Solubilization and partial purification of the high affinity [^{3}H] imipramine binding site from human platelets

Moshe Rehavi[+], Phil Skolnick and Steven M. Paul*

Clinical Neuroscience Branch, NIMH and *Laboratory of Bioorganic Chemistry, NIADDK, National Institutes of Health, Bethesda, MD 20205, USA*

Received 29 October 1982

1. INTRODUCTION

High affinity binding sites for [^{3}H]imipramine have been identified and pharmacologically characterized in brain and platelet membranes [1–4]. Several lines of evidence have suggested that this site is structurally related to the uptake site for serotonin and that high affinity [^{3}H]imipramine binding can serve as a label of the serotonin transporter [4–7]. In order to determine the molecular properties of the high affinity [^{3}H]imipramine binding site and further characterize its structural relationship to the serotonin transporter, we initiated a series of experiments to solubilize and subsequently purify the binding site from human platelet membranes. The present report describes the solubilization of the [^{3}H]imipramine binding site from human platelets. The solubilized binding site has a pharmacological profile and cation requirement which is identical to the membrane-bound binding site. Optimal solubilization was achieved using the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) which is a zwitterionic derivative of cholic acid that has been successfully used for solubilization of other receptors [8,10]. Partial purification of the solubilized [^{3}H]imipramine binding site was carried out by gel filtration chromatography using 2-nitro[^{3}H]imipramine as a radioligand. 2-Nitro[^{3}H]imipramine is one of several derivatives of imipramine synthesized in our laboratory [11], that has both a high affinity for the [^{3}H]imipramine binding site and dissociates very slowly when incubations are done at 0–4°C [7,11,12].

[+] Present address: Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv, Israel

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Preparation of platelets*

Blood was collected from medication-free volunteers by venipuncture and gently mixed with an anticoagulent solution containing 16 mM citrate buffer and 1 mM EDTA. Platelets were isolated and platelet membranes were prepared as in [4,12].

2.2. *Solubilization*

Platelet membranes were disrupted using a Brinkman polytron (setting 7, 20 s) in 50 mM Tris–HCl (pH 7.4) containing 120 mM NaCl, 5 mM KCl (buffer A) and centrifuged at 15000 × *g* for 10 min. The membrane fragments were suspended to 10 mg protein/ml in the above buffer containing 0.5% (w/v) CHAPS. The mixture was homogenized with a glass homogenizer using a glass pestle (5 strokes) and the homogenate was kept for 30 min on ice (0–4°C) and then centrifuged at 100000 × *g* for 60 min. The resulting supernatant was used for the assay of solubilized imipramine binding sites. In other experiments labelling of the imipramine binding site with 2-nitro[^{3}H]imipramine prior to solubilization was carried out. Disrupted platelet membranes (1.5 mg

Published by Elsevier Biomedical Press
00145793/82/0000—0000/$2.75

protein/ml) were incubated with 4 nM 2-nitro[^{3}H]imipramine (spec. act. 73.6 Ci/mmol, NEN, Boston MA) in buffer A with or without 10 μM imipramine at 22°C for 30 min. Following incubation, membranes were centrifuged at 20000 × g for 15 min and the supernatant containing unbound 2-nitro[^{3}H]imipramine was discarded. The pellet was solubilized as above.

2.3. *[^{3}H]Imipramine binding*

To determine [^{3}H]imipramine binding to the solubilized membrane preparation the incubation mixture contained 100 μl solubilized membranes, 100 μl [^{3}H]imipramine (spec. act. 29.8 Ci/mmol, NEN, Boston MA) at final conc. 2 nM (unless otherwise stated), 750 μl buffer A and 50 μl of the same buffer with or without chlorimipramine (500 nM). Chlorimipramine (500 nM) was added to determine non-specific binding. After incubation at 0°C for 60 min, 100 μl 10% (w/v) activated charcoal in buffer A containing 2% (w/v) bovine serum albumin was added and the mixture rapidly mixed. The samples were then maintained for 5 min on ice and subsequently centrifuged at 1500 × g for 10 min. An aliquot (800 μl) of the supernatant was counted for radioactivity using a liquid scintillation counter. Separation of protein, bound from free 2-nitro[^{3}H]imipramine, is efficiently carried out using this procedure (unpublished).

2.4. *Gel filtration chromatography*

Sepharose CL-6B was equilibrated with buffer A containing 0.05% (w/v) CHAPS and packed in a 53 × 1.7 cm glass chromatography column. A 2 ml sample of the soluble fraction obtained, following preincubation of platelet membranes with 2-nitro[^{3}H]imipramine, was applied to the column. The column was eluted in buffer A containing 0.05% CHAPS at 4°C and at a flow-rate of 0.15 ml/min. Fractions (1 ml) were collected using an automatic fraction collector and samples (100 μl) from each fraction were counted for radioactivity in a liquid scintillation counter as in [7,11].

