Identification of a specific, high affinity imipramine-binding protein from human and rat platelet membranes

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A specific, [^{3}H]-imipramine-binding protein (IBP) of high affinity has been identified in human and rat platelets. This binding protein was isolated in active form using digitonin as solubilizing agent in presence of suitable protease inhibitors (leupeptin, PMSF, and EGTA). Digitonin effectively solubilized 60–70% and 55–60% of IBP from human and rat platelet membranes respectively. Displacement of [^{3}H]-imipramine with pharmacological agents was similar in membrane bound and solubilized receptor. The solubilized receptor retained specific imipramine-binding activity after blotting on nitrocellulose. Electrophoretic runs of solubilisate after transfer to nitrocellulose exhibited specific, high affinity binding to a band in the 98 K dalton area. The approach used for the identification of the IBP in this study is not only rapid and convenient but is also highly specific and could be used for identification of other receptor proteins for their preliminary characterization.

SEVERAL investigators have suggested the use of [^{3}H]-imipramine, considered to label the serotonin transporter complex, as a biological marker in major depressive illness and associated states[1]. The physiology[2], localization[3], pharmacology[4], heterogeneity[5] and clinical applications[6, 7] of the imipramine-binding site has been the subject of intensive study by numerous workers during the last decade.

Whether imipramine labels the 5-HT carrier protein directly or a different modulator protein is a matter of debate[8–11] and makes imipramine-binding protein (IBP) an attractive macromolecule for molecular characterization. Among the few attempts made in this direction, gel filtration techniques have not proved fully conclusive[12] and molecular size determination by radiation target analysis which provides molecular dimensions as rough approximations[13] requires confirmation by other procedures. Therefore, in the present investigation we have attempted a more specific approach to identify the agent solubilized native [^{3}H]-imipramine-binding protein from human platelets by radioligand binding and displacement on nitrocellulose. In addition, this binding protein from rat platelets was characterized to investigate whether heterogeneity in protein nature exists in rat and human blood platelets.

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Methods

[^{3}H]-Imipramine hydrochloride (25 ci/mmol) was purchased from Amersham International UK, desipramine, digitonin, sodium cholate, sodium deoxycholate, CHAPS, Triton X-100 and Nonidet P-40 were obtained from Sigma Chemicals Co., USA.

Preparation of platelet rich plasma

Human blood was obtained from medication-free volunteers by venipuncture and mixed with acid citrate dextrose (ACD) as anticoagulant. Rat blood was collected from the jugular vein of adult animals of Wistar strain into polypropylene tubes containing 3.8% sodium citrate (1 ml sodium citrate for 9 ml blood). Platelet rich plasma (PRP) was prepared by centrifuging blood at 200 *g* for 10 min.

Preparation of platelet membrane

PRP was centrifuged (18,000 *g* for 10 min) for platelet isolation and platelets were processed for membrane preparation by the method of Davis *et al.*[14]. The final membrane pellets were suspended in 50 mM Tris, 120 mM NaCl, 5 mM KCl and 5 mM EDTA, pH 7.4. The mean protein concentration was approximately 2–3 mg/ml. Membranes were stored at –20°C till further use. Such storage of the membrane was not found to alter the receptor binding.

Solubilization of protein

All agents were prepared in buffer with mild stirring. The solubilizing buffer was supplemented with 2 mM

