Biochemical Pharmacology, Vol. 36, No. 13, pp. 2095–2099, 1987.
Printed in Great Britain.

0006–2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

DOPAMINE AGONIST HIGH-AFFINITY STATE IN SOLUBILIZED D_2 RECEPTORS IN STRIATUM, BUT NOT IN ANTERIOR PITUITARY

ANNE DUMBRILLE-ROSS* and PHILIP SEEMAN
Department of Pharmacology, University of Toronto, Toronto, Canada M5S 1A8

(*Received* 15 *August* 1986; *accepted* 25 *November* 1986)

Abstract—To determine whether brain and pituitary dopamine D_2 receptors have similar characteristics on solubilization, canine striatal and bovine anterior pituitary tissues were solubilized with digitonin, and their D_2 dopamine receptors were characterized by competition studies and by high pressure liquid chromatography. Solubilized striatal membranes retained the ability to bind agonists with high affinity and retained sensitivity to guanine nucleotides. In contrast, however, solubilized pituitary membranes no longer revealed high-affinity binding of agonists and sensitivity to guanine nucleotide unless they had been solubilized in the presence of agonist. Agonist-pre-labelled pituitary receptors were of larger apparent molecular weight than antagonist-pre-labelled receptors. However, striatal receptors pre-labelled by agonist or antagonist were of similar apparent molecular weight. Thus, unlike the pituitary, striatal receptors probably remain associated with a guanine nucleotide binding protein (N) upon solubilization.

Dopamine D_2 receptors in both the anterior pituitary gland and the brain striatum are associated with dopamine-inhibited adenylate cyclase [1, 2]. It remains to be established whether the D_2 receptors in these different tissues are identical proteins.

The D_2 receptors in both tissues can exist in either a high-affinity state, D_2^{High}, or a low-affinity state, D_2^{Low}, for agonists [3–6]. The D_2^{High} state can be converted to the D_2^{Low} state with sodium ions and a guanine nucleotide [4, 5]. This effect of guanine nucleotide is a result of an association between the receptor and the guanine nucleotide binding protein in the well characterized β-adrenergic system [7]. A similar association has been postulated for the guanine nucleotide sensitivity of α_2-adrenergic [8], adenosine A_1 [9], dopamine D_1 [10] and dopamine D_2 [11] receptors.

The D_2^{High} state is lost, however, in digitonin-solubilized tissue from the anterior pituitary [11]. This also occurs in solubilized α_2- and β-adrenoceptors [8, 12, 13], all of which lose their sensitivity to guanine nucleotide.

This present report indicates, however, that the D_2^{High} state can exist in the digitonin-solubilized striatum, suggesting that there may be different biochemical control mechanisms in central and peripheral tissues.

* Address correspondence to: A. Dumbrille-Ross, Ph.D., Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, Ontario M5S 1A8, Canada.

† Abbreviations: ADTN (±)-6,7-dihydroxy-2-aminotetralin; Gpp(NH)p, guanylylimidodiphosphate; K_D dissociation constant; NPA, *N-n*-propylnorapomorphine; and N_i, guanine nucleotide inhibitory protein.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Spiperone (26.4 Ci/mmol) and [^{3}H]*N*-propylnorapomorphine (66 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{3}H]Spiperone (89 Ci/mmol) was from the Amersham Corp. (UK). Digitonin from the Wako Chemical Co. (Japan). HPLC molecular exclusion columns (Toyosoda, TSK-4000 SW) were purchased from the Beckman Instrument Co. (Berkeley, CA). Sephadex G-50 (fine) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and ADTN† from Research Biochemicals Inc. (Wayland, MA). Dopamine, norepinephrine, serotonin and Gpp(NH)p were from the Sigma Chemical Co. (St. Louis, MO). (+)-Butaclamol was donated by Ayerst Research Laboratories (Montreal, Quebec); SCH 23390 was donated by the Schering Corp. (Bloomfield, NJ).

