Coupling of D₂ dopamine receptors to G-proteins in solubilized preparations of bovine caudate nucleus

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1. The coupling of D₂ dopamine receptors and G-proteins has been examined in cholate-solubilized preparations of bovine caudate nucleus. 2. No receptor–G-protein coupling could be detected in solubilized preparations obtained in 0.3 % cholate, but if this preparation is diluted 5-fold, coupling is re-established. 3. The dilution process was examined, and it was shown that the change in ionic strength was an important factor in modulating the observed receptor–G-protein interaction. 4. Ionic strength was shown, however, not to be the primary determinant of receptor–G-protein coupling. This is likely to be the formation, upon dilution of the preparation, of vesicles in which receptor and G-protein reassociate. 5. The formation of vesicles upon dilution was examined by a variety of techniques, including thermal-stability studies, gel filtration, centrifugation and electron microscopy.

INTRODUCTION

The D₂ dopamine receptor is an important site of action for dopamine in mediating a number of important physiological functions, as well as being a site of action of certain drugs, e.g. anti-Parkinsonian and anti-schizophrenic drugs. Considerable progress has been made recently in understanding this receptor. It has been purified from brain and anterior pituitary (Elazar *et al.*, 1988; Senogles *et al.*, 1988; Williamson *et al.*, 1988), and cDNA species for two isoforms of the receptor have been cloned and sequenced (Bunzow *et al.*, 1988; Dal Toso *et al.*, 1989; reviewed by Strange, 1990). Additional D₂-like receptors have recently been identified by gene cloning and termed the D₃ and D₄ dopamine receptors (Sokoloff *et al.*, 1990; Van Tol *et al.*, 1991). Although these would probably be included in typical assays for D₂ dopamine receptors using ligand binding with [³H]spiperone (as in the present study), the levels of D₃ and D₄ receptors in the caudate nucleus are very low, so that if this tissue is used the contribution of D₃ and D₄ receptors will be small.

The D₂ dopamine receptor elicits several biochemical effects, e.g. inhibition of cyclic AMP production, opening of K⁺ channels, inhibition of Ca²⁺ channels (Vallar & Meldolesi, 1989), and does so via one or more G-proteins. Recent reports on the purification of receptor–G-protein complexes and from reconstitution studies highlight G₀ and G_{i2} in interacting with the D₂ dopamine receptor (Senogles *et al.*, 1987, 1990; Ohara *et al.*, 1988; Elazar *et al.*, 1989).

In the present paper we have attempted to develop solubilized preparations of bovine caudate nucleus containing D₂ dopamine receptors coupled to G-proteins in order to study receptor–G-protein interaction in more detail.

EXPERIMENTAL

Materials

[³H]Spiperone (21.8 Ci/mmol) was from Amersham International. (+)-Butaclamol was from Semat, and (–)-butaclamol (Ayerst Pharmaceuticals), mianserin (Beecham Pharmaceuticals) and haloperidol and spiperone (Janssen Pharmaceutica) were generously given. All other reagents were from commercial sources and were of the highest purity available.

Solubilization of D₂ dopamine receptors from bovine caudate nucleus

D₂ dopamine receptors were solubilized from a mixed mitochondrial–microsomal preparation of bovine caudate nucleus with 0.3 % cholate/1 M-NaCl and assayed by [³H]spiperone binding as described by Leonard *et al.* (1988). Saturation and competition binding data were analysed by non-linear least-squares computer curve fitting as in Withy *et al.* (1981). Various dilutions of the standard solubilized preparation were made with a buffer containing Hepes (20 mM), MgCl₂ (5 mM), EDTA (1 mM) and EGTA (1 mM), pH 7.4. Protein was determined as described by Leonard *et al.* (1988).

Thermal stability of solubilized preparations

Standard solubilized preparations (0.3 % cholate/1 M-NaCl) and 1.33-fold and 5-fold diluted preparations were incubated at 22 °C. At various times samples were removed and assayed for [³H]spiperone binding at 4 °C.

