Antisera specific for D₂ dopamine receptors

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Antisera have been raised against two peptides from the sequence of D₂ dopamine receptors: peptide 1 from the predicted second extracellular loop and peptide 2 from the predicted third intracellular loop. The antisera recognize specifically a 95 kDa band in Western blots of several bovine brain regions, which corresponds to the denatured D₂ dopamine receptor, whereas in recombinant CHO cells expressing D₂ dopamine receptors a 80 kDa band is seen. The antisera

immunoprecipitate 10–20% of the D₂ dopamine receptors from soluble preparations of bovine brain. The antisera recognize D₂ dopamine receptors in immunofluorescence analyses of recombinant CHO cells bearing the receptor gene. The antisera directed against the third intracellular loop, but not those against the second extracellular loop, will interfere with the coupling of D₂ dopamine receptors and G-proteins in bovine brain preparations.

INTRODUCTION

Receptors for the neurotransmitter dopamine have been classified in biochemical and pharmacological studies into two subtypes, D₁ and D₂ (Kebabian and Calne, 1979; Creese and Fraser, 1987). The application of gene cloning to dopamine receptors has, however, identified additional isoforms, and it is now important to understand the function of these isoforms. For example a family of D₂-like dopamine receptors has been recognized by gene cloning, termed D_{2(short)}, D_{2(long)}, D₃ and D₄ receptors (Bunzow et al., 1988; Dal Toso et al., 1989; Sokoloff et al., 1990; Van Tol et al., 1991; reviewed by Strange, 1990, 1991a,b).

Studies are now in progress to determine the properties of these isoforms, their function and their tissue locations. Information on the tissue location may be obtained by examining the distribution of the mRNA for the isoforms by using hybridization *in situ*, the polymerase chain reaction and solution hybridization/protection (see, e.g., Giros et al., 1989; Mansour et al., 1990; O'Malley et al., 1990; Gandleman et al., 1991; Snyder et al., 1991). The distribution of receptor protein may not directly parallel the distribution of mRNA, so that it is necessary also to perform studies at the expressed protein level. Some information on the distribution of the receptor protein may be obtained by using ligand autoradiography (Mansour et al., 1990) or by purifying receptors from different tissues (Chazot and Strange, 1992b; Strange, 1992), but these methods do not directly distinguish the isoforms. Immunological methods with isoform-selective antisera should in principle be very powerful for determining the isoform protein distribution. This paper describes the development of two antisera directed against the D_{2(short)} and D_{2(long)} isoforms.

EXPERIMENTAL

Materials

Peptides were synthesized by Alta Bioscience (Birmingham) and purified as described below. [³H]Spiperone (28 Ci/mmol) was obtained from Amersham International. Peroxidase-labelled swine anti-rabbit immunoglobulin was purchased from DAKO Immunoglobulins (Denmark). Fluorescein isothiocyanate

(FITC)-labelled goat anti-rabbit IgG, *p*-phenylenediamine, *o*-phenylenediamine dihydrochloride, keyhole-limpet haemocyanin, glutaraldehyde [grade 1; 25% (w/v) aq. solution], ovalbumin, thimerizole, 4-chloro-1-naphthol, RPMI 1640 medium and Freund's adjuvants (complete and incomplete) were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). Foetal-calf serum was purchased from Imperial Laboratories (Andover, Hants, U.K.) and heat-inactivated at 56 °C for 30 min before use. All other chemicals used were of analytical grade.

Preparation of peptide conjugates

Crude peptide (30 mg) was dissolved in 2 ml of 0.1 M (NH₄)₂CO₃/2.5 mM mercaptoethanol (for peptide-1 only) and applied to a 20 ml Bio-Gel P2 column, equilibrated in the same buffer. The column was eluted with buffer at a flow rate of 10 ml/h, and 1 ml fractions were collected. The fractions containing peptide were identified by a biuret assay and then amalgamated, and the solvent was removed by freeze drying.

