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Short communication

[^{3}H]GBR 12935 binding in platelets: A possible association with cytochrome P-450IID6?

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Abstract

The nature of [^{3}H] {1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) piperazine dihydrochloride} (GBR 12935) binding to human platelets was investigated. A common property of the inhibitors of this binding was their association with the cytochrome P-450 system. *cis*-Flupenthixol and {1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine dihydrochloride} (GBR 12909) biphasically inhibited the binding. The fraction of [^{3}H]GBR 12935 binding that was inhibited by low concentrations of *cis*-flupenthixol was sensitive to protease treatment. [^{3}H]GBR 12935 binding to this fraction was saturable and of high affinity (K_d 4.5 nM). The present results reveal that [^{3}H]GBR 12935 binds to multiple sites in platelets and suggest that part of the binding is associated with cytochrome P-450IID6.

Keywords: [^{3}H]GBR 12935 binding; Piperazine acceptor site; Cytochrome P-450IID6; Platelet, human

1. Introduction

The binding of the tritiated neuronal dopamine uptake inhibitor {1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) piperazine dihydrochloride} (GBR 12935) in platelets has yielded characteristics indicating that the binding is to a 'piperazine acceptor site', since piperazine derivatives such as, e.g., flupenthixol and trifluperazine inhibit the binding with IC_{50} values in the low nanomolar range (Gordon et al., 1994). [^{3}H]GBR 12935 recognizes the piperazine acceptor site in brain tissue as well. In canine brain this site has been identified as P450IID1, a subform of the biotransformative cytochrome P-450 system (P-450) (Niznik et al., 1990; Tyndale et al., 1991). In human brain, a close association was recently demonstrated between the piperazine acceptor site and the human counterpart of P450IID1, P450IID6 (Allard et al., 1994) (Henceforth, the latter designation will be used irrespective of species).

The pharmacological nature and physiological significance of the piperazine acceptor site in platelets are to a large extent unknown. In the present study, the pharmacological characteristics of [^{3}H]GBR 12935 binding to platelet membranes were investigated. It was revealed that [^{3}H]GBR 12935 labelled multiple binding sites, and that part of the binding exhibited pharmacological characteristics indicating a close association with P450IID6.

2. Materials and methods

2.1. Tissue preparation

The tissue was collected in accordance with the method described by Marcusson and Tiger (1988). Briefly, outdated blood platelet concentrates were collected at the University Hospital of Umeå. Each concentrate represented 4 individual donors. The concentrate was centrifuged at $120 \times g$ for 20 min. The supernatant was then centrifuged at $18\,000 \times g$ for 20 min. The pellets were frozen at −70°C until assay. At the day of assay, the tissue was washed in 10 + 10 ml of assay buffer (50 mM Tris–HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) and centrifuged at $18\,000 \times g$ for 10 min. The pellets were finally homogenized using a Kinematica Polytron homogenizer (Luzern), at a setting of 4, 10 s, and then resuspended to a final tissue concentration of 150–355 μg protein/ml in the binding assay.

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2.2. Compounds

Dopamine, (−)-cocaine, L-lobeline, quinidine, sparteine and protease P 5380 (alkaline protease, activity 7–15 units per mg solid, from *Bacillus licheniformis*) were purchased from Sigma (St. Louis, MO, USA). Debrisoquine, dextromethorphan, 1,3-1,3-di(2-tolyl)guanidine (DTG), dizocilpine maleate [(+)-MK-801)], proadifen (SKF 525 A), (+)-*N*-allylnormetazocine hydrochloride (SKF 10,047) were purchased from Research Biochemicals International (Natick, MA, USA). The following compounds were obtained as gifts: budipine (Byk Gulden Pharmaceutika, Konstanz, Germany), haloperidol (Janssen Pharmaceutica, Beerse, Belgium), *cis*-(*Z*)-flupenthixol (H. Lundbeck, Copenhagen, Denmark), {1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]piperazine dihydrochloride} (GBR 12909) (Novo-Nordisk, Copenhagen, Denmark), mazindol (Sandoz, Basel, Switzerland), *R*(+)-3-(3-hydroxyphenyl)-*N*-propylpiperidine hydrochloride [(+)-3-PPP] and alaproclate (Astra Arcus, Södertälje, Sweden).

