Characterisation of brain D_2 dopamine receptors solubilised by lysophosphatidylcholine

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Brain D_2 dopamine receptors have been solubilised using lysophosphatidylcholine. The inclusion of proteinase inhibitors during solubilisation enables a preparation to be obtained containing a high proportion of solubilised D_2 receptors with pharmacological properties similar to those of membrane-bound D_2 receptors.

D_2 dopamine receptor *Solubilisation* *Lysophosphatidylcholine* *[^{3}H]Spiperone* *Bovine caudate nucleus*

1. INTRODUCTION

Brain dopamine receptors have been studied intensively over the past few years in their native membrane-bound state (e.g., [1]). We have studied in detail one class of dopamine receptor in bovine brain using [^{3}H]spiperone binding [2,3] and the pharmacological characteristics are those of a D_2 receptor. A complete understanding of the mechanism of action of this receptor will be obtained only when the receptor is isolated and fully characterised. A prerequisite for this is solubilisation of active receptors from the membrane using detergents; reports have appeared describing the use of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate [4]) and digitonin [5,6] for solubilisation of active brain D_2 receptors. We have screened a series of ionic, non-ionic and zwitterionic detergents for solubilisation of brain D_2 receptors ([7], J.M. Hall, M.W., P.G.S., unpublished) and have found that lysophosphatidylcholine (LPC) is a useful detergent for this purpose. Here, we describe some of the characteristics of the LPC-solubilised receptors.

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Abbreviation: LPC, lysophosphatidylcholine

2. EXPERIMENTAL

Benzamidine, benzethonium chloride, EGTA, iodoacetic acid, leupeptin, LPC, pepstatin A and phenylmethanesulphonylfluoride were from Sigma (London) (Poole); EDTA from Fisons Ltd (Loughborough, Leicestershire); methylmethanethiolsulphonate from Aldrich Chemical Company (Gillingham, Dorset); all other chemicals were obtained from suppliers given in [2,3].

A mixed mitochondrial–microsomal preparation was made from bovine caudate nucleus as in [3] and D_2 receptors were solubilised from this preparation as in [7]. Mitochondrial–microsomal preparation (~2 mg protein/ml) and LPC at the concentration shown (generally 0.1%) in a buffer containing sucrose (0.32 M), Hepes [4-(2-hydroxyethyl)-piperazine-ethanesulphonate] (20 mM), EDTA (0.1 mM), dithiothreitol (0.1 mM) at pH 7.4 were mixed by homogenisation (Teflon/glass) and the solubilised preparation (~1 mg protein/ml) was obtained after centrifugation (100000 × g, 60 min). In some experiments proteinase inhibitors were included (EDTA (1 mM), EGTA (1 mM), phenylmethane sulphonylfluoride (0.1 mM), benzethonium chloride (0.1 mM) pepstatin A (18 μM)).

Solubilised receptors were assayed by incubating

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0.45 ml of the solubilised preparation with [^{3}H]spiperone (~1 nM for displacement experiments) and pargyline (10 μM) in 0.5 ml final vol. buffer containing proteinase inhibitors and other drugs where indicated at 4°C for the time shown (in most experiments this was 4 h although some were run for 16 h). The incubation was terminated by the addition of 0.1 ml of a suspension of charcoal (Norit GSX, 10%) pre-equilibrated at 4°C with bovine serum albumin (2.2%) in buffer which was mixed and centrifuged (12000 × g, 2.5 min). Aliquots (0.3 ml) of the supernatant were taken for determination of bound radioactivity as in [2,3]. Specific binding of [^{3}H]spiperone was defined as the difference between parallel assays containing 1 μM (−)- and (+)-butaclamol. IC_{50}-Values are the concentrations of substances required to displace 50% of the specifically bound [^{3}H]spiperone. Protein concentrations were determined as in [2,3] except that 3.2% SDS was included as in [7].

