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Identification of a dopamine-binding protein on the membrane of the human platelet

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The binding of [^{3}H]dopamine to platelet membranes has been examined in an attempt to identify the putative dopamine-uptake mechanism of the platelet. [^{3}H]Dopamine has been shown to bind to a 42000 Da glycoprotein in platelet membrane with high affinity (K_d = 22.6 nM) and binding of [^{3}H]dopamine was competed for by dopamine, molecules with catechol moieties, 5-hydroxytryptamine, GSH and ascorbic acid. Differences in pharmacological profile and molecular mass suggest that [^{3}H]dopamine does not bind to a known receptor, a neuronal-type dopamine transporter or the platelet 5-hydroxytryptamine-uptake site. It is proposed that this novel binding site for dopamine, which has been purified 1000-fold from particulate platelet membrane, is likely to be a component of the dopamine-uptake mechanism of the human platelet.

INTRODUCTION

[^{3}H]Dopamine uptake by platelets occurs by a temperature- and energy-dependent process [1] that does not involve a known dopamine receptor [2,3] or a neuronal-like dopamine transporter [4]. Furthermore, altered dopamine uptake by platelets has been reported in subjects with Parkinson's disease [5], Huntington's chorea [6] and schizophrenia [7], diseases thought to be associated with altered dopaminergic activity in the brain. A better understanding of the platelet dopamine-uptake mechanism, which is distinct from the platelet imipramine-sensitive 5-hydroxytryptamine-uptake mechanism [8], could lead to insights into biochemical changes underlying these diseases.

Radioactive derivatives of drugs which bind to dopamine receptors [9] or to the neuronal dopamine transporter [10] have been used to identify these molecules on the neuronal membrane. However, these agents do not affect dopamine uptake by the platelet [3,4] and would therefore be unlikely to bind to the dopamine-uptake site of the platelet. Thus, presuming that radioactive dopaminergic drugs would not be of use in identifying the dopamine-uptake mechanism of the human platelet and, on the assumption that the platelet dopamine-uptake mechanism would bind dopamine, the binding of [^{3}H]dopamine to the platelet membrane was studied.

MATERIALS AND METHODS

Materials

The drugs bromocriptine, mazindol (Sandoz Australia Pty. Ltd), domperidone, spiperone (Janssen Cilag Pty. Ltd.), nomifensine (Hoechst Australia), quinpirole hydrochloride (Eli Lilly and Co.), raclopride (Astra Research Centre), RU 24926 (Roussel Pharmaceuticals Pty. Ltd.) and SCH 23390 (Essex Laboratory Pty. Ltd.) were kindly donated by the companies as indicated. The chemicals benzene-1,2,4-triol, DHPA, hydroquinone and resorcinol were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. EDTA, GSH, Tris and NaCl were obtained from BDH Chemicals, Poole, Dorset, U.K. ADTN, (+)-butaclamol, (−)-butaclamol, 8OH DPAT, fluphenazine, GBR 12909, glutamate, glycine, metoclopramide, pimozide, (+)-SKF 38393, (−)-SKF 38393, (±)-sulpiride, thioridazine, thiothixene and trifluoperazine were purchased from Research Biochemicals Inc., Natick, MA, U.S.A. 3,4-[7-^{3}H]Dihydroxyphenethylamine was purchased from Du Pont (Australia) Ltd., Sydney, N.S.W., Australia. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of crude particulate platelet membrane

Bags of expired platelet concentrates were obtained from the Red Cross Blood Bank, Melbourne, Australia. The contents of the bags were pooled, centrifuged at 100 *g* for 15 min at 4 °C to remove red and white blood cells, and the supernatant of the platelet concentrate was collected. The platelet concentrate was then centrifuged at 9000 *g* ($r_{av.}$ 98 mm) for 6 min at 4 °C, the supernatant was discarded and the platelet pellets were washed with 3 × 10 vol. of Tris buffer (25 mM-Tris, 120 mM-NaCl, 2.5 mM-EDTA, 100 μM-PMSF, pH 7.7). The platelets were lysed by homogenization, with a Janke and Kunkel Ulta-Turrax homogenizer with a S25N-10G dispersing tool, into 10 vol. of lysing buffer (5 mM-Tris, 2.5 mM-EDTA, 100 μM-PMSF, pH 7.7), and the particulate membrane was precipitated by centrifugation at 20000 *g* ($r_{av.}$ 70 mm) for 15 min at 4 °C. The crude particulate membrane was washed with 3 × 10 vol. of Tris buffer, suspended in 2 vol. (w/v) of Tris buffer and stored at −70 °C until required.

