] to the 5HT transporter necessitates the presence of Na $^+$. It should be noted that in our initial strategy chosen (results not shown) the elution was performed in a Na $^+$ -free buffer in the absence of litoxetine. However, because of the very limited degree of [3 H]paroxetine-binding activity recovered, we have decided to include the selective 5HT-uptake inhibitor litoxetine in the elution buffer. In addition, the elution was performed at 30 $^{\circ}$ C as opposed to 4 $^{\circ}$ C, as the higher temperature increased 3-fold the recovery of [3 H]paroxetine-binding sites (results not shown).

Chromatography on the immobilized lectin support WGA-Sephadex was chosen to purify the 5HT transporter further. This affinity-chromatography step permitted a 5.4-fold enrichment of [3 H]paroxetine-binding activity, with a recovery of 93 %. With the WGA-Sephadex chromatography step, the final purification of the 5HT transporter was about 2000-fold, corresponding to a [3 H]paroxetine-binding activity of 1.1 nmol/mg of protein. It should also be noted that the binding of the solubilized 5HT transporter to the lectin support demonstrates that this transporter is a glycoprotein.

The strategy to utilize a second affinity-chromatography step with WGA-Sephadex was chosen because our attempts to purify the transporter on ion-exchange resins (DEAE- and CM-Sephadex) gave almost no purification and very little recovery of [3 H]paroxetine-binding activity (H. Esnaud & D. Graham, unpublished work). For some experiments we also examined the possibility of further purification by high-performance steric-exclusion chromatography. This chromatographic step indicated that [3 H]paroxetine-binding activity was associated with a complex of M_r 260000. Nonetheless, the value does not indicate the true M_r of the native 5HT transporter, as it does not take into account the amount of digitonin bound to this protein, and in addition makes the assumption that the detergent-transporter complex is a globular moiety. However, this step permitted a purification factor of 4–5-fold (H. Esnaud & D. Graham, unpublished work) with a 45–55 % recovery of [3 H]paroxetine binding activity. In several experiments, therefore, an attempt was made to purify the 5HT transporter using the two affinity-chromatography steps, citalopram-based agarose and WGA-Sephadex, together with high-performance steric-exclusion chromatography. Unfortunately, this protocol resulted in very poor recovery of [3 H]paroxetine-binding activity (H. Esnaud & D. Graham, unpublished work), possibly due to the lengthy time factor involved in this procedure.

SDS/PAGE of the affinity-purified 5HT-transporter preparations revealed a number of polypeptide bands upon silver staining. This indicates that the purification protocol adopted provided only a partial purification. Nonetheless, when the detergent extracts were subjected to the purification protocol in the presence of a saturating concentration of free citalopram, a major polypeptide band of M_r 73000 was no longer present on analysis by SDS/PAGE. This suggests strongly that this polypeptide corresponds to the 5HT transporter protein. With an M_r of 73000, the 5HT transporter should bind 13.7 nmol of [3 H]paroxetine/mg of protein, assuming one paroxetine-binding site/molecule of transporter. This value is about 12-fold higher

than that found in our affinity-purified preparation. It is therefore noteworthy that the polypeptide pattern on SDS/PAGE of the affinity-purified preparation indicated that the M_r -73000 band corresponded to about 10% of the total protein stained. In this context, it must be pointed out that a polypeptide of M_r 73000 for the rat brain 5HT transport is of similar size to the recently claimed value for the human platelet 5-HT transporter (M_r = 78000; [17]). Also the glycosylated γ -aminobutyrate transporter, a functionally similar transporter which is probably derived from the same ancestral gene as the 5-HT transporter, is a polypeptide of similar M_r (80000; [18]). In addition, the deduced amino acid sequences from cloned cDNAs of the noradrenaline and γ -aminobutyrate transporters predict proteins of M_r of 69000 and 67000 respectively [19,20].

The integrity of the 5HT transporter after the affinity-chromatography purification protocol was verified by examining the properties of [3 H]paroxetine binding to this preparation. These data demonstrate that the specificity and potency of the selected compounds for the [3 H]paroxetine-binding site were maintained. Thus the substrate and 5HT-uptake-inhibitor binding domains of the 5HT transporter were conserved throughout the purification procedure.

Finally we wish to point out that, since the present work was completed, two papers have appeared describing the cloning of the 5HT transporter [21,22]. The open reading frames of these cDNA clones would predict proteins of M_r 73000 and M_r 68000 for the 5HT transporters present in rat basophilic leukaemic cells [21] and rat brain [22] respectively. The protein purification protocol described in the present study has now been adapted to permit 5HT-transporter purification starting from 36 g of rat cerebral cortex. It is hoped that isolation of the polypeptide band of M_r 73000 separated by SDS/PAGE of this larger-scale preparation will provide sufficient material for partial protein-sequencing data. Comparison of this data with the protein sequence predicted from the rat brain cDNA clone [22] will then permit confirmation that the polypeptide band of M_r 73000 purified by our affinity-chromatography protocol indeed corresponds to the 5HT transporter.

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