The purified peptide (6 mg) and carrier protein (keyhole-limpet haemocyanin or ovalbumin) (6 mg) were dissolved in 3 ml of 0.1 M NaHCO₃ containing 6 μl of glutaraldehyde (final concn. 0.05%). This mixture was incubated in a glass test tube for 16 h at room temperature with vigorous mixing. The reaction was terminated by addition of 0.3 ml of 1 M glycine ethyl ester, pH 8.0; the resulting mixture was adjusted to pH 8.0 where necessary, and left for 30 min at room temperature. The protein was precipitated by addition of 14.4 ml of acetone and kept at –80 °C for 30 min. The protein was then collected by centrifugation (3500 g, 20 min, 4 °C), and the pellet dried under a stream of nitrogen gas. The pellets were then resuspended at 200 μg/ml in phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄) for haemocyanin conjugates, or 0.05 M Na₂CO₃, pH 9.6, for ovalbumin conjugates.

Immunization of rabbits

A 100 μg portion of the peptide–haemocyanin conjugates (500 μl) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously in two or three separate

patches on the back of a rabbit. Subsequent injections were with Freund's incomplete adjuvant and were performed on a 4–6-weekly cycle. Each rabbit was bled from the ear vein at least 1 week before the first injection (pre-immune serum) and 7–10 days after each injection (antiserum). Serum was prepared and stored at -80°C .

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A $10\text{ }\mu\text{g}$ portion of peptide–ovalbumin conjugate in $50\text{ }\mu\text{l}$ of $0.05\text{ M Na}_2\text{CO}_3$, pH 9.6, was added to each well of a 96-well microtitre plate and incubated at 4°C for 15 h. The wells were emptied and washed with $3 \times 200\text{ }\mu\text{l}$ of $0.5\text{ M NaCl}/20\text{ mM Tris}$, pH 7.5 (TBS), containing 0.05% (v/v) Tween 20. Then $200\text{ }\mu\text{l}$ of 1% (w/v) BSA in TBS was added to each well and incubated at room temperature for 90 min, and the wells were emptied and antiserum was added ($50\text{ }\mu\text{l}$ /well, diluted in the range $1/10$ – $1/10000$ in PBS containing 0.05% Tween 20) for 60 min. The wells were emptied and washed as above, and horseradish-peroxidase-linked anti-rabbit IgG was added ($50\text{ }\mu\text{l}$ at $1/500$ dilution in PBS containing 0.05% Tween 20) for 60 min. The wells were emptied and washed as above, but with five changes of buffer and finally with $150\text{ }\mu\text{l}$ of developing solution [citric acid (0.024 M), Na_2HPO_4 (0.05 M), *o*-phenylenediamine dihydrochloride (0.4 mg/ml), H_2O_2 ($0.4\text{ }\mu\text{l/ml}$)] was added to each well. After 30 min the A_{490} was determined. Parallel incubations with ovalbumin alone as the antigen were performed in order to define the background adsorption of antisera to the carrier protein.

Western blotting analysis

Membrane proteins ($500\text{ }\mu\text{g}$) from different tissues were separated by SDS/PAGE under reducing conditions as described in Leonard et al. (1988). The proteins were transferred electrophoretically to nitrocellulose membranes by using an LKB 2117 Multiphor II Electrophoresis unit at a constant current (mA) of $1.2 \times \text{area (cm}^2\text{)}$, for 2 h.

The nitrocellulose was stained in 0.2% (w/v) Ponceau S/ 5% (v/v) acetic acid/ 20% (v/v) propan-2-ol for 1.5 min to reveal the protein bands and molecular-mass markers. The nitrocellulose was cut into appropriate lanes and incubated in 3% (w/v) gelatin in TBS for 90 min at 37°C to decrease non-specific binding of antisera. The lanes were then incubated with the antiserum (diluted $1/50$ – $1/200$) in 1% (w/v) gelatin in TBS with gentle mixing for 15 h at 25°C .