Binding of [^{3}H]dopamine by crude particulate membrane

To determine the effect of incubation temperature, [^{3}H]dopamine (10 nM) binding to platelet membrane was measured in the absence or presence of 50 μM-dopamine after incubations at 37 °C for 30 or 60 min, at 25 °C for 60 or 120 min and at 4 °C for 60 min or overnight. The bound and free [^{3}H]dopamine were separated by filtration through Whatman GF/C filter discs. The filter discs were then washed with 3 × 5 ml of 0.9% NaCl and placed into liquid-scintillation vials; 5 ml of

Abbreviations used: ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene; 8OH DPAT, (±)-2-dipropylamino-8-hydroxy-1,2,3,4-tetrahydronaphthalene; PMSF, phenylmethanesulphonyl fluoride; L-DOPA, L-3,4-dihydroxyphenylalanine; DHPA, dihydroxyphenylacetic acid.

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Table 1. Specificity and class of compounds tested at 50 μM for an effect on the binding of [^{3}H]dopamine to platelet membranes

Dopamine-receptor agonists:	dopamine, ADTN, quinpirole, RU 24926, bromocriptine, (+)-SKF 38393, (−)-SKF 38393
Dopamine-receptor antagonists:	SCH 23390, domperidone, haloperidol, raclopride, spiperone, (+)-butaclamol, (−)-butaclamol, fluphenazine, pimozide, (±)-sulpiride, thioridazine, thiothixene, trifluoperazine
Antagonists of other receptors:	8OH DPAT, clonidine, metoclopramide, propranolol
Inhibitors of neuronal dopamine transport:	cocaine, GBR 12909, maprotiline, mazindol, benztropine, nomifensine
Inhibitors of other neuronal neurotransmitter transporters:	desmethylimipramine, imipramine, reserpine
Neurotransmitters:	adrenaline, noradrenaline, acetylcholine, γ-aminobutyric acid, glutamate, histamine, glycine, 5-hydroxytryptamine
Compounds with some structural similarity to dopamine:	benzene-1,2,4-triol, ethylene glycol, glucose, glycerol, homovanillic acid, L-DOPA, phenol, propranolol, resorcinol, tryptophan, tyrosine, 3-iodotyrosine, 3,5-di-iodotyrosine, indole, indole-3-acetic acid, isoprenaline, hydroquinone, apomorphine, 6-hydroxydopamine, DHPA, catechol
Reductants and oxidants:	GSH, GSSG, ascorbic acid, 2-mercaptoethanol, dithiothreitol

Beckman Ready Protein liquid-scintillation cocktail was added and the vials were left at room temperature for 1 h. The radioactivity in each vial was then counted and specific binding of [^{3}H]dopamine taken as the binding of [^{3}H]dopamine in the absence of unlabelled dopamine minus the binding in the presence of 50 μM-dopamine.

To determine the rate of association of [^{3}H]dopamine with the platelet membrane, the specific binding of [^{3}H]dopamine was measured at 4 °C after various incubation periods ranging from 1 min to 32 h. In addition, the dissociation of [^{3}H]dopamine from the platelet membrane was measured by first incubating platelet membrane and [^{3}H]dopamine overnight at 4 °C. The membrane was then precipitated by centrifugation at 2000 *g* for 15 min at 4 °C and the supernatant removed. The membrane was reconstituted in 10 ml of Tris buffer without or with (500 μM) dopamine, and the [^{3}H]dopamine bound to the membrane was measured after further incubation at 4 °C for periods ranging from 5 min to 32 h. The binding of [^{3}H]dopamine (1 nM–0.1 mM), in the absence or presence of 50 μM unlabelled dopamine, to crude particulate platelet membrane (0.05 mg of protein/ml) was measured after an overnight incubation at 4 °C in Tris buffer. Scatchard analysis of these data was then carried out with the EBDA computer program to determine the dissociation constant (K_d) and maximal binding capacity ($B_{max.}$) for [^{3}H]dopamine binding to platelet membranes.

To determine the effect of varying the pH of the incubation media on [^{3}H]dopamine binding to platelet membranes, samples of membrane (1.0 ml) were thawed and precipitated by centrifugation at 20000 *g* ($r_{av.}$ 70 mm) for 15 min at 4 °C. The membrane was then reconstituted in Tris buffer adjusted with conc. HCl to pH values in the range 5.0–9.0. [^{3}H]Dopamine binding was then measured after an overnight incubation at 4 °C in the different Tris buffers.