Membrane preparation. Frozen brain (canine) and bovine pituitaries were obtained from Pel-Freeze Biologicals (Rogers, AR). All tissues were stored at −70° until used.

Striata were dissected from partially thawed brains and immediately homogenized (Brinkmann Polytron, 20 sec at setting #6) in 20 ml of 50 mM Tris–HCl buffer containing: 1 mM EDTA (acid), 5 mM KCl, 1.5 mM $CaCl_2$ and 4 mM $MgCl_2$, pH 7.4, at 4°. All procedures were carried out at 0–4°. Homogenates were centrifuged for 20 min at 48,000 *g*, and the resulting pellets were resuspended in an original volume of buffer and recentrifuged. Tissue was kept on ice until used.

Anterior pituitaries were dissected from thawed whole pituitaries and minced finely with scissors, suspended in chilled buffer as above (pH 7.4 at 4°, 66 mg tissue/ml), disrupted with a Brinkmann Polytron (20 sec at setting #7), and passed through two

layers of cheesecloth. The homogenate was centrifuged at 480 *g* for 10 min at 4°. The supernatant fraction was kept aside, and the pellet was resuspended and centrifuged again similarly in buffer. The combined supernatant fractions were centrifuged at 30,000 *g* for 30 min at 4°. The resulting pellet was resuspended in buffer (600 mg original wet weight/ml). This was kept frozen at −70° until used.

Membranes were preincubated at 37° for 10 min for HPLC experiments.

Receptor solubilization. Striatal membrane pellets were resuspended in Tris-ion buffer (as above) containing 1% digitonin (wt/v), 0.1% ascorbate and 12.5 μM nialamide, pH 7.4 (4°), with a membrane to detergent ratio of 100 mg tissue/10 mg digitonin/ml solution. Pituitary membranes were centrifuged at 30,000 *g* for 10 min and resuspended to a membrane to detergent ratio of 600 mg tissue/10 mg digitonin/ml solution, and the following protease inhibitors were present: bacitracin (100 μg/ml), benzamidine (10^{-4} M), trypsin inhibitor (10 μg/ml) and phenylmethylsulfonyl fluoride (10^{-5} M). The suspension was stirred slowly for 30–40 min at 4° with the solution subsequently centrifuged for 60 min at 110,000 *g*. The supernatant fraction containing soluble receptors was aspirated and placed on ice for immediate use. During incubation with the ^{3}H-labeled ligand, membranes or soluble preparations were concomitantly incubated with 100 nM SCH-23390 (final concentration) to occlude binding to membrane dopamine D_1 receptors.

Soluble receptor binding assays. For saturation experiments, 0.3 ml (0.45 ± 0.03 mg protein) of solubilized receptor preparation was incubated in duplicate with increasing concentrations (0.075 ml) of [^{3}H]spiperone or [^{3}H]*N*-propylnorapomorphine for 90 min at 22° in a total assay volume of 0.45 ml Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, and 4 mM $MgCl_2$. Steady-state binding conditions were attained during this time. Bound receptor was separated from free ^{3}H-labeled ligand by Sephadex G-50 (fine) chromatography. Briefly, 0.3 ml of the assay mixture was applied to a 4-ml column (0.5 × 12 cm) of Sephadex G-50, preequilibrated at 4° with 50 mM Tris–HCl buffer (as above) and 0.1% digitonin. Receptors were eluted in the void volume at a flow rate of 0.5 ml/min at 4°. Fractions (0.5 ml) were collected and monitored for tritium on a Beckman 460 liquid scintillation spectrometer at 43% efficiency. Non-specific binding to soluble D_2 receptors was defined as that binding which occurred in the presence of 10 μM (+)-butaclamol.