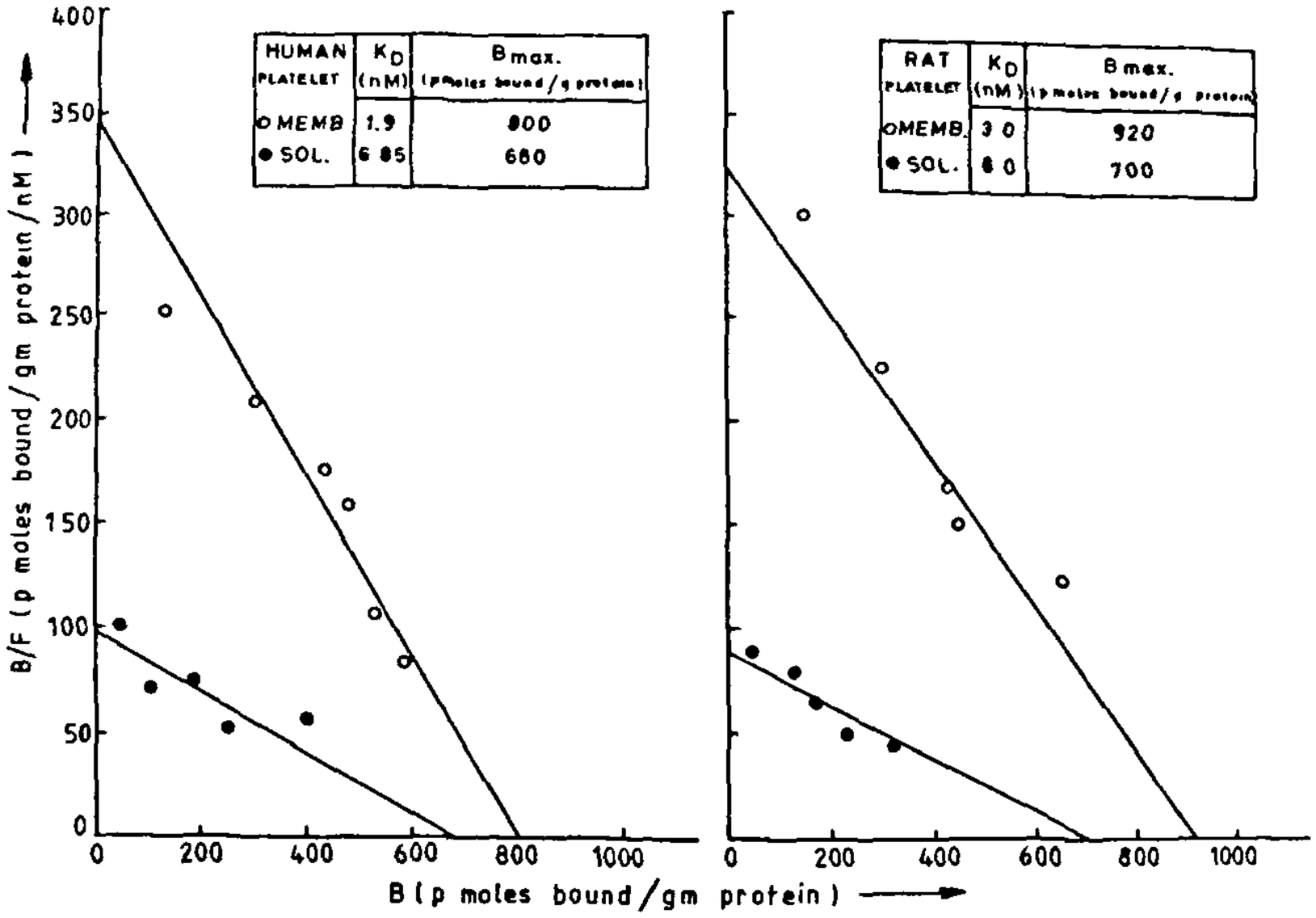


Figure 1. Scatchard plots for membrane and digitonin solubilized fractions of human and rat platelets.