To determine the effect of heat treatment, platelet membrane was incubated at a concentration of 1 mg/ml at 37 °C, 56 °C, 80 °C or 100 °C for 30 min. To determine the effect of exposure to proteolytic enzymes on the ability of platelet membrane to bind [^{3}H]dopamine, membrane was incubated without or with 5 units of papain, 100 units of α-chymotrypsin, 1.25 mg of soya-bean trypsin inhibitor/ml or 100 units of α-chymotrypsin plus 1.25 mg of soya-bean trypsin inhibitor/ml for 60 min at 37 °C. Membranes were then centrifuged at 10000 *g* ($r_{av.}$ 70 mm) for 5 min, the supernatant was discarded, and the membrane pellets were washed with 5 × 5 vol. of Tris buffer, reconstituted to the original volume with Tris buffer and then used to bind [^{3}H]dopamine.

The effect of 50 μM of a variety of neurotransmitters, neurotransmitter-receptor agonists and antagonists and neuronal neurotransmitter-uptake inhibitors (Table 1) on the binding of [^{3}H]dopamine (10 nM) to platelet membrane was measured. At the same concentration, the effect on [^{3}H]dopamine binding of compounds with some structural similarity to dopamine was also measured. In addition, as reductants and oxidants have been shown to affect dopaminergic neurons [11], the effect of some oxidants and reductants on [^{3}H]dopamine binding to platelets was measured. Finally, the effect of a range of concentrations, 1 nM–0.1 mM, of dopamine, adrenaline, noradrenaline, 5-hydroxytryptamine, L-DOPA, (+)- and (−)-SKF 38393, ADTN, catechol, isoprenaline, GSH, ascorbic acid, hydroquinone, apomorphine, DHPA and 6-hydroxydopamine on [^{3}H]dopamine binding was measured. K_i values for the effects of these compounds on [^{3}H]dopamine binding to platelet membranes were calculated by using the Ligand computer program.

Solubilization and chromatography of the [^{3}H]dopamine-binding site on platelet membranes

Platelet membranes at a final concentration of 8 mg/ml were rotated in Tris buffer containing 1% digitonin for 1 h at 4 °C. After centrifugation of the lysate at 100000 *g* ($r_{av.}$ 63.4 mm) at 4 °C for 60 min, solubilized protein was collected as the supernatant. The ability of the solubilized membrane to bind [^{3}H]dopamine was measured as described for particulate membrane.

To determine if the dopamine-binding site is glycosylated, platelet membranes (500 μg of protein in 3 ml) were cycled over a 10 ml column of wheat-germ agglutinin–Sepharose 4B (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in Tris buffer containing 0.1% digitonin (equilibration buffer), and the unbound fraction was collected. The column was washed with 5 × 5.0 ml of equilibration buffer and the wash fractions were collected and pooled. The column was then eluted with 3 × 3.0 ml of equilibration buffer containing 250 μM-*N*-acetylglucosamine. The *N*-acetylglucosamine eluate and the post-elution washes were dialysed overnight in 100 vol. of equilibration buffer by using a 10000 Da-cut-off dialysis membrane. Unbound proteins (flow-through and wash) and bound proteins (eluate and post-elution wash) were each concentrated to 3.0 ml in Centriprep 10 concentrators (Amicon) by repeated centrifugation at 2700 *g* at 4 °C until the desired volume was reached. At a concentration of 0.05 mg of protein/ml, the ability of each preparation to bind [^{3}H]dopamine without and with 50 μM-dopamine was measured.

As the identification of the dopamine-binding site on platelet membranes was dependent on the maintenance of [^{3}H]dopamine-binding activity, determination of the molecular mass of the binding site could not involve denaturing techniques such as polyacrylamide-gel electrophoresis. Thus digitonin-solubilized platelet membrane (100 μg) was chromatographed at a flow rate of 1.0 ml/min on a Zorbax GF-250 9.4 mm (internal diam.) × 25 cm size-exclusion h.p.l.c. column (Phenomenex, Torrance, CA, U.S.A.) equilibrated with equilibration buffer. The eluate from the column was monitored at 254 nm and collected as 0.25 ml fractions. The ability of each fraction to bind

[^{3}H]dopamine without or with unlabelled dopamine was measured as described for particulate membrane. The column was calibrated by chromatography of a mixture of Dextran Blue, apoferritin, β-amylase, alcohol dehydrogenase, albumin, carbonic anhydrase, cytochrome *c* and [^{3}H]dopamine.

For partial purification of the dopamine-binding site, digitonin-solubilized platelet membrane was chromatographed on a 20 cm × 3 cm column containing hydroxyapatite equilibrated with equilibration buffer. The bound components of the membrane were eluted at a flow rate of 1.0 ml/min by increasing the concentration of sodium phosphate in the equilibration buffer to 1 M over 90 min by using a Bio-Rad Econopump and controller. The eluate was collected as 5 ml fractions, and the [^{3}H]dopamine-binding capacity and protein concentration in each fraction were then measured.