For competition experiments, 0.3 ml of soluble receptor was incubated in duplicate with increasing concentrations of ADTN and 0.075 ml of [^{3}H]spiperone (0.5 nM, final concentration) for 90 min at 22°, as described above. Agonist/[^{3}H]spiperone competition experiments were done as described above except for the addition of 0.1% ascorbate and 12.5 μM nialamide in either the absence or presence of 120 mM NaCl and/or 100 μM Gpp(NH)p, where indicated. Assays were terminated by Sephadex G-50 chromatography at 4° as described above.

Data analysis. All saturation and competition data were analyzed using the non-linear least-squares curve-fitting program LIGAND [14]. Scatchard analyses were performed for each assay condition to be tested for agonist/^{3}H-labeled antagonist interaction. The dissociation constant obtained was subsequently used in the determination of agonist competition parameters. Both saturation and competition data were fit using the extra sum of squares principle to allow objective analysis. Data were routinely fit to both single- and multiple-site models, and compared for statistically significant differences. The model chosen was that which best described the data at a 0.01 level of significance. A more complex model was chosen only if it represented a statistically significant improvement in fit between the estimated parameters and the raw data from two or more independent experiments as described in Ref. 5.

HPLC of solubilized receptor preparation on steric exclusion columns. For experiments with "pre-labelled" receptors, membrane preparations were first labelled with [^{3}H]*N*-propylnorapomorphine or [^{3}H]spiperone in the absence and presence of (+)-butaclamol for 60 min at 22°, centrifuged (15 min, 30,000 *g*) to remove free ligand, and then solubilized with 1% digitonin. The parallel "post-labelling" experiments were treated in an identical manner but no ^{3}H-labeled ligand was added. These membranes were solubilized and then incubated with ^{3}H-labeled ligands.

With the exception of experiments involving the pre-labelling of some preparations, solubilized receptors were concentrated 2- to 4-fold on an Amicon concentrating cell with a YM-30 membrane. The solubilized preparations were passed through a 220 nM Millipore filter and incubated with either [^{3}H]spiperone (3 nM) or [^{3}H]*N*-propylnorapomorphine (5 nM) for 60 min at 22° in the absence or presence of 10 μM (+)-butaclamol. Each solubilized sample (0.5 ml) was chromatographed on two tandem-linked TSK-4000 (7.5 × 300 mm) steric exclusion columns at a flow rate of 1 ml/min at 4° using a Waters HPLC pump (model 510) and u.v. detector (Series 440). The mobile phase consisted of 50 mM Tris–H_2SO_4, 10 mM $MgCl_2$ and 0.1% digitonin at pH 7.0 (4°). Twelve minutes after sample injection, 0.2-ml fractions were collected and monitored for tritium by liquid scintillation spectrometry. Continuous protein profiles were obtained at a fixed wavelength of 280 nm. TSK columns were calibrated with protein standards of known molecular weights (Pharmacia): thyroglobin (M_r = 669,000), ferritin (M_r = 440,000), catalase (M_r = 232,000), aldolase (M_r = 158,000), and bovine serum albumin (M_r = 67,000).

RESULTS

Anterior pituitary. The D_2^{High} state was lost in digitonin-solubilized anterior pituitary tissue. This is shown in Fig. 1 and Table 1, where ADTN recognized only a single population of [^{3}H]spiperone binding sites in the solubilized tissue. These sites correspond to the agonist low-affinity form (D_2^{Low}) in