The antiserum solution was then decanted and stored at 4°C in the presence of 0.05% (w/v) thimerizole. This solution could be used for two additional incubations. The lanes were washed for $2 \times 10\text{ min}$ in 0.05% Tween 20 in TBS, and twice in TBS. The lanes were then incubated with horseradish-peroxidase-conjugated swine anti-rabbit immunoglobulin (diluted $1/1000$ in 1% gelatin in TBS) for 2 h at room temperature with gentle mixing. The second antibody solution was decanted and the lanes were washed as above. The proteins recognized by the primary antiserum were then detected by addition of colour reagent. The colour reagent was prepared by dropwise addition to 8 ml of TBS of a solution of 6 mg of 4-chloro-1-naphthol in 2 ml of methanol with continuous stirring. Then $10\text{ }\mu\text{l}$ of $100\text{-vol. H}_2\text{O}_2$ was added and the freshly prepared colour reagent was immediately added to each lane and incubated in the dark for 30 min. After colour development, the lanes were washed in distilled water for 30 min in the dark.

Immunoprecipitation of D_2 dopamine receptors

A soluble preparation of bovine caudate nucleus membranes (2 ml for antiserum 1A or 3 ml for antiserum 1B) prepared in 0.3% cholate/ 1 M NaCl as in Leonard et al. (1988) was incubated with $100\text{ }\mu\text{l}$ of Protein A (*Staphylococcus aureus* dead cells) (25 mg/ml in $20\text{ mM Hepes}/1\text{ mM EDTA}/1\text{ mM EGTA}$, pH 7.5) for 60 min at 4°C with gentle mixing. The suspension was centrifuged (1700 g , 10 min, 4°C) and the supernatant treated with antiserum (1 – $100\text{ }\mu\text{l}$) and incubated at 4°C for 24 h with gentle mixing. Goat anti-rabbit immunoglobulin ($50\text{ }\mu\text{l}$) was then added to the mixture and incubated for 4 h at 4°C , after which $100\text{ }\mu\text{l}$ of Protein A was added and incubated at 4°C for 1 h with gentle mixing. After centrifugation (1700 g , 10 min) the soluble D_2 dopamine receptors in the supernatant were assayed by [^3H]spiperone binding as described by Leonard et al. (1988) and Williamson et al. (1988). Specific immunoprecipitation was determined from the difference between the [^3H]spiperone binding in this incubation compared with parallel incubations with preimmune serum.

Immunofluorescent localization of D_2 dopamine receptors

CHO cells (parent untransfected and recombinant cells transfected with the $\text{D}_{2(\text{long})}$ receptor gene) were cultured on glass coverslips (13 mm -diameter) in RPMI 1640 medium containing 10% (v/v) heat-inactivated foetal-bovine serum and 2 mM glutamine at 37°C in an humidified atmosphere of air/ CO_2 ($19:1$). For experiments, the cell monolayers were washed with $3 \times 1\text{ ml}$ of PBS and fixed in methanol (20 min, -20°C). Fixed cells were washed three times in PBS containing 10 mg of BSA/ ml and 0.05% (v/v) Tween 20 (PBS/BSA) and placed cell-side down on a $25\text{ }\mu\text{l}$ drop of either primary antiserum or PBS/BSA placed on a strip of Parafilm in an humidified box and incubated for 15 h at 4°C . After incubation, cover slips were floated off the antiserum by pipetting $200\text{ }\mu\text{l}$ of TBS under the coverslip and washed for $2 \times 5\text{ min}$ in PBS/BSA and for $2 \times 5\text{ min}$ in TBS. The coverslips were next incubated with FITC-labelled goat anti-rabbit IgG in the same manner as above for 90 min. Coverslips were removed and washed as above and mounted on microscope slides by using a glycerol-based mountant containing 1 mg/ml *p*-phenylenediamine to slow the decay of the FITC label. The slides were viewed at $40 \times$ magnification on a Zeiss Axiomatic microscope under phase contrast with normal and u.v. light. Photographs were taken on Ilford XP2 film, developed with an Ilford photographic chemistry kit and printed on Ilford Multigrade III paper.

Effects of antiserum on receptor/G-protein coupling

Soluble preparations of D_2 dopamine receptors were obtained by using 0.3% cholate/ 1 M NaCl as in Leonard et al. (1988). Soluble receptor preparation was incubated with antiserum at a $1/50$ dilution for 16 h at 4°C with gentle mixing. This preparation was then diluted 5-fold with $20\text{ mM Hepes}/1\text{ mM EDTA}/1\text{ mM EGTA}$, pH 7.5, to which MgCl_2 was added to a final concentration of 5 mM . Dopamine/[^3H]spiperone competition experiments were performed in the presence and absence of $100\text{ }\mu\text{M}$ GTP as described by Chazot and Strange (1992a). Parallel incubations were performed with preimmune serum.