Gel filtration of solubilized preparations on Sepharose CL 4B

The standard solubilized preparation was diluted 1.33-fold (0.225 % cholate/0.75 M-NaCl) or 5-fold (0.06 % cholate/0.2 M-NaCl), and 3 ml samples of either preparation were applied to a column of Sepharose CL 4B (1.5 cm × 45 cm, 100 ml packed volume; flow rate 10 ml/h). The column had been pre-equilibrated with buffer containing 20 mM-Hepes, 1 mM-EDTA, 1 mM-EGTA, 0.75 M- or 0.2 M-NaCl, 0.225 % or 0.06 % cholate, 2 mM-sodium acetate and 0.04 % or 0.01 % soybean phosphatidylcholine. Fractions of volume 3 ml were collected, and [³H]spiperone binding and protein were assayed. The column was calibrated with standard proteins [cytochrome *c* (*M_r* 12400), BSA (*M_r* 66000); alcohol dehydrogenase (*M_r* 150000)] and Dextran Blue (void volume) and cyclic AMP (total included volume).

Sedimentation of solubilized preparations

Standard solubilized preparation (0.3 % cholate/1 M-NaCl) was diluted to different extents (1.1-, 1.2-, 1.33- and 5-fold) and 8 ml samples of each preparation were centrifuged (100000 *g*, 90 min, 4 °C). The supernatants were collected, and any pellet was resuspended with a hand-held glass/glass homogenizer in 8 ml of buffer (21.25 % sucrose, cholate and NaCl as in the

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starting preparation, 20 mM-Hepes, 1 mM-EDTA, 1 mM-EGTA, pH 7.4). [^3H]Spiperone binding and protein levels were assayed in the supernatant and resuspended pellet. The sucrose was included in order to decrease any tendency of vesicles to sediment in subsequent binding assays (Wheatley & Strange, 1984).

Electron microscopy

Samples of standard solubilized and 5-fold-diluted preparations were applied to electron-microscope grids and negatively stained with uranyl acetate and examined in a Philips 400 electron microscope.

RESULTS

Effect of dilution on guanine nucleotide sensitivity of agonist binding to solubilized D_2 dopamine receptor

Dopamine/[^3H]spiperone competition experiments were performed in the standard solubilized preparation of bovine caudate nucleus membranes obtained with 0.3% cholate/1 M-NaCl and in this preparation diluted 5-fold (Fig. 1). In the standard solubilized preparation, dopamine competition curves fitted well to a one-binding-site model, and addition of GTP (0.1 mM) did

Fig. 1. Dopamine/[^3H]spiperone competition binding data in solubilized preparations of bovine caudate nucleus

Dopamine/[^3H]spiperone competition curves are shown in (a) the standard solubilized preparation (0.3% cholate/1 M-NaCl) and (b) this preparation diluted 5-fold, in the absence (○) and presence (□) of 0.1 mM-GTP. Data are the means of triplicate determinations (variation < 10% of mean) from representative experiments replicated as in Table 1, and the best fit curves to one-site (a) and two-site (b) models are shown. The parameters of the best-fit curves were: (a) no-GTP, IC_{50} 17.6 μM ; +GTP, IC_{50} 16.9 μM ; (b) no-GTP, IC_{50}^h 290 nM (69%), IC_{50}^l 210 μM (31%); +GTP, IC_{50}^h 2900 nM (57%), IC_{50}^l 360 μM (43%).

not affect the curves. In the 5-fold-diluted preparation the dopamine competition curve was shifted to the left and was fitted best by a two-binding-site model (Fig. 1). Upon addition of GTP (0.1 mM) a substantial shift to the right in the dopamine competition curve was seen, and, although a two-binding-site model was still the best description of the data, the proportion of higher-affinity sites was decreased in the presence of GTP and there were small changes in the affinities of the two sites (Table 1). The affinity of dopamine in the standard soluble preparation was similar to the affinities observed for the lower-affinity site in the 5-fold-diluted preparation.

In control experiments (results not shown), antagonist (spiperone and haloperidol) competition curves were unaffected by GTP in the 5-fold-diluted preparation, and there was no evidence in competition experiments with selective antagonists for any 5HT_2 , 5-hydroxytryptamine or α_1 -adrenergic components of [^3H]spiperone binding, so that specific [^3H]spiperone binding is solely to D_2 dopamine receptors. Saturation analysis of [^3H]spiperone binding in the 5-fold-diluted soluble preparation gave values of B_{max} 383 ± 72 fmol/mg of protein and K_d 0.4 ± 0.09 nM (mean $\pm$ S.D., $n = 3$). For the standard soluble preparation the corresponding values are B_{max} 217 ± 31 fmol/mg, K_d 1.13 ± 0.17 nM (Leonard, 1987).