2.3. [^{3}H]GBR 12935 binding

The binding of [^{3}H]GBR 12935 (40–45.7 Ci/mmol) (New England Nuclear, Boston, MA, USA) was carried out for 60 min at 25°C in a total volume of 2.0 ml. The incubation volume contained 250 μl of tissue homogenate, 250 μl of radioligand, 250 μl of inhibitory drugs or buffer and 1250 μl binding buffer (50 mM Tris–HCl, 120 mM NaCl, 0.01% bovine serum albumin, pH 7.4). The experiments were carried out using duplicate assay tubes. After the addition of 6 ml of ice-cold assay buffer, the homogenates were filtered through Whatman GF/C filters using a 24-channel cell harvester (Brandel, Gaithersburg, MD, USA) Finally, the filters were washed with three 6-ml rinses of the buffer and the radioactivity determined by liquid scintillation spectroscopy.

2.4. Data analysis and statistics

The binding data were analyzed using the iterative curve fitting program LIGAND (Munson and Rodbard, 1980). The fit to different binding models was compared by an *F*-test with the previous model tested to determine whether, for example, a two-site model fitted the data significantly better ($P < 0.05$) than a single-site model.

3. Results

cis-Flupenthixol, an inhibitor of [^{3}H]GBR 12935 binding to P450IID6 in canine brain and in bovine liver (Niznik et al., 1990) and the dopamine uptake inhibitor/P450IID6 ligand GBR 12909 (Niznik et al., 1990) biphasically inhibited [^{3}H]GBR 12935 binding with the highest affinities of the inhibitors investigated (Table 1). Likewise, alaproclate, an *N*-methyl-D-aspartate (NMDA) compound and inhibitor of 5-hydroxytryptamine (5-HT) re-uptake (Svensson et al., 1994) and of P-450 (Ross et al., 1987), inhibited the [^{3}H]GBR 12935 binding with an affinity constant below 1 μM. Proadifen, an inhibitor of some subforms of P-450 (Testa and Jenner, 1982) and σ-ligand (Klein et al., 1991; Ross, 1991), haloperidol, a σ-ligand and inhibitor of P450IID6 (Tyndale et al., 1991; Otton et al., 1992) and sparteine, a prototype substrate for P450IID6, inhibited the binding in the range of 3–9 μM. The latter compound exhibited inhibitory properties reminiscent of a two-site model with a small high-affinity fraction around 30 nM. The two-site model was however not statistically significant (see Table 1). The P450IID6 inhibitors budipine (Fonne-Pfister et al., 1987; Otton et al., 1992), lobeline (Fonne-Pfister and Meyer, 1988) and quinidine (Otton et al., 1992), were less potent inhibitors, although 50–60% of the total binding was displaced by these drugs. No or marginal activity were displayed by the σ-ligands DTG, (+)-3-PPP and SKF 10.047, by dextromethorphan, a compound associated with receptors of the NMDA and σ-systems and with P450IID6 (Klein et al., 1991) or by the NMDA antagonist (+)-MK-801.

Table 1
Inhibition of [^{3}H]GBR 12935 binding in platelets by various compounds

	K_i (μM) (range)	% inhibition of total binding	*n*
cis-flupenthixol	0.027 (0.013–0.055)	50	3
	10 (6–17)	40	
GBR 12909	0.175 (0.110–0.240)	55	2
	26 (19–33)	25	
alaproclate	0.74 (0.68–0.81)	75	3
proadifen	3 (0.2–6)	75	4
haloperidol	4.5 (3–6)	70	2
sparteine	9 (6–14) [a]	55	4
lobeline	18 (5–31)	50	2
budipine	33 (28–37)	50	3
quinidine	110	75	1