The binding activity, previously fractionated with the hydroxyapatite column, was dialysed overnight at 4 °C in 25 mM-Tris containing 2.5 mM-EDTA, 100 μM-PMSF and 0.1% digitonin (DEAE equilibration buffer). Then 1 ml of the dialysed hydroxyapatite fraction was chromatographed on a Waters Protein-Pak DEAE 5PW h.p.l.c. column equilibrated in DEAE equilibration buffer. Bound protein was eluted at a flow rate of 1.0 ml/min by increasing the concentration of NaCl in the DEAE equilibration buffer to 1 M over 30 min. The eluate was collected in fractions at 1 min intervals, and the [^{3}H]dopamine-binding capacity and protein concentration of each fraction were measured.

RESULTS AND DISCUSSION

[^{3}H]Dopamine binding by platelet membranes

Specific binding of [^{3}H]dopamine by platelet membranes was highest (3.0±0.16 pmol/mg of protein) after an overnight incubation at 4 °C compared with incubations at 37 °C for 30 min (1.2±0.07 pmol/mg of protein), 37 °C for 60 min (1.4±0.17), 25 °C for 60 min (1.9±0.1), 25 °C for 120 min (2.3±0.14) or 4 °C for 60 min (1.1±0.3). At 4 °C overnight, binding of [^{3}H]dopamine to platelet membrane was maximal at pH 7.6 and pH 7.8 (2.5±0.23 pmol/mg of protein) and decreased as pH increased (binding at pH 9.0 = 0.96±0.21 pmol/mg of protein) or decreased (binding at pH 5.0 = 0.05 pmol/mg of protein). Thus, although significant amounts of [^{3}H]dopamine were bound by platelet membrane after incubation at 37 °C for 30 min, in further experiments [^{3}H]dopamine binding was measured after incubation with membranes overnight at 4 °C to maximize the binding of [^{3}H]dopamine.

The time course of specific binding of [^{3}H]dopamine to platelet membranes at 4 °C revealed that steady-state binding was achieved after 12 h (Fig. 1). Analysis of the linear portions of the association and dissociation curves indicated that the on-rate for [^{3}H]dopamine of 0.422 pmol/h per mg of protein was significantly higher than the off-rate in the absence (0.207 pmol/h per mg) or presence (0.215 pmol/h per mg) of unlabelled dopamine. The demonstration of similar off-rates for [^{3}H]dopamine in the presence and absence of unlabelled dopamine would suggest that there is no co-operativity between [^{3}H]dopamine-binding sites on the platelet membrane [12].

[^{3}H]Dopamine bound to platelet membrane in a dose-dependent manner (Fig. 2). A Scatchard plot of [^{3}H]dopamine binding was linear, consistent with binding to a single class of binding site on the platelet membrane (Fig. 2 insert). The dissociation constant (K_d) for the binding of [^{3}H]dopamine to platelet membrane was 22.6 nM and maximal binding capacity ($B_{max.}$) was 23.8 pmol/mg of protein.

Heating platelet membranes to 37 °C for 30 min did not affect the binding of [^{3}H]dopamine (1.9±0.4 pmol/mg of protein, versus control 2.0±0.1 pmol/mg of protein). However, heating the membrane to 56 °C, 80 °C or 100 °C for 30 min decreased binding of [^{3}H]dopamine to 1.0±0.3, 0.7±0.25 and 0.2±0.2 pmol/mg respectively, i.e. by 50%, 65% and 90%. Binding of [^{3}H]dopamine was also decreased from 1.7±0.5 to 0.4±0.1 and 0.5±0.1 pmol/mg of protein after exposure of the membrane to the proteolytic enzymes papain and chymotrypsin respectively. Soya-bean trypsin inhibitor had no effect on binding, but partially reversed the effect of chymotrypsin (1.3±0.1 pmol/mg of protein). The demonstration that the [^{3}H]dopamine-binding site is sensitive to both heat and pro-

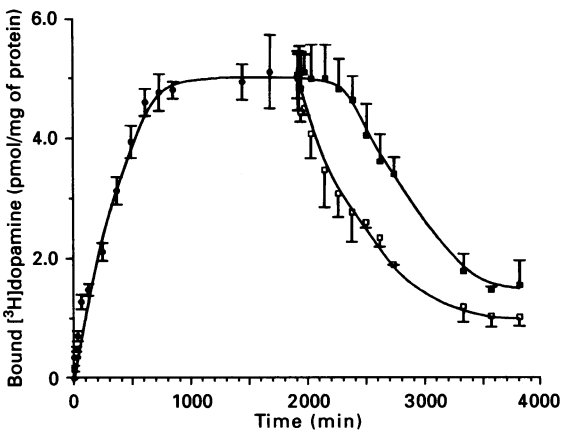

Fig. 1. Association and dissociation of [^{3}H]dopamine and human platelets

The association (●) and dissociation [in the absence (■) or presence (□) of 500 μM-dopamine] of [^{3}H]dopamine and platelet membrane was measured as described in the Materials and methods section. Means±S.D. of triplicate results from three experiments are shown.