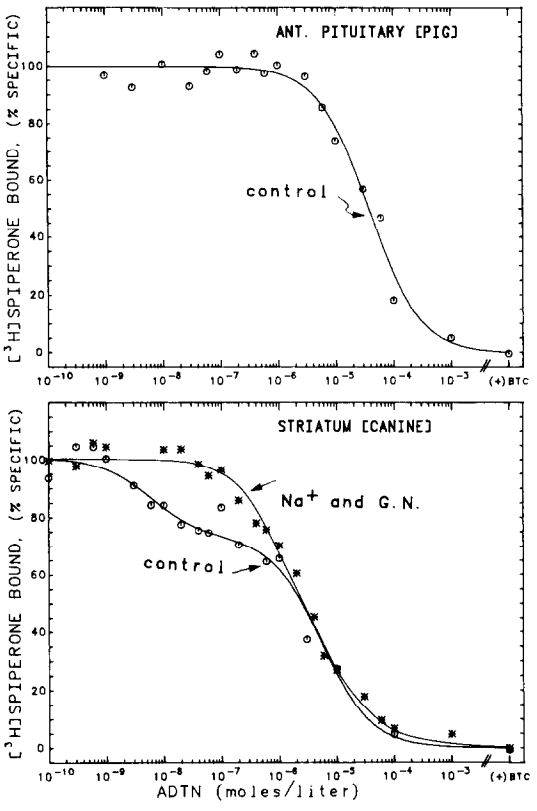

Fig. 1. Ability of the dopamine agonist ADTN to compete for the binding of [^{3}H]spiperone to soluble preparations of striatal and anterior pituitary dopamine receptors. Anterior pituitary: Increasing concentrations of ADTN were incubated with anterior pituitary tissue, 500 pM [^{3}H]spiperone (in the absence of sodium ions) and Gpp(NH)p [G.N.], and the binding of [^{3}H]spiperone was assayed as described in Experimental Procedures. Brain striatum: ADTN was incubated with striatal tissue and [^{3}H]spiperone in the absence of sodium ions (control) or in the presence of 120 mM sodium chloride and 100 μM Gpp(NH)p. Non-specific binding was determined in the presence of 10 μM (+)-butaclamol. The data were analyzed by computer as described above. Dissociation constants are listed in Table 1. The results shown are from experiments conducted in duplicate.

the native membrane. This suggests that, upon solubilization, the pituitary receptor has lost its ability to bind agonists with high affinity. A similar loss of high-affinity binding and of sensitivity to guanine nucleotide of agonist competition curves for ^{3}H-labeled antagonist upon solubilization has been reported previously.

The apparent molecular size of agonist pre-labelled, and antagonist pre- or post-labelled, receptors was similar in the pituitary: [^{3}H]NPA did not bind specifically to agonist post-labelled receptors (Fig. 2).

Brain striatum. Competition experiments with solubilized striatal preparations revealed that ADTN competed with [^{3}H]spiperone binding at both the high- and low-affinity forms of the receptor (Fig. 1). The high-affinity component was lost on addition of 100 μM Gpp(NH)p in the presence or absence of sodium (Table 1), indicating that sensitivity to guanine nucleotide was retained on solubilization of this tissue.

When striatal tissue was solubilized, subsequently labelled with ^{3}H-labeled agonist in the presence of guanine nucleotide, and chromatographed on steric exclusion columns, the binding was decreased 50% but was not lost completely. The apparent molecular weight of the receptors remaining in the presence of guanine nucleotide was not detectably different from that in control preparations. The presence of sodium ions did not alter the apparent molecular size of the binding complex.

The apparent molecular size of agonist pre-labelled, and antagonist pre- or post-labelled, receptors was similar in the striatum (Fig. 3). There was a small but consistent decrease in the apparent molecular size of agonist post-labelled receptors. This difference corresponds to a calculated molecular weight of approximately 50,000.

DISCUSSION

The main finding of this study was that the agonist still bound with high and low affinity to solubilized striatal receptors, but only with low affinity to solubilized receptors of the anterior pituitary. The high-affinity agonist state of the solubilized striatal receptors was still capable of being modulated by guanine nucleotide.