Data are mean (range) values of the affinity constant (K_i) for the drugs investigated and the percentage inhibition of [^{3}H]GBR 12935 (1 nM) binding. The inhibition of [^{3}H]GBR 12935 binding was also investigated using cocaine ($n = 2$), dopamine ($n = 2$), mazindol ($n = 2$), debrisoquine ($n = 1$), DTG ($n = 1$), (+)-3-PPP ($n = 1$), SKF 10.047 ($n = 1$), dextromethorphan ($n = 1$) and (+)-MK-801 ($n = 1$). In these experiments, inhibition was less than 15% at 10 μM of inhibitor or data too scattered to allow determination of K_i by LIGAND. n = number of experiments.
[a] In three of the experiments, inhibition curves reminiscent of two-site binding models appeared with a high affinity binding fraction (10–15% of total binding) around 30 nM, although the two-site model was not statistically significant.

The *cis*-flupenthixol inhibition of [^{3}H]GBR 12935 was further examined in tissue samples that were treated with a protease. Homogenates obtained after the first homogenization were incubated for 3 h at 37°C in the presence of the protease. Control tissue was simultaneously kept on ice. The homogenate was then treated as described above and used in the binding assay. A reduction by 50% of the total binding occurred (Fig. 1A). The protease sensitive

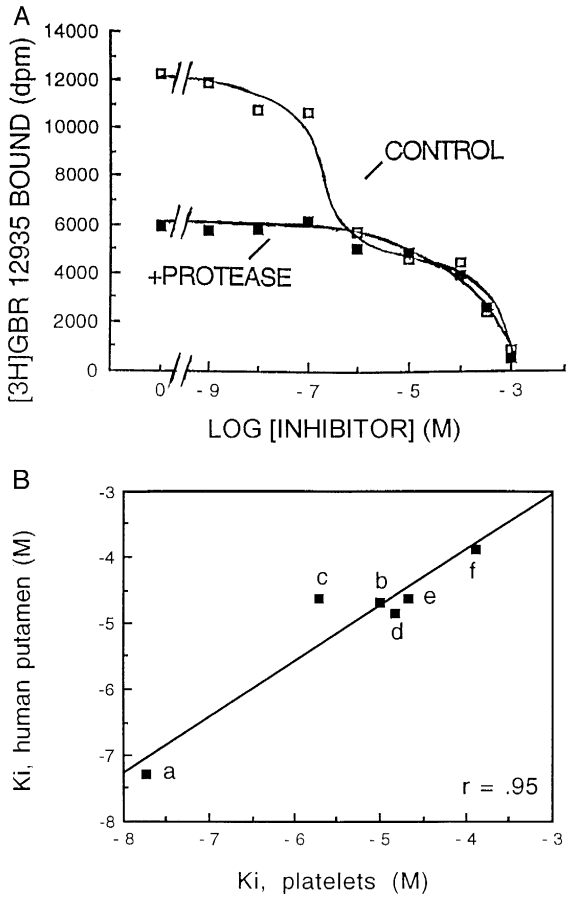

Fig. 1. (A) Inhibition by *cis*-flupenthixol of [^{3}H]GBR 12935 (1 nM) binding in platelets. Homogenates obtained after the first homogenization were incubated for 3 h at 37°C in the presence of a protease. Control tissue was simultaneously kept on ice. The figure shows one of 2 similar experiments. (B) Correlation plot of K_i values for the inhibition of [^{3}H]GBR 12935 binding in the human putamen (Allard et al., 1994) and in platelets (present study). (a) *cis*-flupenthixol $^{\text{high affinity}}$; (b) *cis*-flupenthixol $^{\text{low affinity}}$; (c) proadifen; (d) lobeline; (e) budipine; (f) quinidine.

[^{3}H]GBR 12935 binding fraction was identical with the fraction sensitive to low concentrations of the inhibitor. Protease sensitivity experiments were conducted also with proadifen, lobeline, budipine and quinidine as inhibitors ($n = 2$). In these experiments the binding was reduced by 55–60% of the total binding, i.e., almost the entire [^{3}H]GBR 12935 binding fraction that was displaced by the inhibitor (figure not shown). These experiments revealed that the inhibitors labelled also a marginal fraction of the protease insensitive [^{3}H]GBR 12935 binding fraction.