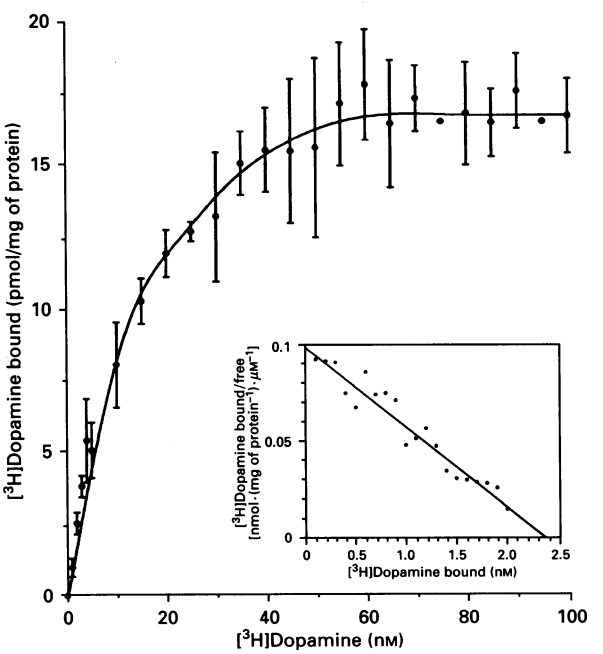

Fig. 2. Saturation binding isotherm of [^{3}H]dopamine with human platelet membranes

Specific binding of [^{3}H]dopamine (means of triplicate results from three experiments) to platelet membranes was measured as described in the Materials and methods section. Means±S.D. of triplicate results from three experiments are shown. Insert: Scatchard plot of the binding of [^{3}H]dopamine with platelet membrane.

teolytic enzymes would suggest it is a protein component of the platelet membrane.

Binding of [^{3}H]dopamine to platelet membrane was competed for by unlabelled dopamine in a dose-dependent manner (Fig. 3*a*), being inhibited by 97% at 50 μM. Binding of [^{3}H]dopamine was also competed for by noradrenaline, adrenaline, 5-hydroxytryptamine, ADTN, apomorphine, (+)- and (−)-SKF 38393, L-DOPA (Fig. 3*a*), isoprenaline, 6-hydroxydopamine, catechol, hydroquinone, DHPA, GSH and ascorbic acid (Fig. 3*b*). However, only dopamine and ADTN were able to decrease [^{3}H]dopamine binding by more than 90% at 0.1 μM, the other compounds causing decreases of 55–90%. None of the other neurotransmitters, drugs or compounds with structural similarities to dopamine had any effect on the binding of [^{3}H]dopamine.

Thus, of the 15 compounds which decreased the binding of [^{3}H]dopamine to platelet membrane, 10 have a 1,2-dihydroxyphenyl (catechol) moiety. This moiety has been shown to be important in facilitating binding to and subsequent activation of known dopamine receptors [13]. The presence of the catechol moiety seems essential to maintain binding activity, as changing a hydroxyl group on the catechol moiety [e.g. in (+)-SKF 38393 to a chloride ion in SCH 23390, in catechol to a hydrogen in phenol or in L-DOPA to a hydrogen ion in tyrosine] results in loss of competition for dopamine binding. In addition it has been shown that, as the complexity of the amine terminal on the catechol moiety increases (e.g. adrenaline > noradrenaline > dopamine), the ability to compete for the dopamine-binding site decreases (K_i: dopamine 18 nM, noradrenaline 71 nM and adrenaline 2590 nM). On the other hand, dopamine and noradrenaline were more potent at displacing [^{3}H]dopamine than were catechol, DHPA and benzene-1,2,4-triol. Thus it would appear that the amine terminus of a catechol molecule also modulates binding to the dopamine-binding site.

Hydroquinone, ascorbic acid and GSH, which do not contain a catechol moiety, also decreased [^{3}H]dopamine binding. These compounds, like the catechols, can act as reducing agents [14,15]. It therefore seemed possible that the interaction between dopamine and the platelet membrane was mainly influenced by the redox state of the membrane and was independent of the structural selectivity for catechol compounds. Against this argument was the demonstration that more potent reducing agents such as resorcinol (another dihydroxyphenyl molecule), ascorbic acid and GSH [14,15] were less potent than some catechols in decreasing dopamine binding to platelet membranes. Furthermore, two other reducing agents, dithiothreitol and 2-mercaptoethanol, did not alter dopamine binding (results not shown).

