# Reconstitution of solubilised brain $D_2$ dopamine receptors into phospholipid vesicles

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$D_2$ dopamine receptors have been solubilised from bovine caudate nucleus using cholate/sodium chloride in the presence of soyabean phospholipid. Reconstitution of the receptors into soyabean phospholipid vesicles has been achieved by dialysis to remove detergent and salt. The receptors are truly reconstituted as judged by sedimentation, electron microscopy, heat stability and analysis on sucrose density gradients. The ligand-binding properties of the reconstituted receptors resemble those of the solubilised preparation.

*$D_2$ dopamine receptor* *Solubilisation* *Reconstitution* *[$^3$H]Spiperone binding*

## 1. INTRODUCTION

In vitro techniques have recently been used intensively for studying brain dopamine receptors and the $D_2$ class, which shows a high affinity for dopamine antagonists, has been implicated in many of the important physiological functions of dopamine [1–4]. More recently brain $D_2$ dopamine receptors have been solubilised in an active form using a variety of detergents and purification studies are in progress [5,6]. The integrity of the solubilised receptors can partly be ascertained from the ability of the solubilised receptors to bind dopamine receptor-specific ligands but it will be desirable, however, to demonstrate functional interaction of the solubilised receptors with the other components of the dopamine transmembrane signalling system and one approach to this is to reconstitute the solubilised receptors into defined phospholipid vesicles [7]. This will also enable the influence of the membrane environment on receptor properties to be established. Here we describe the first report of reconstitution of solubilised dopamine $D_2$ receptors.

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## 2. EXPERIMENTAL

Mixed mitochondrial-microsomal membrane preparation from bovine caudate nucleus, obtained as in [8,9] was sedimented (130000 × *g*, 60 min) and the supernatant discarded. The pellet was resuspended at a protein concentration of 1 mg/ml (Teflon/steel homogeniser, 0.18 mm radial clearance, 800 rpm, 5 strokes) in an ice-cold medium containing cholate (5.7 mg/ml), sodium chloride (1 M), crude soyabean phosphatidylcholine (5 mg/ml, Sigma, type 11-S), Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulpho-nic acid) (20 M, pH 7.4), EDTA (1 mM), EGTA (1 mM), $MgCl_2$ (20 mM), dithiothreitol (0.1 mM). Phenylmethylsulphonyl fluoride (0.1 mM) was added just prior to solubilisation. This medium was sonicated before use to clarify the solution. The resuspended material was stirred gently at 4°C for 60 min and then centrifuged (135000 × *g*, 90 min) and the upper 75% of the supernatant taken as the solubilised preparation. The solubilised preparation was dialysed in Visking 8/32 tubing at 4°C against approximately 100 vols of buffer (Hepes 20 mM), EDTA (1 mM), EGTA (1 mM), magnesium chloride (20 mM), sodium chloride (1 M), dithiothreitol (0.1 mM, pH

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7.4) for 28 h changing the buffer 3 times, followed by the same buffer minus the sodium chloride for 12 h. The turbid dialysate was centrifuged (136 000 × *g*, 90 min) and the pellet resuspended in the low salt buffer used for dialysis by gentle homogenisation (Teflon/steel) to give the reconstituted preparation (protein concentration approx. 0.6 mg/ml).

[$^3$H]Spiperone (~ 1 nM) binding was assayed essentially as in [10] except that assays on reconstituted material were performed at 25°C, 30 min and the density of the preparation was raised to about 20% sucrose (equivalent to about 17% sucrose in the binding assay) in order to prevent sedimentation of liposomes during the centrifugation stage of the assay. This did not hinder centrifugation of the charcoal during the assay. Specific [$^3$H]spiperone binding was defined as the difference in binding between assays containing 1 $\mu$M (-)- and (+)-butaclamol.

## 3. RESULTS AND DISCUSSION

Initially $D_2$ receptors from bovine caudate nucleus, assayed by [$^3$H]spiperone binding [8] and solubilised with cholate (0.2%), sodium chloride (1 M) [8] were mixed with sonicated crude soyabean phosphatidylcholine and dialysed to remove the detergent. No incorporation of protein or $D_2$ receptors into the vesicle preparation formed on dialysis was achieved in this way and it seems that it is important that detergent, lipid and protein are thoroughly co-solubilised prior to dialysis in order to achieve incorporation into the vesicles formed upon dialysis. In order to achieve this, $D_2$ receptors from bovine caudate nucleus membranes were solubilised by cholate (0.57%), sodium chloride (1 M) in the presence of 0.5% crude soyabean phosphatidylcholine and using this method approx. 60% solubilisation of protein is achieved and a preparation containing active solubilised $D_2$ receptors is obtained (table 1). Displacement experiments using selective substances indicated that the specific [$^3$H]spiperone binding was solely to $D_2$ receptors with no $S_2$ serotonin receptor component. The receptors are truly solubilised as judged by ultrafiltration through 200 nm filters (110% recovery of $D_2$ receptors, 93% recovery of protein, 95% recovery of lipid) and electron microscopy. When this preparation was dialysed against detergent-free low ionic strength buffer, however, there was still very little incorporation of protein or receptors into the vesicles formed.