Table 1. Regulation of agonist affinity for solubilized dopamine receptors in the striatum and pituitary

	K_D (nM) of ADTN				Proportions (%)			
	Striatum		Pituitary		Striatum		Pituitary	
	D_2^{High}	D_2^{Low}	D_2^{High}	D_2^{Low}	D_2^{High}	D_2^{Low}	D_2^{High}	D_2^{Low}
Control	24	2,700	—	15,800	35	65	0	100
G.N.	—	1,750	—	—	0	100	—	—
G.N. + Na^+	—	1,600	—	—	0	100	—	—

Soluble preparations of canine striatal or bovine pituitary membranes were incubated with 10^{-14} to 10^{-3} M ADTN in the absence or presence of 120 mM sodium chloride and/or 100 μM Gpp(NH)p [G.N.], and 500 pM [^{3}H]spiperone for 90 min at 22°. Assays were conducted and later analyzed by computer for both one- and two-site fits as described in Experimental Procedures. Values are means of two to four independent experiments performed in duplicate. The SEM was < 15%.

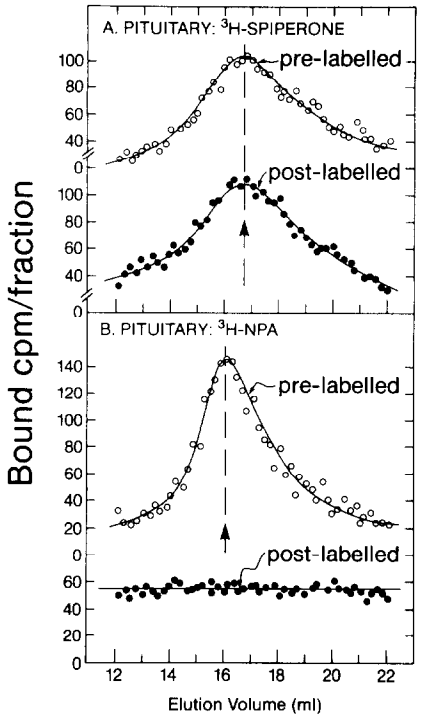

Fig. 2. Molecular exclusion HPLC elution profiles of pre- and post-labelled solubilized anterior pituitary dopamine receptors. (A) Pre-labelled receptors were incubated with 2 nM [^{3}H]spiperone in the presence of sodium chloride (120 mM) and, then, solubilized with 1% digitonin at 4°, in the absence or presence of 120 mM NaCl. Post-labelled receptors were initially solubilized with 1% digitonin and then incubated with [^{3}H]spiperone. All samples were incubated in the presence of 100 nM SCH-23390 which occluded D_1 receptors. Similar to Fig. 2, each sample was applied to two TSK-4000 HPLC steric exclusion columns and chromatographed at a flow rate of 1 ml/min at 4°. Non-specific binding was defined in the presence of 10 μM (+)-butaclamol. Unlike striatal tissue, in the pituitary there was a small non-specific peak (up to 70 cpm) which occurred at an elution volume of 18.3 ml. Specific binding was determined by subtracting the counts in the elution profiles of soluble preparations in the presence of butaclamol from the total profile. Total binding was between 800 and 1000 dpm in all cases. Elution volumes (ml) of the peaks were as follows: [^{3}H]spiperone pre-label, 16.6; [^{3}H]spiperone post-label, 16.6. (B) Receptors were pre-labelled or post-labelled with 5 nM [^{3}H]*N*-propylnorapomorphine ([^{3}H]NPA), and experiments were performed as described for [^{3}H]spiperone. The elution volume of the [^{3}H]NPA pre-label peak was 16.0. There was no [^{3}H]NPA post-label peak. All four preparations were from the same tissue batch and run the same day. Experiments were performed in the presence of sodium as elution peaks were 40% smaller in its absence. Elution volumes were identical in the presence or absence of sodium ions.