3. RESULTS

The binding of [^{3}H]imipramine to the solubilized platelet membranes is saturable and of high affinity (fig. 1). In contrast, non-specific binding increased linearly with increasing concentrations of [^{3}H]imipramine (not shown). At 2 nM, the specific binding of [^{3}H]imipramine was 60% of the total binding. Scatchard analysis indicated a single population of binding sites with an apparent dissociation constant (K_d) of 1.2 nM and maximal number of binding sites (B_{max}) of 175 fmol/mg protein. The apparent K_d value of [^{3}H]imipramine binding to solubilized membranes (1.2 nM) is identical to the value obtained in intact platelet membranes (1.2 nM) [4], whereas the B_{max} value observed in the solubilized fraction was 30% of the value obtained in platelet membranes [4]. Specific [^{3}H]imipramine binding to the soluble fraction was heat-sensitive, since preheating the solubilized binding site to 50°C for 15 min prior to assay reduced specific binding by >90%.

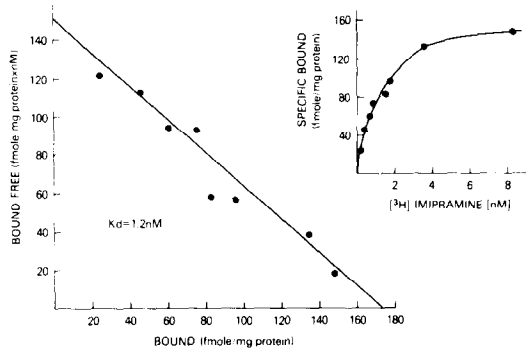

Fig. 1. Scatchard analysis of [^{3}H]imipramine binding to CHAPS-solubilized platelets. Binding of various concentrations of ligand was carried out at 0°C for 60 min; non-specific binding was measured in the presence of 500 nM chlorimipramine. Bound [^{3}H]imipramine was separated from free [^{3}H]imipramine using the charcoal method as described in the text. Inset: saturation isotherm of specific [^{3}H]imipramine binding. Values are from a representative exp. repeated 3-times with identical results.

[^{3}H]Imipramine binding to the solubilized fraction was also found to be sensitive to trypsin. Preincubation of the solubilized membrane fraction with trypsin (1 mg/ml) for 30 min at 25°C resulted in a 60% decrease of specific binding. Specific binding was also found to be sodium-dependent, since increasing the [Na^+] from 10–320 mM resulted in a highly significant increase (fig. 2) in specific

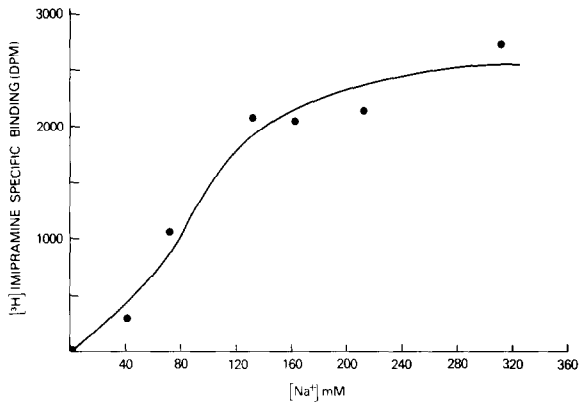

Fig. 2. Na^+ dependency of [^{3}H]imipramine binding to CHAPS-solubilized platelets. Specific binding was measured in the presence of various concentrations of sodium using 2 nM [^{3}H]imipramine and 500 nM chlorimipramine for measuring non-specific binding. The potassium ion concentration was kept constant (5 mM). Points represent the means of 2 exp. each done in triplicate.

Table 1

Effects of drugs on specific [^{3}H]imipramine binding to solubilized and membrane-bound binding sites [IC_{50} (nM)]

Compound	Solubilized	Membrane-bound
Imipramine	7	3
Chlorimipramine	7	2
Amitriptyline	10	17
2-Hydroxy-imipramine	15	44
Norzimelidine	27	64
Nortriptyline	70	74
Desipramine	120	130
Zimelidine	7000	1500
Atropine	>10000	>10000
Phentolamine	>10000	>10000

Specific [^{3}H]imipramine binding to solubilized and membrane-bound binding sites [4] was carried out as described (text, [6,7]). IC_{50} values, representing the concentration of unlabelled drug which inhibited 50% of the specific binding of [^{3}H]imipramine (2 nM), were calculated from displacement curves using 6 concentrations of unlabelled drug. Values represent the mean of 3 separate exp. with SEM < 20%

[^{3}H]imipramine binding. These results are identical to those observed using intact membranes, and represent an increase in the apparent affinity (decreased K_d) of [^{3}H]imipramine in the presence of sodium (unpulished).