Table 1. Binding of [^{3}H]-imipramine to human and rat platelet membranes and solubilisates (Sol.) obtained with various agents

Fraction	p moles [^{3}H]-imipramine bound/g protein Human	Rat
Membrane	565 ± 48 0	651 ± 51 4
Digitonin Sol	353 ± 23.3	388 ± 29 1
Na cholate Sol.	182 ± 12 3	152 ± 12 92
CHAPS Sol.	63 ± 5 48	64 ± 6 01
Triton X-100 Sol	ND	ND
Na deoxycholate Sol	ND	ND
Nonidet P-40 Sol.	ND	ND

Represented values are mean ± S.E. of three separate experiments
ND, Binding not detectable

PMSF, 50 µg/ml leupeptin and 2 mM EDTA. Platelet membrane aliquots were gently agitated after addition of solubilizing buffer at 4°C for 60 min. Final concentration of agents ranged from 1 to 2% to screen optimum agent concentration. Solubilisates were obtained after removal of unsolubilized material by ultracentrifugation at *105,000 g* for *1* h at 4°C. *Supernatant solubilisates* were immediately used for binding.

[^{3}H]-Imipramine binding to membranes and *solubilisates*

[^{3}H]-Imipramine binding was done following the procedure of Davis *et al* [14], with minor alterations. The binding assay (final volume 300 µl) consisted of 50 µl assay buffer, 50 µl of [^{3}H]-imipramine (5 nM) and 200 µl membrane (approx. 300–500 µg protein) or solubilisate (approx. 160–180 µg) protein. Parallel incubations were carried out with tubes also containing desipramine (100 µM) as the displacing agent to determine non-specific binding. After one hour of incubation at 4°C, 5 ml cold assay buffer was added and contents were rapidly filtered over Whatman GF/B glass fibre filters. The filters were washed twice with 5 ml of cold assay buffer. Solubilisates were incubated overnight and recovery of solubilized protein with bound radioactivity was measured as described by Bruns *et al.*[15], employing GF/B filters presoaked in polyethylenimine.

Radioactivity was determined after extraction into 10 ml scintillation cocktail using a Rack β-scintillation counter having a 50% efficiency for [^{3}H].

Blot analysis of solubilisates on nitrocellulose

To further analyse the retention of imipramine-binding activity of solubilisate, blot analysis was performed using 2 cm^2 nitrocellulose squares. These were incubated with solubilisate (200 µl) for 30 min at 4°C. The squares were dried for 1–2 h followed by repeated buffer washing to remove excess unbound protein and then incubated at 4°C with 5 nM [^{3}H]-imipramine, overnight. In parallel experiments, radiolabelled imipramine was mixed with cold desipramine (100 µM). After three washings in incubation buffer, the squares

were suspended in scintillation cocktail and counted. Squares of nitrocellulose without solubilisate were similarly incubated as above which served as controls to define non-specific binding of imipramine to nitrocellulose and was subtracted from radioactivity bound by solubilisate.

Polyacrylamide gel electrophoresis

Solubilized protein was subjected to electrophoresis on native gels as described by Davis[16]. After the electrophoretic run, proteins were electrically transferred to nitrocellulose sheets in Tris-glycine buffer, pH 8.3 for 20 h at 25 mA at 4°C. These sheets were treated with [^{3}H]-imipramine (5 nM) alone, or along with an excess of cold desipramine (100 µM). Further processing for the binding assay was as described above.

Protein contents were estimated by the method of Markwell *et al*[17] using bovine serum albumin as standard.

Results

Among the various agents screened in this study, digitonin proved to be the most appropriate for human and rat blood platelets as indicated by yield of the solubilized specific binding sites as a function of protein (Table 1). Sodium cholate and CHAPS were not as effective as digitonin. Digitonin could solubilize approximately 60–70% and 55–60% of IBP in active form in human and rat platelet membranes respectively, whereas solubilization of the binding protein by cholate was 20% and 25% and by CHAPS was 10% and 9% respectively from human and rat platelet membranes.

Scatchard analysis of membrane fractions and digitonin solubilisates indicated a single population of binding sites with K_D and B_{max} values as given in Figure 1.

A decrease in ligand affinity and maximum number of binding sites (B_{max}) was observed in human and rat digitonin solubilisates. However, the displacement profile of specific [^{3}H]-imipramine binding in presence of various pharmacological agents in membrane bound and solubilized fractions of human and rat platelet confirms the intactness of ligand-binding site as IC_{50} values obtained were not markedly different (Table 2).