Although D_2 receptors solubilized from anterior pituitary membranes lost high-affinity agonist binding and sensitivity to guanine nucleotide, this was not the case with striatal receptors. The high-affinity agonist interaction with soluble striatal receptors, the sensitivity to guanine nucleotide, as well as the finding that the receptor complex has the same apparent molecular size regardless of whether it was pre-labelled with agonist, or pre- or post-labelled with

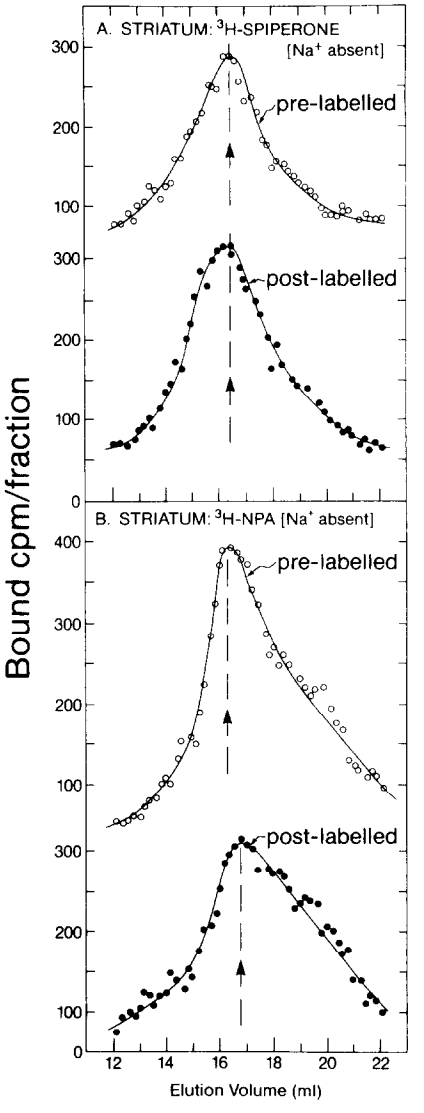

Fig. 3. Molecular exclusion HPLC elution profiles of pre- and post-labelled solubilized striatal dopamine receptors. (A) Pre-labelled receptors: receptors were pre-labelled with 2 nM [^{3}H]spiperone and solubilized with 1% digitonin at 4°. Post-labelled receptors: receptors were solubilized with 1% digitonin and subsequently incubated with 2 nM [^{3}H]spiperone. SCH-23390 (100 nM) was present in all tubes to occupy D_1 sites. Each solubilized sample (0.5 ml) was applied to two tandem-linked TSK-4000 molecular exclusion columns and chromatographed at a flow rate of 1 ml/min at 4°. Non-specific binding, determined in the presence of 10 μM (+)-butaclamol, represented less than 10% of the signal. Total binding was between 900 and 1100 dpm in all cases. Experiments were in the absence of sodium ions. Elution volumes (ml) of the peaks were as follows: [^{3}H]spiperone pre-labelled, 16.5; [^{3}H]spiperone post-labelled, 16.5. (B) Receptors were pre- or post-labelled with 5 nM ([^{3}H]NPA as described for [^{3}H]spiperone. Elution volumes (ml) of the peaks were as follows: [^{3}H]NPA pre-labelled, 16.5; [^{3}H]NPA post-labelled, 16.9. All four preparations were from the same tissue batch and run the same day.

antagonist, suggest that the striatal receptor–N protein complex may be stable to solubilization even without agonist occupation of the receptor. Similarly, while peripheral α_2- and β-adrenergic receptors lose the agonist high-affinity state upon solubilization, central dopamine D_1, muscarinic cholinergic and adenosine A_1 receptors do not [8, 9, 12, 13]. The fact that there was no detectable change in the molecular weight after addition of guanine nucleotide suggests that the N protein was closely associated with the receptor both after solubilization and in the presence of guanine nucleotide. The fact that the agonist high-affinity form of the receptor was diminished suggests that there was a change, possibly allosteric in nature, in the receptor-N complex. Similar to our results, Kuno *et al.* [15] observed that a high- and low-affinity state of the D_2 receptor was detectable after solubilization with CHAPS. Contrary to these studiess, Leff and Creese [16] could not detect high-affinity binding in solubilized striatal tissue. This may have been a result of preparing the tissue in the presence of sodium, which dissolves the N protein from the receptor.