To characterize the pharmacological profile of the solubilized binding site, a series of antidepressants and other drugs were studied for their potency in inhibiting [^{3}H]imipramine binding to the soluble fraction. Table 1 shows the IC_{50}-values for 10 drugs in inhibiting the binding of [^{3}H]imipramine (2 nM) to the solubilized binding site. The relative potencies of these drugs are very similar to that obtained (table 1) using intact platelet membranes [4].

An estimation of the molecular size of the detergent-solubilized binding site was obtained by gel filtration chromatography of the receptor–ligand complex using a calibrated Sepharose CL-6B column. Fig. 3 shows the elution profile of the solubilized fraction obtained from 2-nitro[^{3}H]-imipramine preincubated platelets. The bulk of bound 2-nitro[^{3}H]imipramine radioactivity, which was sensitive to displacement by an excess of unlabelled imipramine (10 μM), migrated in a position betweeen that of the marker protein thyroglobulin (M_r 660000) and the void volume, indicating a relatively large M_r complex. When preincubation of platelets with 2-nitro[^{3}H]imipramine was carried out in the presence of 10 μM unlabelled imipramine, the radioactivity of this peak was dramatically diminished while the radioactivity of the second peak remained unchanged (fig. 3)

4. DISCUSSION

This study reports the successful solubilization of high affinity [^{3}H]imipramine binding sites from human platelets. The solubilized binding sites have virtually identical properties and characteristics of the membrane-bound binding sites. Specific binding in the solubilized preparation is of high affinity with an app. K_d which is identical to that found in intact platelet membranes [4]. Furthermore, the pharmacological profile of the solubilized binding site is also very similar to that of the membrane-bound binding site. The sodium