3. RESULTS

Increasing concentrations of LPC gave increasing solubilisation of protein and [^{3}H]spiperone binding sites from bovine caudate nucleus membranes (fig.1). LPC at 0.1% was used for subsequent experiments as this gave a high degree of solubilisation. Under these conditions roughly 10% of [^{3}H]spiperone binding sites are extracted assuming that the solubilised binding is to D_2 receptors (see below). The time course of [^{3}H]spiperone binding to the solubilised preparation is shown in fig.2 and in studies reported below an incubation time of 4 h was generally used. [^{3}H]Spiperone specifically bound to the solubilised preparation was defined as the difference in binding in parallel assays con-

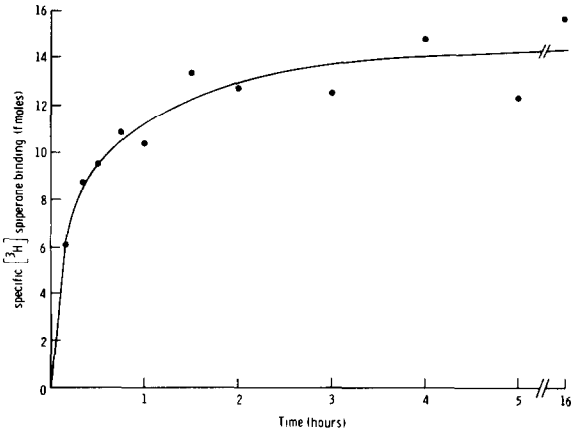

Fig.2. Time-course for specific binding of [^{3}H]spiperone to LPC-solubilised preparation of bovine caudate nucleus. Proteinase inhibitors were present as in fig.4.

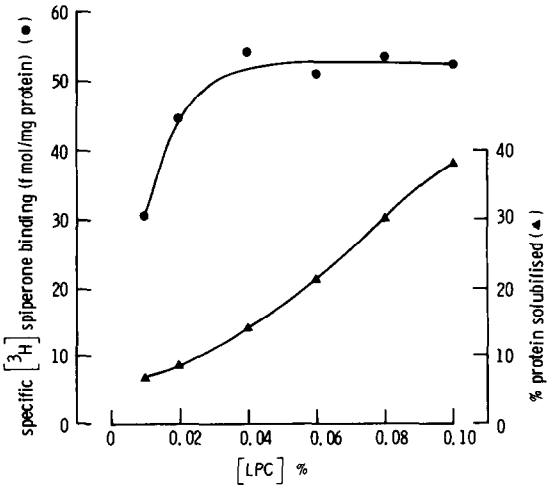

Fig.1. Solubilisation of specific [^{3}H]spiperone binding and protein from bovine caudate nucleus by LPC. Supplementary proteinase inhibitors were absent.

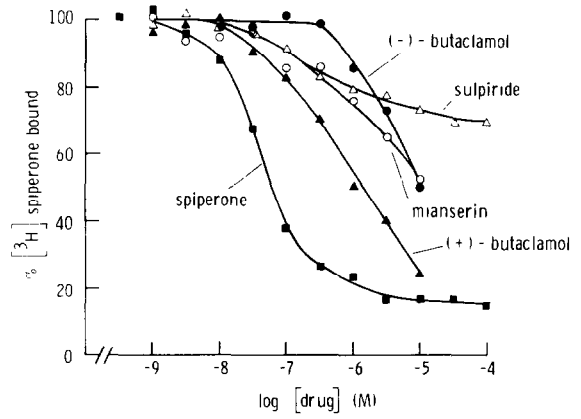

Fig.3. Displacement of [^{3}H]spiperone (1 nM) binding from an LPC-solubilised preparation of bovine caudate nucleus by various competing substances. Supplementary proteinase inhibitors were absent.

taining 1 μM (−)- and (+)-butaclamol, respectively and the precise characteristics of the solubilised preparation were determined in displacement experiments (fig.3).