When a lower dilution (1.33-fold) of the standard soluble preparation was examined and dopamine/[^3H]spiperone competition curves were analysed, dopamine competition occurred at higher concentrations compared with the 5-fold-diluted preparation, and the competition curve was steeper. However, compared with the standard soluble preparation, addition of GTP did shift the dopamine competition curve to the right, but not as much as in the 5-fold-diluted preparation (Table 1). In the presence of GTP, competition curves modelled best to a single class of sites.

In order to examine the factors involved in the appearance of guanine nucleotide sensitivity of agonist binding upon dilution, either cholate or NaCl was added to the 5-fold-diluted preparation (0.06% cholate/0.2 M-NaCl) and dopamine/[^3H]spiperone competition curves were obtained. Increasing the cholate to 0.2% had no effect on the dopamine competition curves or the effect of GTP (results not shown). We were unable to use 0.3% cholate, as this rendered the preparation unstable. Increasing the NaCl concentration to 1.0 M had a profound effect (Fig. 2), shifting the dopamine competition curve to the right. A further shift to the right was seen upon addition of GTP (0.1 mM), but the apparent effect of GTP was decreased in the presence of 1 M-NaCl compared with that seen with 0.2 M-NaCl in the 5-fold-diluted preparation. The dopamine competition curves were fitted best by two-binding-site models, except in the presence of NaCl and GTP, where a one-binding-site model was the best fit. The proportion of higher-affinity sites was progressively decreased by the increase to 1 M-NaCl and the subsequent addition of GTP, with changes in the affinities of the sites as well. In comparison with the standard solubilized preparation (0.3% cholate/1 M-NaCl), where dopamine competition curves fit best to one-binding-site models, increasing the NaCl to 1 M in the 5-fold-diluted preparation in the absence of GTP fails to eliminate the higher-affinity binding sites fully.

Supplementation of the 5-fold-diluted preparation with 1 M-NaCl and 0.2% cholate together gave results (not shown) essentially identical with those obtained with addition of 1 M-NaCl alone, further supporting the contention that the dopamine competition curves are more sensitive to changes in the NaCl concentration than in the cholate concentration.

Similar effects were seen when KCl (0.8 M), sodium acetate (0.8 M) or the cation substitute *N*-methyl-D-glucamine hydrochloride (0.47 M) was added to the 5-fold-diluted preparation

Table 1. Dopamine/[³H]spiperone competition data at D₂ dopamine receptors in bovine caudate nucleus

Dopamine/[³H]spiperone competition data in various solubilized preparations of bovine caudate nucleus, supplemented as indicated, were analysed by non-linear least-squares computer curve fitting as in Withy *et al.* (1981), which gives IC₅₀^h and IC₅₀^l, the corrected IC₅₀ values for higher- and lower-affinity states, and the percentage of the higher-affinity form (% R_h) when data fit best to a two-site model. Some data fit best to a one-site model, in which case only IC₅₀^l is recorded. Data are expressed as means ± S.D. for *n* experiments. The K_d values for [³H]spiperone used to correct IC₅₀ values were 0.4 nM (5-fold- and 2-fold-diluted preparation) and 1.13 nM (1.33-fold-diluted preparation). The large errors in some cases reflect the pooling of data obtained with different preparations of receptors and the variations in the properties (IC₅₀ values) of different preparations. This makes it difficult to assess the significance of changes in IC₅₀ values. Where there were changes in mean IC₅₀ or % values, these always reflected clear effects of GTP in an experiment such as Fig. 1(b).