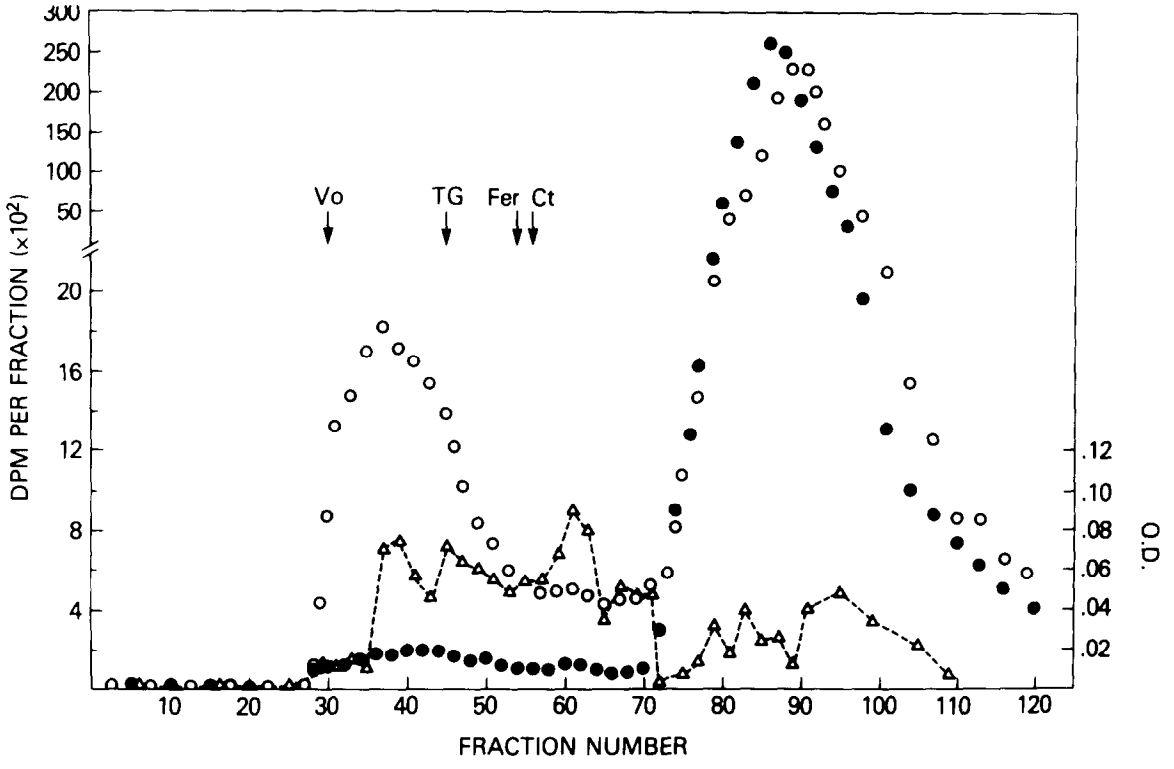

Fig. 3. Sepharose CL-6B gel filtration chromatography of the CHAPS-solubilized extract of 2-nitro[^{3}H]imipramine-preincubated platelet membranes: 2 ml of the 0.5% CHAPS extract was applied to Sepharose CL-6B column and eluted at 0°C with 0.05% CHAPS in buffer A as described in section 2. Platelet membranes were preincubated with 2 nM 2-nitro[^{3}H]imipramine with (●) or without (○) 10 μM unlabelled imipramine, prior to the solubilization. Protein concentrations (Δ) were measured as in [13]. V_0 is the void volume, and elution volumes for thyroglobulin (TG), ferritin (Fer) and catalase (Ct) are shown. The figure represents a typical elution profile of 3 separate exp.

dependency of [^{3}H]imipramine binding to both the soluble and membrane-bound sites [5] suggests that the sodium recognition or regulatory site associated with the high affinity [^{3}H]imipramine binding site is retained in an active form during solubilization. These data suggest that many (if not all) of the properties of this binding site are unaltered during solubilization and that the detergent CHAPS is capable of substituting for the membrane lipids in maintaining the active conformation of the site. Since the binding is very sensitive to both heat and proteolytic enzyme(s) it is likely that a protein is a major constituent of the [^{3}H]imipramine binding site. The size of this protein, estimated by gel filtration chromatography, is $>M_r$ 700 000. However, detergent molecules, lipids, and associated proteins, other than the imipramine binding site, may contribute to the large size of this species.

Using digitonin as detergent and a flow-dialysis method for measuring [^{3}H]imipramine binding, solubilization of the serotonin 'transporter' from porcine platelets was reported [14]. It is unclear however, if the solubilized site reported here is identical to the site reported by these investigators since there is a large difference in the affinities of [^{3}H]imipramine for the two sites. Here, we observed very high affinity binding to the solubilized site with an app. K_d = (1.2 nM) which is identical to the apparent dissociation constant of the membrane-bound site. In contrast, [14] reported the apparent affinity of [^{3}H]imipramine for the solubilized site

to be almost 40-times lower (K_d = 45 nM). This discrepency may also relate to a species difference between porcine and human platelet membranes. Nevertheless, in human platelets we have observed a much more efficient solubilization of [^{3}H]imipramine binding sites with CHAPS, as compared to digitonin (unpublished). Reconstitution of the solubilized sites into liposomes followed by measuring both [^{3}H]imipramine binding and [^{3}H]serotonin uptake in the reconstituted organeles is important in understanding the molecular components of the serotonin transporter complex.

REFERENCES

[1] Raisman, R., Briley, M.S. and Langer, S.Z. (1979) Nature 281, 148–150.
[2] Rehavi, M., Paul, S.M., Skolnick, P. and Goodwin, F.K. (1980) Life Sci. 26, 2273–2279.
[3] Langer, S.Z., Briley, M.S., Raisman, R., Henry, J.F. and Morselli, P.L. (1980) Naun. Schmied. Arch. Pharmacol. 313, 189–194.
[4] Paul, S.M., Rehavi, M., Skolnick, P. and Goodwin, F.K. (1980) Life Sci. 26, 953–959.
[5] Langer, S.Z., Moret, C., Raisman, R., Dubocovich, M.L. and Briley, M., (1980) Science 210, 1133–1135.
[6] Paul, S.M., Rehavi, M., Rice, K.C., Ittah, Y. and Skolnick, P. (1981) Life Sci. 28, 2753–2760.
[7] Rehavi, M., Ittah, Y., Rice, K.C., Skolnick, P., Goodwin, F.K. and Paul, S.M. (1980) Biochem. Biophys. Res. Commun. 99, 954–959.
[8] Hjelmeland, L.M. (1980) Proc. Natl. Acad. Sci. USA 77, 6368.
[9] Sigmonds, W.F., Koski, G., Streaty, R.A., Hjelmeland, L.M. and Klee, W.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4623–4627.
[10] Lew, J.Y., Fong, J.C. and Goldstein, M. (1981) Eur. J. Pharmacol. 72, 403–405.
[11] Rehavi, M., Ittah, Y., Skolnick, P., Rice, K.C. and Paul, S.M. (1982) Naun. Schmied. Arch. Pharmacol. 320, 45–49.
[12] Rehavi, M., Tracer, H., Rice, K., Skolnick, P. and Paul, S.M. Life Sci. in press.
[13] Costa, J.L., Murphy, D.L. and Kafka, M.S. (1977) Biochem. Pharmacol. 26, 517–521.
[14] Lowery, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[15] Talvenheimo, J. and Rudnick, G. (1980) J. Biol. (1983) Chem. 255, 8606–8611.