Dopamine binds to known dopamine receptors by hydrogen bonding to two serine residues on the receptor protein [13]. If [^{3}H]dopamine binding to platelet membranes also involved hydrogen bonding, this may account for the effects of compounds such as hydroquinone, ascorbic acid and GSH. Furthermore, if binding involved both stereospecific structural recognition and hydrogen bonding, this could explain why catechol compounds compete better for [^{3}H]dopamine binding compared with hydroquinone, ascorbic acid and GSH. Resorcinol may not be able to influence dopamine binding, because its conformation prevents it from gaining access to the dopamine-binding site.

How 5-hydroxytryptamine inhibits the binding of dopamine to platelet membrane remains unclear, as does its ability to decrease [^{3}H]dopamine uptake by platelets [8]. However, as phenol and tyrosine do not affect the binding of [^{3}H]dopamine, it seems unlikely that the monohydroxyphenyl moiety of 5-hydroxytryptamine is involved. Furthermore, as neither indole nor the indole-3-acetic acid affected dopamine binding, it seems most likely that amine group on the 5-hydroxytryptamine side chain is responsible for competition for [^{3}H]dopamine binding.

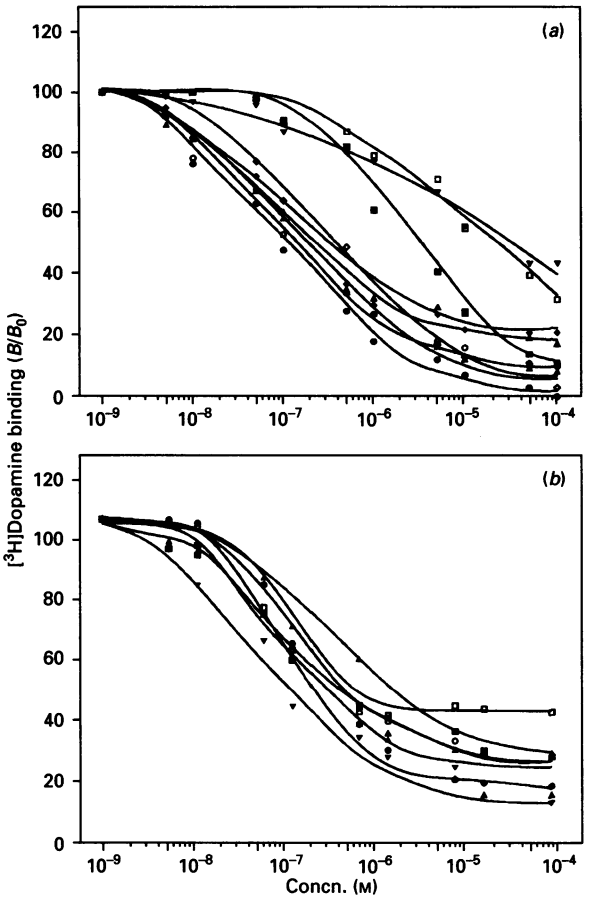

Fig. 3. Competition of [^{3}H]dopamine with a variety of agents for binding to human platelet membranes

Binding of [^{3}H]dopamine (mean of triplicate results from three experiments) by platelet membrane in the presence of increasing concentrations of: (*a*) the neurotransmitters dopamine (●), noradrenaline (○), adrenaline (■) and 5-hydroxytryptamine (□), the dopamine-receptor agonists ADTN (◇), apomorphine (▼), (+)-SKF 38393 (△) and (−)-SKF 38393 (◆), and the dopamine precursor L-DOPA (▲); (*b*) the reductants GSH (○) and ascorbic acid (□), and isoprenaline (△), 6-hydroxydopamine (▼), catechol (●), DHPA (▲) and hydroquinone (■), agents with some structural similarities to dopamine. The binding of [^{3}H]dopamine in the presence of each concentration of effector (*b*) is expressed as a percentage of [^{3}H]dopamine binding in the absence of that agent (B_0). The results shown are means from triplicate measurements in three experiments. At no point did the S.D. exceed ±15% of the mean value shown.

This hypothesis is made more attractive because the amine side chain of dopamine and 5-hydroxytryptamine is identical.

Solubilization and partial purification of the [^{3}H]dopamine-binding site from platelet membranes

The particulate membrane used to carry these series of experiments bound 1.62 pmol/mg of protein, whereas the digitonin-solubilized fraction of platelet membrane bound 1.07 pmol of [^{3}H]dopamine/mg of protein. Although the digitonin-solubilized fraction was used in all the chromatography procedures, purification yields were related to the binding activity of the crude particulate membrane (Table 2).