Table 3 shows specific [^{3}H]-imipramine binding to digitonin solubilisates of human and rat platelet membranes after blot analysis. Background counts were reduce
buffer

Table 2. Displacement of [^{3}H]-imipramine from membrane bound [M] and solubilized [S] binding sites of human and rat platelets using pharmacological agents (IC_{50} values represent concentration of the drug required to inhibit 50% of the specific binding of [^{3}H]-imipramine (5 nM) and were calculated from displacement curves obtained from 5 separate platelet preparations)

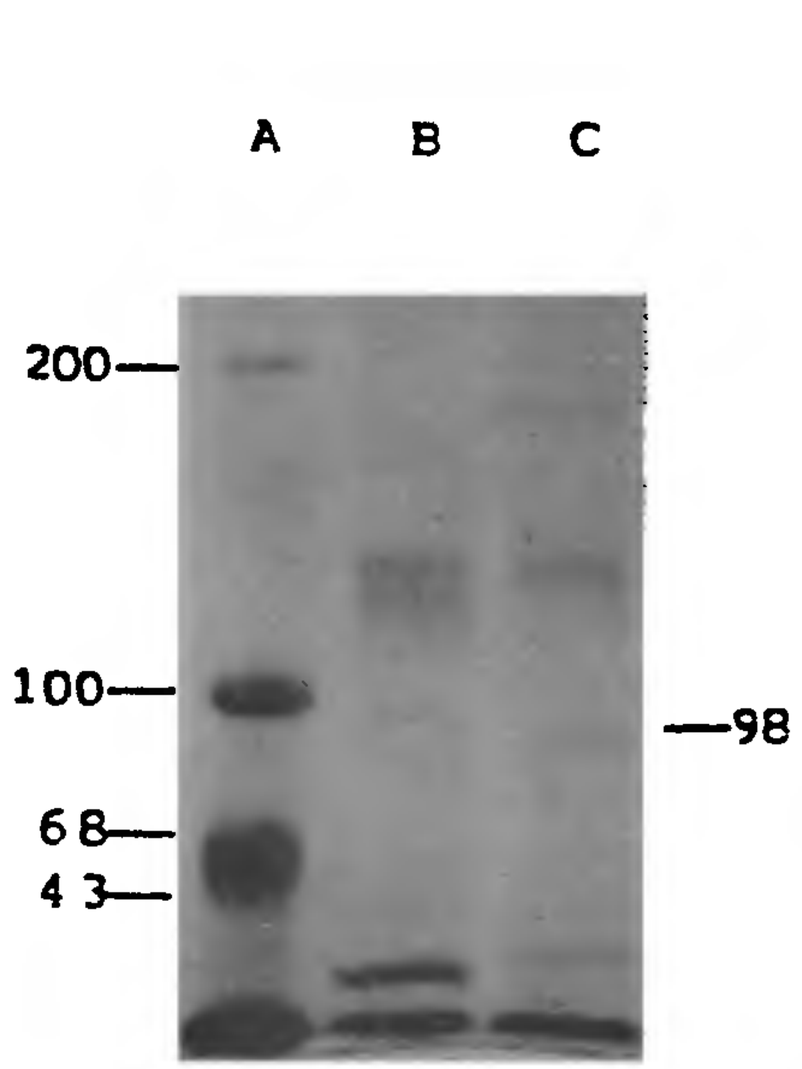
	IC_{50} (nM)			
	Human		Rat	
Drug	M	S	M	S
Imipramine	12	23	8	21
Amitryptaline	35	50	28	42
Nortryptaline	80	95	22	88
Desipramine	130	390	179	401
5-Hydroxy-tryptamine	5200	6500	4900	6320
Haloperidol	> 100,000	>100,000	>100,000	>100,000

Standard errors were [< 10%] of the mean

Table 3. [^{3}H]-Imipramine binding to digitonin solubilisate after blotting on nitrocellulose

	(cpm* on nitrocellulose)		
Solubilisate	Total	Non-specific	Specific
Human platelet	3421 ± 307	1600 ± 120	1821 ± 147
Rat platelet	2909 ± 258	1421 ± 109	1488 ± 122

*Values are mean ± S.E. of 5 blot replicates and represent counts obtained after pretreatment of nitrocellulose with 5% casein, which reduces the non-specific binding of [^{3}H]-imipramine to nitrocellulose by 40–50%.