Table 1

Displacement of specific [$^3$H]spiperone binding from solubilised and reconstituted preparations of $D_2$ dopamine receptors

| Substance | $IC_{50}$ (nM) | |
|---|---|---|
| | Solubilised | Reconstituted |
| (+)-Butaclamol | 132 | 224 |
| (-)-Butaclamol | >10000 | >10000 |
| Domperidone | 219 | 263 |
| Mianserin | >10000 | >10000 |
| Pipamperone | 1514 | 447 |
| Spiperone | 12 | 11 |
| Sulpiride | 1413 | 537 |

$IC_{50}$ (concentration of substance giving half-maximal displacement of specific [$^3$H]spiperone binding) values were determined in solubilised and reconstituted preparations

The procedure was, therefore, modified by including 20 mM $Mg^{2+}$ during solubilisation and dialysis, using a two stage dialysis against high and low salt buffer in order to ensure removal of detergent before salt and using as rapid a dialysis time as possible (using small diameter dialysis tubing) and these changes achieved incorporation (34 ± 7% incorporation, mean ± SD, based on solubilised preparation). The vesicles formed upon dialysis were collected by centrifugation and contained about half of the protein from the solubilised preparation. Active receptor were found only in the vesicle preparation, none being found in the supernatant, so that the presence of receptors in the vesicle preparation is unlikely to be due to occlusion or sticking. Receptors in the vesicle preparation may be assayed by [$^3$H]spiperone binding either by the charcoal adsorption technique, modified as stated above, or by trapping on glass fibre (GF/C) filters. No specific [$^3$H]spiperone binding is observed in a control vesicle preparation formed by a dialysis of solubilised lipid in the absence of added protein from the solubilised receptor preparation.

The $D_2$ receptors are likely, therefore, to be reconstituted into vesicles and we have verified this in several ways. The reconstituted $D_2$ receptors sediment upon centrifugation (200 000 × *g*, 1 h)

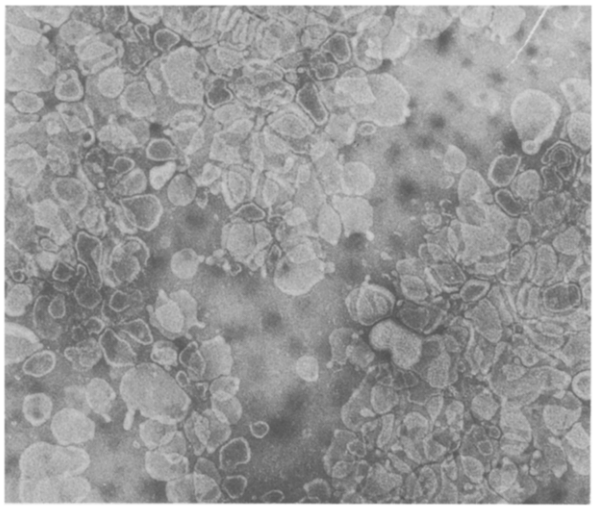

Fig. 1. Electron micrograph of reconstituted preparation. Reconstituted preparation was examined by negative staining (phosphotungstic acid) in a Philips 410 electron microscope. The magnification is 58 000 ×.

whereas solubilised receptors do not under these conditions. Examination of the reconstituted preparation reveals a heterogeneous population of mainly unilamellar vesicles (diameter 118 ± 37 nm, mean ± SD) (fig.1) whereas the solubilised preparation shows no such vesicles. The reconstituted preparation is stable at 25°C for 30 min whereas the solubilised preparation is not. If tracer quantities of [$^{14}$C]phosphatidylcholine are included with the bulk lipid during solubilisation and reconstitution, and the reconstituted preparation analysed on a 5–25% sucrose density gradient (fig.2) comigration of lipid and receptors is observed. Thus the receptors are truly reconstituted rather than aggregated. The small amount of receptors in the 18% sucrose region of the gradient may represent limited aggregation of receptors as more receptor material (non-vesicular by electron microscopy) is found in this region if a lower phospholipid/protein ratio is employed during solubilisation and reconstitution.