A significant proportion (66%) of the [^{3}H]dopamine-binding activity in the digitonin-solubilized platelet membrane was retained on the wheat-germ agglutinin–Sepharose 4B column which, when eluted with *N*-acetylglucosamine, bound 0.943 pmol

Table 2. Binding activity in various platelet membrane preparations

Membrane preparation	[^{3}H]Dopamine binding (pmol/mg of protein)	Purification (fold)
Crude particulate	1.62	1.0
Digitonin solubilized	1.07	0.66
Wheat-germ agglutinin	0.95	0.58
Zorbax G-250	3.5	2.2
Hydroxyapatite	15.0	9.0
DEAE-Protein Pak	2000.0	1200.0

of [^{3}H]dopamine/mg of protein. In addition, the binding activity which did not bind to the wheat-germ agglutinin–Sepharose 4B column on the first passage could be extracted by subsequent passage through and elution from a regenerated column (results not shown). This suggests that the binding activity which passed through the column on the first application was unable to be retained because the active sites on the wheat-germ agglutinin were saturated. Furthermore, as all the [^{3}H]dopamine-binding activity was eventually retained by the wheat-germ agglutinin–Sepharose 4B column, it appears that the platelet dopamine-binding site is glycosylated.

Specific [^{3}H]dopamine-binding activity was eluted from the Zorbax GF-250 column at an elution time of 8.8 min; a minor fraction (approx. 5%) was eluted at 11 min (Fig. 4*a*). Virtually all (97%) of the binding activity applied to the column was recovered. A comparison of the elution of [^{3}H]dopamine-binding-site activity with the elution of molecules of known molecular mass indicated that the [^{3}H]dopamine-binding site had a molecular mass of 42000 Da. The greatest binding of [^{3}H]dopamine by a column fraction was 3.5 pmol/mg of protein, which was a 2.2-fold purification of the binding site from the levels present in the particulate platelet membrane.

Some 90% of the [^{3}H]dopamine-binding activity and 20% of the protein applied to the hydroxyapatite column was eluted as a discrete peak (Fig. 4*b*), in which maximal binding activity reached 15.0 pmol/mg of protein, a significant purification of the [^{3}H]dopamine-binding site (Table 2). When the fraction from the hydroxyapatite column with maximal binding activity was chromatographed on a DEAE-Protein Pak column, 95% of the [^{3}H]dopamine-binding activity was eluted from the column in a single fraction containing 1% of the protein (Fig. 4*c*). This fraction bound 2000 pmol of [^{3}H]dopamine/mg of protein; this represented a major purification of the binding site (Table 2). Finally, neither 1 M-sodium phosphate nor 1 M-NaCl added to equilibration buffer affected [^{3}H]dopamine binding by either particulate or solubilized platelet membrane.

Comparison of the platelet dopamine-binding protein with other known dopamine-binding sites

[^{3}H]Dopamine has been shown to bind to a number of binding sites on neuronal membranes. Two of these binding sites have been shown to be discrete receptors, called the dopamine D_2-receptor and dopamine D_1-receptor [9]. Cloning strategies have shown that these receptors comprise 'classes' of receptors which exist in a number of forms (dopamine D_2-receptor class = D_{2S} [16], D_{2L} [17], D_3 [18] and D_4 [19]; dopamine D_1-receptor class = D_1 [20] and D_5 [21]). Of these receptors, only the dopamine D_{2L}, D_3 and D_4 receptors have an affinity for dopamine in the low-nanomolar range like the dopamine-binding site in platelet membrane. However, unlike the dopamine-binding site in the platelet, all these receptors show a high affinity for