Figur
C) platelets electrophoresed in native gels (7%) and then transferred to nitrocellulose sheets, 98 K band demonstrated specific, displaceable binding (50–60%) of [^{3}H]-imipramine (5 nM) [total binding (cpm): 2855.6 (B), 2167.2 (C); non-specific binding (cpm) in presence of 100 µM desipramine; 1134.4 (B), 998.7 (C)] while other bands demonstrated negligible displaceable binding. Lane A shows non-denatured molecular weight standards.

Figure 2 shows distribution of protein bands in digitonin solubilisates of human and rat platelets. The band corresponding to an approximate molecular weight of 98 kD exhibited appreciable (50–60%) displaceable, high affinity [^{3}H]-imipramine binding after transfer on nitrocellulose.

Discussion

Several workers have attempted to isolate the IBP by solubilization with agents like CHAPS[18], cholate[12] and digitonin[14], but no data are available on the comparative effects of these agents. In this study using six different agents, we obtained maximum recovery of IBP activity in digitonin solubilisates and therefore further experiments were conducted with these fractions. The decrease in ligand affinity observed in digitonin solubilisates might be due to conformational changes in the receptor molecule as a result of alterations in its micro environment, as suggested by Shreeve for decrease in affinity following agent solubilization of alpha-adrenoceptor[12]. However, the specificity of solubilized receptor protein to bind radiolabelled imipramine was not affected as evident from pharmacological displacement data and blot analysis. Previously, we have also observed similar temperature sensitivity and Na^+ dependency of digitonin solubilized and membrane bound protein for binding [^{3}H]-imipramine[19], indicating preservation of binding criteria of native protein after solubilization. Davis and coworkers used gel filtration techniques to characterize the binding protein after solubilization, using irreversible ligand, with results not to their satisfaction since data indicated over estimation[20]. Use of a ligand which may firmly bind to the receptor molecule may not help in characterization of the specific receptor protein alone, since the labelling of non-specific sites cannot be ruled out. Therefore, an ideal approach would be to use a ligand which binds specifically with high affinity and can be displaced. The use of radioligand binding for identification of specific protein band(s) on nitrocellulose has been employed before for estimation of approximate molecular weight of a particular protein[21]. In our investigation, we could successfully utilize this technique to identify the approximate molecular weight of IBP of two different species.

The approximate molecular weight of IBP appears to be 98,000 daltons, as an appreciable (50–60%) high affinity, displaceable binding was obtained to this band in both human and rat platelet preparations. Approximate size of human IBP as determined by Mellerup and coworkers[13] by irradiation technique was 86,000 daltons, which is closely consistent with the value obtained in our investigation.

Conclusion

In a step towards molecular characterization of the imipramine binding protein, we have attempted to identify its approximate molecular weight by a convenient and sensitive procedure, irrespective of the requirement of an irreversible binding ligand of high energy and minimizing the artifacts of non-specific binding. A necessary precaution involves, the exclusion of denaturing conditions such as SDS, pH extremes and high temperatures. Such a procedure/technique could be utilized for estimating approximate molecular weights of native receptor proteins effectively before proceeding further for purification to homogeneity.

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ACKNOWLEDGEMENTS We thank the Director, Industrial Toxicology Research Centre, Lucknow, for his keen interest in this work Renu Sah is a recipient of Senior Research Fellowship from CSIR, New Delhi

Received 8 September 1993, accepted 18 August 1994