The retention of the high-affinity form of the receptor upon solubilization may be a characteristic of brain receptors. It may be due to residual dopamine in the solubilized striatal preparations (as dopamine is present in pre-synaptic striatal neurons) but not in the pituitary. However, it is unlikely that sufficient endogenous dopamine would remain after the tissue was washed, preincubated at 37°, and the membranes subsequently disrupted by solubilization.

There may be a factor or membrane component other than N_i which stabilizes the receptor form which has high affinity for agonist upon solubilization in the brain but not in the periphery. The identity of this component is not known, but there is evidence of non-N_i proteins associated with other brain receptors. A guanine nucleotide binding protein of unknown function, G_o, has been found in the brain in association with the muscarinic cholinergic receptor [17, 18]. This protein is structurally similar to N_i, and also increases agonist affinity for the receptor only in the absence of guanine nucleotide. Unlike N_i, the G_o protein is not removed from the membrane by GTPγ S [18]. It is not yet known if this protein is present in peripheral tissues. Thus, a guanine nucleotide binding protein similar to this G_o protein may be tightly associated with striatal D_2 receptors (but perhaps not pituitary receptors) which maintains the agonist high-affinity state in the absence of guanine nucleotide. However, this is only speculative. Different proteins have been found associated with the thyrotropin [19] and insulin [20] receptors which appear to regulate ligand binding to these receptors.

Acknowledgements—This work was supported by grants from the Medical Research Council (Canada) and The Ontario Mental Health Foundation. A. Dumbrille-Ross is a Post-doctoral Fellow supported by the Ontario Mental Health Foundation. We thank H. B. Niznik, D. Grigoriadis and A. Tirpak for their help and discussion.

REFERENCES

1. P. De Camilli, D. Macconi and A. Spada, *Nature, Lond.* **278**, 151 (1979).
2. S. Weiss, M. Sebben, J. A. Garcia-Sainz and J. Bockaert, *Molec. Pharmac.* **27**, 595 (1985).
3. D. R. Sibley, A. DeLean and I. Creese, *J. biol. Chem.* **257**, 6351 (1982).
4. K. A. Wreggett and P. Seeman, *Molec. Pharmac.* **25**, 10 (1984).
5. D. Grigoriadis and P. Seeman, *J. Neurochem.* **44**, 1925 (1985).
6. M. Watanabe, S. R. George and P. Seeman, *Biochem. Pharmac.* **34**, 2459 (1985).
7. L. E. Limbird, D. M. Gill and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **77**, 775 (1980).
8. L. E. Limbird, *Endocr. Metab.* **10**, E59 (1984).
9. G. L. Stiles, *J. biol. Chem.* **260**, 6728 (1985).
10. H. B. Niznik, N. Y. Otsuka, A. Dumbrille-Ross, D. Grigoriadis, A. Tirpak and P. Seeman, *J. biol. Chem.* **261**, 8397 (1986).
11. B. F. Kilpatrick and M. G. Caron, *J. biol. Chem.* **258**, 13528 (1983).
12. K. H. Jakobs, M. Minuth and K. Aktories, *J. Receptor Res.* **4**, 443 (1984).
13. A. DeLean, B. F. Kilpatrick and M. G. Caron, *Molec. Pharmac.* **22**, 290 (1982).
14. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
15. T. Kuno, K. Saijoh and C. Tanaka, *J. Neurochem.* **41**, 841 (1983).
16. S. E. Leff and I. Creese, *Biochem. biophys. Res. Commun.* **108**, 1150 (1982).
17. V. A. Florio and P. C. Sternweis, *J. biol. Chem.* **260**, 3477 (1985).
18. P. C. Sternweis and J. D. Robishaw, *J. biol. Chem.* **259**, 13806 (1984).
19. T. B. Nielsen, Y. Totsuka, E. S. Kempner and J. B. Field, *Biochemistry* **23**, 6009 (1984).
20. J. T. Harmon, J. A. Hedo and C. R. Kahn, *J. biol. Chem.* **258**, 6875 (1983).