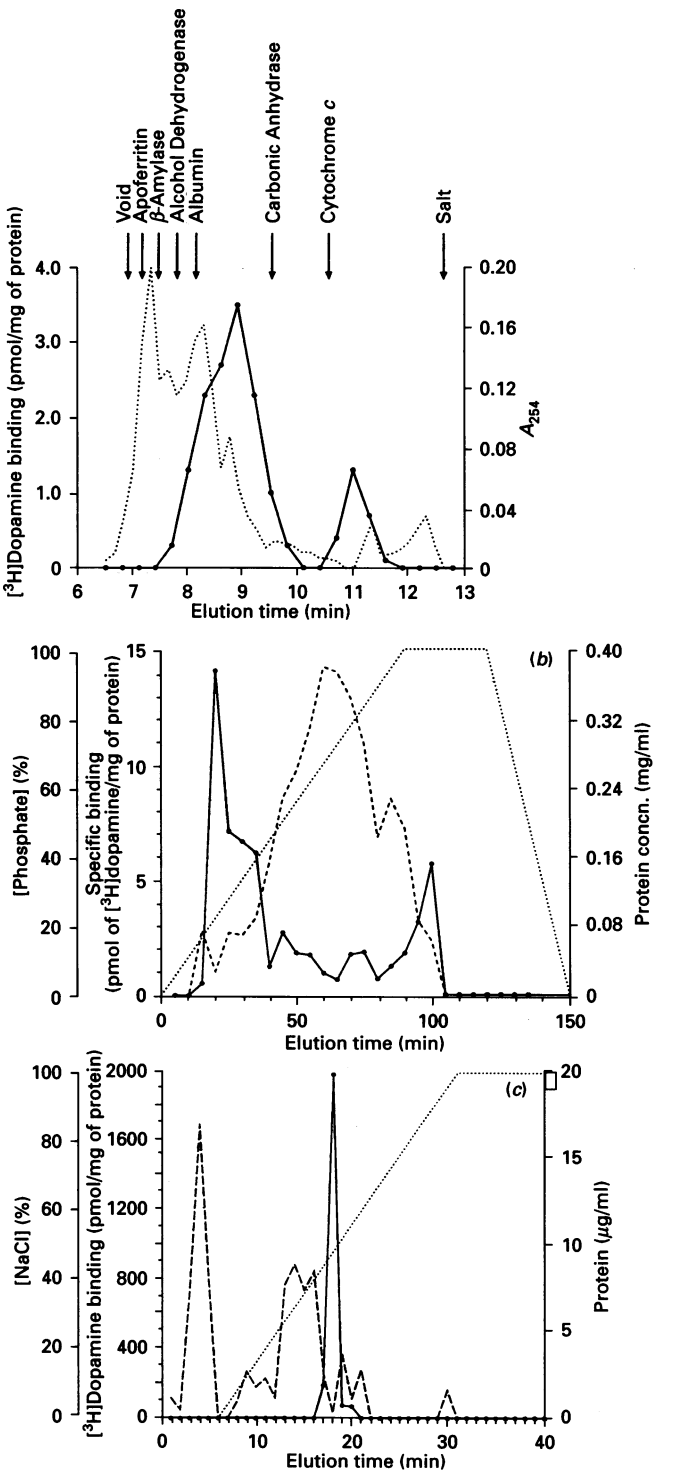

Fig. 4. Chromatography of digitonin-solubilized platelet membranes

(*a*) Chromatography of digitonin-solubilized platelet membrane on a Zorbax GF-250 size-exclusion column calibrated by chromatography of proteins of known molecular mass. The A_{254} of the column eluate (· · · · · ·) and the specific binding of [^{3}H]dopamine (●) by each column fraction were measured. (*b*) Chromatography of digitonin-solubilized platelet membrane on a hydroxyapatite column. Absorbed protein was eluted with increasing concentrations of sodium phosphate (· · · · · ·). The protein concentration (----) and the [^{3}H]dopamine-binding activity (●) of each column fraction were measured. (*c*) Chromatography of protein from the hydroxyapatite column on a DEAE Protein-Pak column. Absorbed protein was eluted by increasing concentrations of NaCl (· · · · · ·). The protein concentration (————) and the [^{3}H]dopamine-binding activity (●) of each column fraction were measured.

dopamine D_2-receptor antagonists and agonists. These marked differences in pharmacological profile make it unlikely that the binding protein present on the platelet membrane is a known dopamine receptor.

Another binding site to which dopamine binds on neuronal membrane is the dopamine transporter. Cocaine, mazindol and GBR 12909 bind to the neuronal dopamine transporter with high affinity [10], but do not affect [^{3}H]dopamine binding to platelet membranes. Furthermore, unlike the platelet dopamine-binding site, the neuronal dopamine transporter has been shown to have a low affinity for dopamine [22] and the cloning of the transporter protein has shown it to be a larger molecule of 69000 Da [23,24]. These differences suggest that the platelet dopamine-binding site is not the neuronal dopamine transporter.

In summary, dopamine binds to a glycoprotein in the platelet membrane which differs from reported dopamine receptors, the neuronal dopamine transporter and the imipramine-sensitive platelet 5-hydroxytryptamine-uptake site. The dopamine-binding site, although measured under non-physiological conditions, has been purified 1200-fold and has been shown to be structurally selective. It therefore seems unlikely the binding site is an artefact due to the conditions in which binding of [^{3}H]dopamine was measured. Whether the dopamine-binding site is present in other tissues, including brain, and is a receptor capable of activating second-messenger systems remains to be determined. However, logically, the uptake of dopamine by platelets would involve the specific binding of dopamine by the platelet membrane. As there is only one dopamine-binding site on the platelet membrane, it seems likely it must be associated with the dopamine-uptake mechanism of the platelet.

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