Preparation	No GTP				+ GTP			
	<i>n</i>	IC ₅₀ ^h (nM)	IC ₅₀ ^l (μM)	% R _h	<i>n</i>	IC ₅₀ ^h (nM)	IC ₅₀ ^l (μM)	% R _h
5-fold-diluted								
Control	23	73.4 ± 110	26.4 ± 49.6	55 ± 12	23	209 ± 287	18.2 ± 15.7	36 ± 20
Cholate (0.2 %)	5	140 ± 197	30.5 ± 28.8	73 ± 6	5	750 ± 1190	19.7 ± 12.4	38 ± 30
NaCl (1 M)	7	875 ± 1220	35.0 ± 6.0	31 ± 20	7	—	15.1 ± 4.8	0
NaCl (1 M) + cholate (0.2 %)	2	300 ± 417	8.6 ± 3.4	17 ± 2	2	—	14.3 ± 2.7	0
KCl (0.8 M)	3	1020 ± 1130	49.9 ± 60.7	53 ± 18	3	—	11.5 ± 2.8	0
MgCl ₂ (0.5 M)	5	—	19.8 ± 9.8	0	5	—	17.7 ± 6.1	0
No MgCl ₂	4	—	12.0 ± 5.1	0	4	—	16.5 ± 5.9	0
Sodium acetate (0.8 M)	2	59.4 ± 8.3	3.1 ± 0.1	28 ± 1	2	—	7.2 ± 4.2	0
<i>N</i> -Methyl-D-glucamine (0.54 M)	3	304 ± 465	48.7 ± 71.0	33 ± 33	3	—	24.6 ± 6.1	0
NaCl (0.5 M) + cholate (0.2 %)	3	284 ± 390	16.6 ± 21.7	44 ± 37	3	184 ± 157	12.1 ± 1.4	24 ± 12
2-fold-diluted								
NaCl (0.5 M) + cholate (0.2 %)	3	238 ± 317	12.7 ± 10.4	42 ± 36	3	680 ± 486	19.0 ± 4.4	53 ± 19
1.33-fold-diluted	3	14.2 ± 0.1	6.7 ± 0.3	17 ± 10	3	—	8.8 ± 4.3	0

Fig. 2. Effect of NaCl on dopamine/[³H]spiperone competition binding data in diluted solubilized preparations of bovine caudate nucleus

Dopamine/[³H]spiperone competition curves are shown for the 5-fold-diluted solubilized preparation with no addition (○), with 0.1 mM-GTP (□), with NaCl (to 1 M) (●) and with NaCl (1 M) and GTP (0.1 mM) (■). Data are the means of triplicate determinations (variation < 10% of mean) from representative experiments replicated as in Table 1, and the best fit curves to one-site (■) and two-site (○, ●, □) models are shown. The parameters of the best-fit curves were: ○, IC₅₀^h 120 nM (66%), IC₅₀^l 25.4 μM (34%); □, IC₅₀^h 840 nM (46%), IC₅₀^l 42.7 μM (54%); ●, IC₅₀^h 160 nM (20%), IC₅₀^l 32 μM (80%); ■, IC₅₀ 51.8 μM.

(0.2 M-NaCl/0.06 % cholate) (Fig. 3, Table 1). The bivalent cation Mg²⁺ was also tested by the addition of MgCl₂, but here the effects are more complex, as the previous experiments have all been performed in the presence of MgCl₂ (5 mM). In the absence of this MgCl₂, the dopamine competition curves are best

Fig. 3. Effect of *N*-methyl-D-glucamine on dopamine/[³H]spiperone competition binding data in diluted solubilized preparations of bovine caudate nucleus

Dopamine/[³H]spiperone competition curves are shown for the 5-fold-diluted solubilized preparations with no addition (○), or with addition of 0.1 mM GTP (□), *N*-methyl-D-glucamine (0.47 M) (●) or both *N*-methyl-D-glucamine and 0.1 mM-GTP (■). The data are the means of triplicate determinations (variation < 10% of mean) from representative experiments replicated as in Table 1, and the best-fit curves to one-site (■) and two-site (○, ●, □) models are shown. The parameters of the best-fit curves were: ○, IC₅₀^h 220 nM (68%), IC₅₀^l 36 μM (32%); □, IC₅₀^h 77 nM (19%), IC₅₀^l 17.8 μM (81%); ●, IC₅₀^h 230 nM (18%), IC₅₀^l 51.2 μM (82%); ■, IC₅₀ 140 μM.

fitted by a one-binding-site model and there is only a very small shift to the right with GTP. This is in marked contrast with the data obtained in the 5-fold-diluted preparation in the presence of