From the inhibition experiments, a definition of specific [^{3}H]GBR 12935 binding to this protease sensitive population of binding sites was chosen, i.e., the difference between [^{3}H]GBR 12935 binding in the absence and presence of 0.3 μM *cis*-flupenthixol. Saturable [^{3}H]GBR 12935 binding (0.3–40 nM) of high affinity was obtained (B_{max} 1425 ± 219 fmol/mg protein (mean ± S.E.M.); K_d 4.5 nM (range 3.5–5.8 nM), $n = 3$) (figure not shown).

4. Discussion

A common property of the compounds that inhibited the [^{3}H]GBR 12935 binding is their association with P-450, in contrast with most of the compounds that did not inhibit the [^{3}H]GBR 12935 binding. The presence of P-450 has been demonstrated in virtually all tissues, including platelets (Haurand and Ullrich, 1985; Alonso et al., 1991). In canine brain, several of the compounds used in the present study have been examined with regard to their affinity for the piperazine acceptor site. Niznik et al. (1990) found that GBR 12909, *cis*-flupenthixol, proadifen, sparteine, budipine, lobeline and quinidine inhibited [^{3}H]GBR 12935 with affinity constants that correlated well with their respective K_i values for liver P450IID6 activity, indicating the identity of the piperazine acceptor sites as P450IID6. Furthermore, the K_i values for *cis*-flupenthixol (high and low affinity values), proadifen, lobeline, budipine and quinidine in the present study are strongly correlated ($r = 0.95$) with the K_i values obtained for the same compounds in the inhibition of [^{3}H]GBR 12935 binding to the piperazine acceptor/P450IID6 binding site in the human putamen (Allard et al., 1994) (Fig. 1B). In the present study it was demonstrated that these compounds have the ability to inhibit a considerable fraction of [^{3}H]GBR 12935 from binding sites of protein nature, in line with the suggestion that these sites are P450IID6.

Some of the compounds that inhibited the [^{3}H]GBR 12935 binding are ligands that also bind to the σ-receptor binding sites, defined as the haloperidol-sensitive binding of SKF 10,047 which are insensitive to opioid antagonists and different from dopamine D2 and phencyclidine receptors (Ross, 1991, and references therein). In the present study, the prototype σ-ligands SKF 10,047, (+)-PPP and DTG were inactive as inhibitors, like dextromethorphan, while the σ-ligand haloperidol displayed moderate potency. However, as indicated above it has been demonstrated that haloperidol displays affinity also for P450IID6. In rat brain and liver, tritiated σ-ligands have yielded binding characteristics quite different from [^{3}H]GBR 12935 binding (Ross, 1991), suggesting that [^{3}H]GBR 12935 is a ligand that is relatively unsensitive to σ-binding sites. However, in that study, as in others, the pharmacological heterogeneity of the σ-receptor binding sites was demonstrated, and the possibility was indicated that some subforms of σ-receptors and of P-450 were identical.

In conclusion, [^{3}H]GBR 12935 bound to multiple sites in platelet membranes. One of these sites was of protein nature and exhibited pharmacological characteristics indicative of P450IID6, since the binding to this site was inhibited by compounds that are known as inhibitors of [^{3}H]GBR 12935 binding to P450IID6 in liver and brain. Secondly, there was a strong correlation between K_i values for inhibitors of [^{3}H]GBR 12935 binding to platelets and to piperazine acceptor/P450IID6 binding sites in the human putamen. Whether the P-450 binding sites in

platelets are identical with some subforms of σ-binding sites is a matter of further investigation. The identification of [^{3}H]GBR 12935-sensitive P450IID6 binding sites in platelets could bring increased potentials in the development of peripheral marker systems in diseases in which P-450 related dysfunctions have been suggested.

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