Non-radioactive spiperone displaced 84% of the [^{3}H]spiperone binding indicating that true non-specific binding was 16% of the total. However, the drug butaclamol, which exists in (+)- and (−) stereoisomeric forms, offers a more discriminating test of the character of the binding. At membrane-bound D_2 receptors (+)-butaclamol is roughly 800-times more potent than (−)-butaclamol in displacing [^{3}H]spiperone binding [2]. In the LPC-solubilised preparation the potency ratio (IC_{50} (−)-butaclamol/IC_{50} (+)-butaclamol) is only 12. This could be because upon solubilisation the D_2 receptors partly lose their ability to discriminate between the isomers of butaclamol or that [^{3}H]spiperone binding in the LPC-solubilised preparation is to D_2 receptors (with a high ability to discriminate between the isomers of butaclamol) and to a class of sites with little or no ability to discriminate between the isomers of butaclamol (non-stereospecific sites). Examination of the displacement curves reveals a portion of the binding (~30%) where (+)-butaclamol (10–300 nM) displaces [^{3}H]spiperone binding but (−)-butaclamol does not. The selective D_2 receptor antagonist sulpiride also displaces ~30% of the binding, whereas the specific serotonergic antagonist mianserin shows no high affinity displacement indicating the absence of serotonergic sites. This latter finding is important as the bovine caudate nucleus membranes used for solubilisation contain comparable numbers of dopaminergic and serotonergic sites [2]. Assuming this analysis, the remaining binding is displaced with roughly equal affinities by the two isomers of butaclamol. Hence the total solubilised [^{3}H]spiperone binding consists of 30% D_2 receptors, 54% non-stereospecific sites (spirodecanone sites [6,8]) and 16% non-specific binding. This analysis also validates the use of 1 μM (+)- and (−)-butaclamol for defining specific D_2 receptor binding in the LPC-solubilised preparation.

The proportion of specific binding observed in these experiments is rather low so we have investigated the effect of adding proteinase inhibitors during solubilisation as such inhibitors have been shown to influence receptor solubilisation in some systems [9]. When inhibitors of the 4 main classes of proteinase [10] (EDTA, EGTA, phenylmethanesulphonylfluoride, benzethonium chloride, pepstatin A) were included during solubilisation the potency ratio for the isomers of butaclamol increased to 170 and the pharmacological profile changed considerably. The major contributor to this change was benzethonium chloride (see section 4).

In displacement experiments on this preparation (fig.4), non-radioactive spiperone gave a biphasic displacement curve, ~60% of the bound [^{3}H]spiperone being displaced with high affinity. Displacements by (+)-butaclamol and the specific D_2 receptor antagonist, domperidone were also biphasic whereas (−)-butaclamol was virtually inactive at the concentrations used. Mianserin showed no high affinity displacement indicating the absence of a significant serotonergic component of binding. The high affinity components of binding of (+)-butaclamol and domperidone indicate that the total [^{3}H]spiperone binding consists of ~40% D_2 receptor binding, the remainder consisting of non-stereospecific sites and non-specific binding sites. A comparison of IC_{50} values for displacing

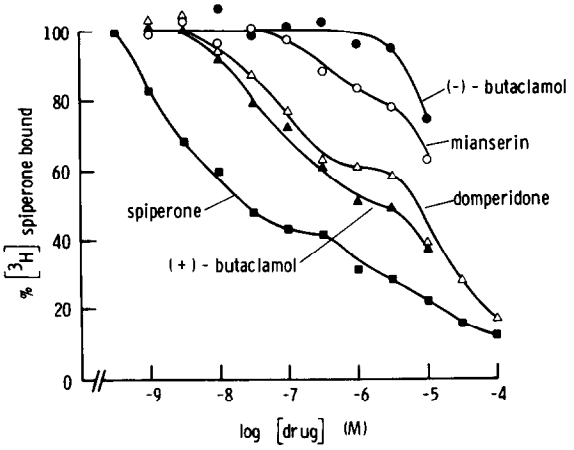

Fig.4. Displacement of [^{3}H]spiperone (1 nM) binding from an LPC-solubilised preparation of bovine caudate nucleus (obtained in the presence of proteinase inhibitors) by various competing substances. EDTA (1 mM), EGTA (1 mM), phenylmethanesulphonylfluoride (0.1 mM) and benzethonium chloride (0.1 mM) were present during solubilisation and assay of [^{3}H]spiperone binding sites – pepstatin A was not included in these experiments as it was without effect (section 4).