Fig. 4. Dopamine/[³H]spiperone competition binding data in diluted solubilized preparations of bovine caudate nucleus

Dopamine/[³H]spiperone competition curves are shown for a 5-fold-diluted solubilized preparation supplemented to 0.2% cholate/0.5 M-NaCl with (●) and without (○) 0.1 mM-GTP (a) and a 2-fold-diluted solubilized preparation supplemented to 0.2% cholate/0.5 M-NaCl with (■) and without (□) 0.1 mM-GTP (b). Data are the means of triplicate determinations (variations < 10% of mean) from representative experiments replicated as in Table 1, and the best-fit curves to two-site models are shown. The parameters of the best-fit curves are (a): no-GTP IC_{50}^h 1.7 μ M (70%), IC_{50}^l 97.5 μ M (30%); +GTP IC_{50}^h 0.22 μ M (15%), IC_{50}^l 40 μ M (85%); (b) no-GTP IC_{50}^h 1.4 μ M (64%), IC_{50}^l 46.7 μ M (36%); +GTP IC_{50}^h 3.1 μ M (56%), IC_{50}^l 42.6 μ M (44%).

MgCl₂ (5 mM). Addition of 0.5 M-MgCl₂, however, shifted the competition curves to the right and eliminated the effects of GTP, these effects being similar to those of addition of high concentrations of univalent cations.

We also compared the effects of GTP on dopamine competition curves in the 5-fold-diluted preparation supplemented to 0.5 M-NaCl/0.2% cholate and a 2-fold-diluted preparation supplemented to the same concentrations of NaCl and cholate (Fig. 4). Dopamine competition curves in the absence of GTP were very similar, but the addition of GTP produced a greater rightward shift in the 5-fold-diluted preparation compared with the 2-fold-diluted preparation, despite the concentrations of NaCl and cholate being identical. The competition curves were all fitted best by two-binding-site models.

Effect of dilution on physical state of solubilized D₂ dopamine receptors

Ultrafiltration through 200 nm-pore-size filters of the standard (0.3% cholate/1 M-NaCl) solubilized preparation of bovine caudate nucleus and the same preparation diluted 5-fold showed

Fig. 5. Thermal stability of solubilized preparations of bovine caudate nucleus

Standard solubilized preparation (○) and this preparation diluted 1.33-fold (□) or 5-fold (△) were incubated at 22 °C, and specific [³H]spiperone binding was determined at different times. The data are given as means $\pm$ S.D. for three experiments.

that in neither case was there major retention of either protein or [³H]spiperone-binding sites (results not shown). Thus the species present in each preparation are smaller than 200 nm.

Thermal-stability studies were performed on the standard soluble preparation and several diluted preparations by incubating them at 22 °C (Fig. 5). The rate of loss of [³H]spiperone-binding activity decreased as the preparation was diluted, and this can be assessed qualitatively from the half-lives of [³H]spiperone binding in the different preparations (30 min, 65 min and 470 min for the standard, 1.33-fold-diluted and 5-fold-diluted preparations respectively). It is difficult to provide a more quantitative assessment of the change in thermal stability, as the decay process seems to consist of more than one phase. If the data are transformed into a semi-log plot, then a fast initial decay and a slower later decay are seen (results not shown), and in the 5-fold-diluted preparation about half the receptors appear to be very stable for at least 24 h. Overall, however, the thermal stability of the D₂ dopamine receptors increased as the preparations were diluted more.

Gel filtration on a Sepharose CL 4B column was used to characterize the species in the different preparations. This technique could not be used for the standard soluble preparation, as it was found to be rather unstable to gel filtration. Gel filtration was possible for the 1.33-fold-diluted preparation, which was more stable, as shown in the thermal-stability experiments. The results are shown in Fig. 6, and show that for the 1.33-fold-diluted preparation most of the [³H]spiperone binding recovered migrated as a peak of Stokes radius 6.3 $\pm$ 0.3 nm (mean $\pm$ S.D., 5 observations), although a small amount appeared in the void volume (recoveries were 45.7 $\pm$ 11.9% and 17.8 $\pm$ 0.4% respectively; means $\pm$ S.D., n = 5). For the 5-fold-diluted preparation, 51.1 $\pm$ 9.7% (n = 3) of the applied [³H]spiperone-binding sites were recovered, and all were found in the void volume. There was also a general shift to a distribution of protein of larger apparent Stokes radius.