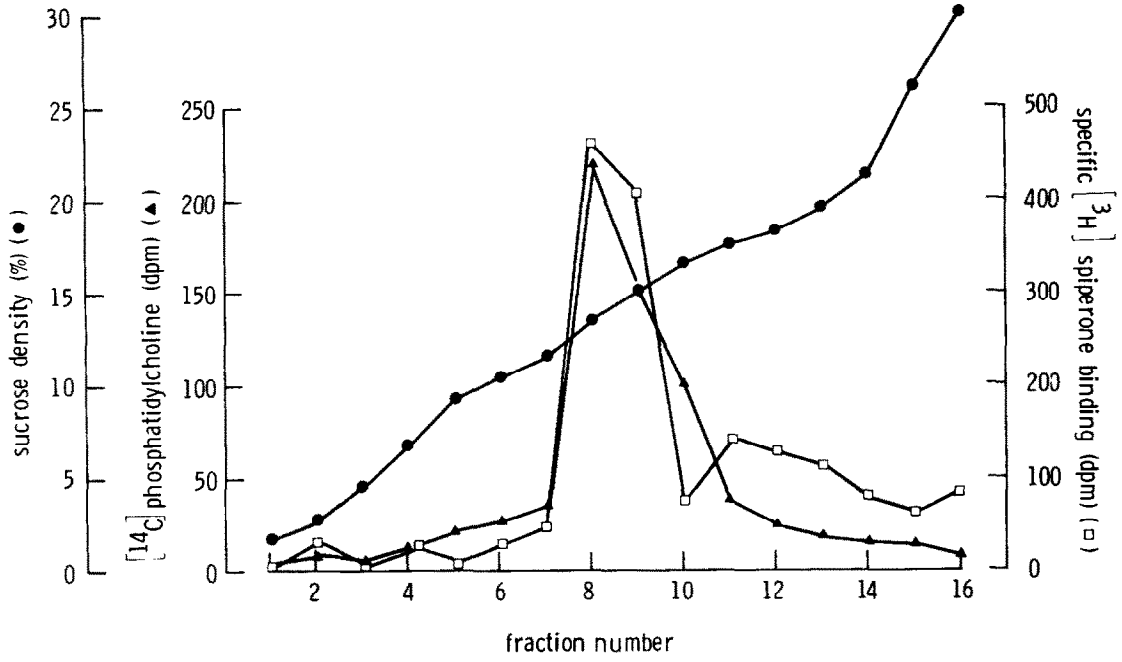


Fig. 2. Sucrose density gradient centrifugation of reconstituted receptors. Reconstitution of solubilised $D_2$ receptors was performed as described except that 1.7 nCi of [$^{14}$C]dioleoylphosphatidylcholine was included during solubilisation. Two-ml samples of reconstituted material were loaded on to sucrose density gradients (5–25%, w/v, sucrose in Hepes (20 mM), EDTA (1 mM), EGTA (1 mM), magnesium chloride (20 mM) (total vol. 14 ml) and centrifuged (18 h, 94 500 × *g*, 4°C). The gradients were unloaded by displacement with 35% sucrose, 1 ml fractions collected and assayed for sucrose concentration, $^{14}$C radioactivity, and specific [$^{3}$H]spiperone binding (after increasing the sucrose concentration to 20%).

The reconstituted $D_2$ receptors have been analysed pharmacologically (table 1) in displacement experiments in order to determine the effect of the manipulations on the properties of the receptors. Specific [$^3$H]spiperone binding represents about 40% of the total binding and the high non-specific binding is likely to be associated with the large amounts of phospholipid present. Pharmacologically the reconstituted receptors resemble the solubilised preparation quite closely. Thus specific [$^3$H]spiperone binding is displaced by the selective $D_2$ receptor antagonists domperidone and sulpiride whereas the selective $S_2$ serotonin receptor antagonist mianserin is virtually inactive. The affinity for the selective $S_2$ antagonist pipamperone increases slightly on reconstitution but this is unlikely to be due to $S_2$ receptors in the preparation owing to the data obtained with the other ligands.

Reconstitution into vesicles of crude soyabean phospholipid does not, therefore, elicit any major changes in the properties of the receptors relative to those in the solubilised preparation and ligand-binding affinities are still somewhat lower than in the corresponding membrane-bound preparations [5,8]. The receptor may show a specificity for particular phospholipids but this question remains unanswered until the effect of a range of phospholipids on the properties of the reconstituted receptors can be studied. Nevertheless, the approach described here for the first time should be very powerful in determining the mechanism of action and constitution of the $D_2$ receptor.

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