Table 1

IC_{50} values (nM) for displacement of specific [^{3}H]spiperone binding to LPC-solubilised preparations of bovine caudate nucleus

Displacing substance	− Proteinase inhibitors	+ Proteinase inhibitors
(+)-Butaclamol	129	56
(−)-Butaclamol	1514	9550
Domperidone	229	79
(±)-Mianserin	355	2818
Spiperone	15	2
(±)-Sulpiride	631	3400

IC_{50} values (concentrations of substance giving 50% inhibition of specific [^{3}H]spiperone (~1 nM) binding were determined as in section 2 to LPC-solubilised preparations obtained in the presence or absence of proteinase inhibitors. Pepstatin A was not included (see fig.4)

drugs in the preparations obtained with and without proteinase inhibitors is given in table 1. Saturation analysis of [^{3}H]spiperone binding to the preparation obtained in the presence of proteinase inhibitors indicated a dissociation constant of 0.24 ± 0.09 nM (mean ± SD, 5 expts) (J.M. Hall, P.G.S., unpublished); there were slight indications of the binding of [^{3}H]spiperone to additional lower affinity sites in some preparations.

4. DISCUSSION

These experiments validate the use of LPC as a useful detergent for solubilisation of active D_2 receptors. In the preparation obtained using LPC alone [^{3}H]spiperone binding is to D_2 receptors but a large number of sites is also present which show low stereospecificity for binding the isomers of butaclamol (non-stereospecific or spirodecanone sites). If proteinase inhibitors are added during solubilisation specific [^{3}H]spiperone binding to D_2 receptors represents a larger proportion of the total [^{3}H]spiperone binding and the yield of solubilised receptors is also slightly increased (by 18% over the control value). The major effect, however, is that the pharmacological profile is much clearer and the presence of D_2 receptors with properties similar to those of membrane-bound receptors can easily be discerned (fig.3,4; table 1). The relative importance of the different proteinase inhibitors has been determined in separate experiments (not shown). Pepstatin A, an inhibitor of aspartic proteinases [10] does not contribute to these changes whereas benzethonium chloride, reported to be an inhibitor of cysteine proteinases [11] and arylaminopeptidases [12] is essential for observing these effects. Serine proteinase and metalloproteinase inhibition are unimportant as EDTA, EGTA and phenylmethanesulphonylfluoride are themselves without effect. Other inhibitors of cysteine proteinases (benzamidine, iodoacetate, leupeptin, methylmethanethiolsulphonate) were without effect so that the effects of benzethonium may be unrelated to proteinase inhibition or alternatively inhibition of a specific benzethonium sensitive proteinase may be occurring. Benzethonium is also reported to be a detergent [13] and to interact with other detergents [14] so that the combination of LPC and benzethonium may solubilise a different combination of proteins or a different conformational state of the same proteins. Alternatively, benzethonium may alter non-specific and non-stereospecific binding of [^{3}H]spiperone. It is not possible at present to distinguish these alternatives but in digitonin solubilisation of bovine caudate nucleus D_2 receptors (not shown), benzethonium does not improve the binding profile suggesting that proteinase inhibition is a less likely explanation.

The affinities of substances for binding to the LPC-solubilised preparation (table 1) show a good correlation with binding affinities at membrane-bound receptors supporting the idea that D_2 receptors are being studied. Also we have verified by electron microscopy, ultrafiltration and sucrose density gradient centrifugation that the receptors are truly solubilised rather than present on small membrane fragments.

The use of LPC in the presence of proteinase inhibitors as above, therefore, provides a useful method for solubilisation of brain D_2 receptors. LPC, therefore, offers an alternative to digitonin which has been widely used for this purpose and overcomes some of the problems inherent in the use of digitonin such as its solubility and the variability of composition of commercial preparations. Here it seems that as the starting membrane preparation contains a mixture of serotonergic and dopaminergic sites, whereas the solubilised

preparation contains only dopaminergic sites, LPC is specifically extracting dopaminergic sites or alternatively the serotonergic sites are also extracted but not detected in the assay conditions. Although the LPC-solubilised preparation offers an attractive preparation for work on solubilised D_2 receptors, specific [^{3}H]spiperone binding still comprises only about half the total [^{3}H]spiperone binding. However, we have managed to increase the proportion of specific binding considerably by carrying out the binding assays at 25°C rather than 4°C (unpublished).

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