Further evidence for an increase in particulate material upon dilution was obtained when the standard soluble preparation and various diluted preparations were centrifuged at 100 000 g for 90 min and the distributions of [³H]spiperone-binding sites and protein in the supernatant and pellet were determined (Table 2). For the standard soluble preparation and a 1.1-fold-diluted preparation there was no evidence for sedimentation of

Fig. 6. Gel filtration of solubilized preparations of bovine caudate nucleus

Samples of the 1.33-fold-diluted (a) and 5-fold-diluted (b) preparations were chromatographed on Sepharose CL 4B columns as described in the Experimental section. Specific [³H]spiperone binding (○, ●) and protein (■, □) were determined in the eluted fractions. V_0 represents the void volume and 1, 2, 3 represent the elution volumes for alcohol dehydrogenase, albumin and cytochrome *c* (Stokes radii 4.50, 3.55, 1.85 nm respectively). Each point is the mean of triplicate determinations, and each experiment has been replicated three times with similar results.

[³H]spiperone-binding sites or protein, but for greater dilutions there was a progressive appearance of [³H]spiperone-binding sites in the pellet and a corresponding loss from the supernatant. For the 5-fold-diluted preparation 93% of the [³H]spiperone binding was found in the pellet. When total protein was analysed, a similar trend was seen, although the extent of sedimentation was less.

Fig. 7. Electron microscopy of 5-fold-diluted preparations of bovine caudate nucleus (magnification $\times 33\,000$)

The bar represents 100 nm.

Electron-microscopic analysis of the 5-fold-diluted preparation demonstrated the presence of heterogeneous unilamellar vesicles (25–75 nm diameter, mean 51 ± 12 nm) (Fig. 7). The standard soluble preparation did not show such vesicles, and instead appeared to consist of amorphous material, as we have reported previously for a similar preparation (Hall *et al.*, 1983; Strange, 1983). Further examination of a range of dilutions of standard soluble preparation (1.33–5-fold) showed the presence of vesicles, but it was not possible to quantify the proportion of vesicles in the different preparations.

DISCUSSION

In this report we have attempted to examine the factors that regulate the appearance of receptor–G-protein (R–G) coupling in detergent-solubilized preparations of the D₂ dopamine receptor. We show that, in cholate-solubilized preparations of D₂ dopamine receptors from bovine caudate nucleus, R–G coupling can only be observed after apparent reconstitution of receptor and G-protein into vesicles has occurred.

We examined R–G coupling in cholate-solubilized preparations of bovine caudate nucleus membranes, using dopamine/

Table 2. Centrifugation of solubilized preparations of bovine caudate nucleus

Standard solubilized preparation and various dilutions thereof were centrifuged (100 000 *g*, 90 min), and specific [³H]spiperone binding and protein were determined in the supernatant and resuspended pellet. Values are means $\pm$ S.D. (3 or more experiments) or mean $\pm$ range (2 experiments) and are expressed as percentages of the values in the starting preparations: *n* is the number of experiments.

(fold) Dilution	[³ H]Spiperone binding (% recovery)				Protein (% recovery)			
	Pellet	Supernatant	Total	<i>n</i>	Pellet	Supernatant	Total	<i>n</i>
None	2.3 $\pm$ 1.7	84.7 $\pm$ 3.9	87	4	5.9 $\pm$ 0.4	101.8 $\pm$ 8.2	108	4
1.1	1.8 $\pm$ 1.2	100.0 $\pm$ 14.9	102	3	6.6 $\pm$ 1.4	103.6 $\pm$ 4.6	110	3
1.2	28.5 $\pm$ 2.1	74.0 $\pm$ 3.4	103	3	13.3 $\pm$ 0.9	86.3 $\pm$ 18.4	100	3
1.33	60.6 $\pm$ 5.1	39.2 $\pm$ 4.5	100	5	19.6 $\pm$ 2.0	82.6 $\pm$ 14.8	102	5
5	56.9 $\pm$ 11.0	4.1 $\pm$ 3.8	61	6	41.8 $\pm$ 2.6	56.5 $\pm$ 0.9	98	2

[³H]spiperone competition binding as our assay system. Evidence for R–G coupling was obtained from analysis of the dopamine/[³H]spiperone competition curves and the effects of GTP. The competition curves were analysed by using one- and two-binding-site models, and the best fit to the data was determined. Where the two-binding-site model was the best fit, we have taken this as evidence for R–G coupling, as the presence of higher- and lower-affinity binding sites is considered to be a reflection of R–G coupling. There is, however, no simple relationship between the two affinity states obtained from such an analysis and the coupled and uncoupled forms of the receptor (Wreggett & De Lean, 1984; Strange, 1987). An exception to this is when competition curves model to a single class of low-affinity sites, where the affinity of the agonist reflects its affinity for the uncoupled form of the receptor. The effects of GTP on dopamine/[³H]spiperone competition curves were also assessed. This is expected to be seen as a change in the proportion of higher- and lower-affinity agonist-binding sites, resulting in agonist competition occurring at higher concentrations, and these effects will reflect R–G coupling and its modulation by GTP.

In our standard solubilized preparation (Williamson *et al.*, 1988) (0.3 % cholate/1 M-NaCl) there was no effect of GTP on dopamine competition curves, which also followed one-binding-site models, and this agrees with other reports on similar preparations (Ohara *et al.*, 1988). A 5-fold dilution of this preparation (0.06 % cholate/0.2 M-NaCl) gave dopamine competition curves which fitted two-binding-site models and which were substantially shifted to lower affinity upon addition of GTP. These findings are similar to those reported by Kazmi *et al.* (1986). The proportion of apparently higher-affinity binding sites was decreased, but not eliminated, upon addition of GTP, and the effects of GTP were larger than those observed in unsolubilized membranes (Leonard *et al.*, 1987). From these data we can conclude that R–G coupling is clearly detectable in the 5-fold-diluted preparation, but not in the standard soluble preparation. The inability of GTP to eliminate completely the higher-affinity binding sites in the diluted preparation is similar to observations reported on membrane-bound (De Lean *et al.*, 1982; De Keyser *et al.*, 1985; Leonard *et al.*, 1987) and solubilized (Kazmi *et al.*, 1986) preparations of D₂ dopamine receptors. It presumably reflects the complexities of R–G interaction in the Ternary Complex Model (Wreggett & De Lean, 1984) and should not be over-interpreted (Strange, 1987). Intermediate dilutions of the standard solubilized preparation also gave preparations in which R–G coupling could be demonstrated, but the extent of the GTP effects was decreased, again supporting the idea that the dilution state of the preparation determines the apparent R–G coupling.

We attempted to determine the factors responsible for the effects of dilution, concentrating on the principal species cholate and NaCl, by supplementing the 5-fold-diluted preparation. These experiments showed that cholate had little effect, whereas NaCl alone substantially affected competition curves. In the presence of NaCl the proportion of higher-affinity agonist-binding sites was decreased and the competition curves were substantially shifted to the right, reflecting this decrease in higher-affinity sites as well as changes in their affinities. There was still a GTP effect on the agonist competition curve, although this was decreased in size, and in the presence of GTP the dopamine competition curve modelled best to a single class of lower-affinity sites.

The effects of NaCl could be mimicked in part by KCl, sodium acetate, MgCl₂ or the cation substitute *N*-methyl-D-glucamine, so that the observed effects are likely to be due to changes in the ionic strength rather than specific effects of NaCl. For most of

these substances their addition decreased the number of apparent higher-affinity sites and shifted the dopamine competition curves to the right, this latter effect including decreases in the affinities of the higher- and lower-affinity states. The size of the GTP effects was variable, depending on the salt added, and in some cases was similar to the control value (KCl, sodium acetate, *N*-methyl-D-glucamine), whereas for MgCl₂ there was no GTP effect. In the presence of the salts and GTP, only a single class of lower-affinity sites was seen. Similar effects of NaCl on other receptors have been reported for A₁ adenosine receptors (Stiles, 1985) and D₂ dopamine receptors (Watanabe *et al.*, 1985), but from the present experiments this seems to be more an effect of ionic strength.

Therefore the addition of different salts to the 5-fold-diluted preparation decreases the number of apparent higher-affinity agonist-binding sites, and thus modulates R–G coupling. Competition curves are shifted to the right, reflecting this change, but there is an additional effect in some cases on the affinities of the higher- and lower-affinity sites, which may reflect effects at the receptor itself. GTP effects are still retained in the presence of the salts, although sometimes these are decreased, but in the presence of the salt and GTP a complete shift to lower-affinity states is seen. Therefore in some respects the addition of salt renders the 5-fold-diluted preparation similar to the undiluted preparation, but the retention of GTP effects is a major difference. It seems that some other factor is important as well, and this may be the actual dilution of the preparation.

The importance of the dilution state of the preparation is highlighted by the experiment where 2-fold- and 5-fold-diluted preparations were compared under identical NaCl (0.5 M) and cholate (0.2 %) concentrations. Greater effects of GTP were seen in the 5-fold-diluted preparations, supporting the idea that some other factor related to the actual dilution of the solubilized preparation affects R–G coupling, rather than the concentrations of NaCl and cholate.

We therefore examined the effects of dilution on the physical state of the preparations. Centrifugation, gel filtration and electron-microscopic studies suggested that, whereas the standard solubilized preparation is indeed truly solubilized, dilution of this preparation creates vesicles (mean diameter 51 nm) which are sedimentable (100 000 *g*, 90 min) and appear in the void volume of a Sepharose CL 4B gel-filtration column. In the 5-fold-diluted preparation vesicularization is complete, whereas for intermediate dilutions partial vesicularization occurs. For example, in the 1.33-fold-diluted preparation, upon gel filtration 18 % of the receptors appear in the void volume and 46 % behave as a 6.3 nm (soluble) species. From the same preparation 61 % of the receptors are sedimentable (corresponding to the vesicle form), the remainder remaining soluble (corresponding to the 6.3 nm species).

The thermal-stability experiments show that there is a general increase in stability as the preparation is diluted, as judged from the half-life of [³H]spiperone binding in the different preparations. The decay kinetics of [³H]spiperone binding in the different preparations are complex and consist of at least two processes. The faster process predominates in the standard solubilized preparation, but as the preparation is diluted the slower process becomes a more significant part of the decay process. It is tempting to suggest that the faster process represents solubilized receptor and the slower process the vesicle-incorporated receptor. The data show, if this proposal is correct, that the solubilized receptor itself becomes more stable as the preparation is diluted (as judged by the rate for the fast decay phase), and this presumably reflects the lower cholate concentration.

It therefore seems reasonable to propose that the extent to which the R–G coupling is observable in different preparations

depends on the extent of formation of vesicles. The 6.3 nm species would then represent receptor in a state where R-G coupling is not observable and the 51 nm vesicular species would represent a state with R-G-coupled receptor. This would then also explain the different extents of GTP effects in the 5-fold- and 2-fold-diluted preparations when tested under identical conditions, as the two preparations will contain different proportions of the two species.

We have not made a detailed comparison of the ligand-binding properties of the fully vesicular species (5-fold-diluted preparation) and native membrane-bound receptors, but affinities for antagonists are comparable. For example, the dissociation constant for spiperone was 0.4 nM in the 5-fold-diluted preparation and 0.17 nM in membrane preparations (Leonard *et al.*, 1987). Agonist affinities were, however, substantially increased in the 5-fold-diluted preparation when compared with the membrane preparation. The basis for these differences is unclear.

We can then conclude that the undiluted solubilized preparations show no R-G coupling because the receptor is solubilized free of G-proteins. Dilution of this preparation forms vesicles into which receptor and G-protein can insert and couple, and then changes in the salt concentration affect the observed R-G coupling, but do not eliminate this. Therefore the effects of the dilution are on the physical state of the preparation, and the concomitant change in NaCl serves only to modulate the observed R-G coupling. These observations are relevant to attempts to purify D₂ dopamine receptors by affinity chromatography from highly diluted soluble preparations (Ramwani & Mishra, 1986), where all the D₂ dopamine receptors will be contained in vesicles. Similarly, the apparent retention of R-G coupling in solubilized D₂ dopamine receptor preparations (Kazmi *et al.*, 1986) reflects the diluted state of the preparation.

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