RESULTS

Titres of anti-peptide antisera

Two peptides were used to obtain antisera, one (NNT-DQNECIA) (peptide-1) from the predicted second extra-

Figure 1 Western-blot analysis of membranes from different bovine brain regions analysed as described in the Experimental section

Lanes: 1, antiserum 1B, cerebellum; 2, antiserum 1B, caudate nucleus; 3, antiserum 2B, cerebellum; 4, antiserum 2B, caudate nucleus; 5, antiserum 2B pretreated with peptide 2, cerebellum; 6, antiserum 2B pretreated with peptide 2, caudate nucleus; 7, antiserum 2B, putamen; 8, antiserum 2B, olfactory tubercle. The antisera were used at 1/50 (1B) and 1/100 (2B) dilutions. The positions of the molecular-mass markers (kDa) are also shown. The arrows indicate the 95 kDa and 40 kDa bands.

Figure 2 Western-blot analysis of membranes from recombinant CHO cells expressing the D_{2(long)} dopamine receptor, analysed as described in the Experimental section

Lanes: 1, untransfected CHO cells; 2, CHO (D_{2(long)}) cells. Antiserum 2B was used at a dilution of 1/100. The positions of the molecular-mass markers (kDa) are also shown and the arrow indicates the band of 80 kDa.

cellular loop and one (AARRAQELEMEL) (peptide-2) from the predicted third intracellular loop of D₂ dopamine receptors. Each peptide after coupling to haemocyanin was used to immunize two rabbits, and the titres of the anti-peptide responses were determined from solid-phase e.l.i.s.a. with peptide ovalbumin conjugate as antigen. The titres from different bleeds were in the range 1/500–1/2000. When different bleeds from the same rabbit were used, the results obtained were similar. In subsequent sections the antisera obtained with peptide-1 and the two rabbits

Figure 3 Immunoprecipitation of [³H]spiperone-binding sites from soluble preparations of bovine caudate nucleus by antisera

Different concentrations of antiserum 1A (○) or 2B (●) were incubated with a soluble preparation of bovine caudate nucleus [2 ml (○) or 3 ml (●); specific [³H]spiperone binding approx. 70 fmol/ml] and immune complexes were removed as described in the Experimental section. The depletion of [³H]spiperone-binding sites shown was defined as the difference between the specific [³H]spiperone binding in the remaining supernatant when the immune serum was used and when an equivalent volume of preimmune serum was used. Preimmune serum did not, however, lead to any significant depletion of [³H]spiperone binding. The data are means ± S.D. for triplicate determinations, and the experiment has been replicated three times.

will be designated 1A and 1B, and the antisera obtained with peptide-2 will be designated as 2A and 2B.

Western blot analysis

Western blot analysis was performed with the various antisera by using a D₂-dopamine-receptor-rich brain region (caudate nucleus) and one with few or no D₂ dopamine receptors (cerebellum) as a comparison. When antisera from some rabbits (1A, 2A) were used, multiple bands were labelled in analyses of both brain regions. Some bands were specific to the caudate nucleus, but overall the patterns were not informative. Other antisera (1B, 2B) provided much simpler patterns (Figure 1). Antiserum 1B labelled a major species of 95 kDa in caudate nucleus, but not in cerebellum. Antiserum 2B identified 95 kDa and 40 kDa bands as major species in caudate nucleus, and these bands were also seen when other D₂-dopamine-receptor-rich brain regions were tested (olfactory tubercle, putamen). When the cerebellum was tested, the 40 kDa species was present, but less strongly, and for the 95 kDa species a very faint band was visible. Neither the 95 kDa nor the 40 kDa species was seen in the different brain regions when preimmune serum was used in place of antiserum.

When the experiments were repeated with antiserum 2B pretreated with the corresponding peptide-2 (250 μg/ml), the labelling of the 95 kDa species was decreased considerably in caudate nucleus. The above experiments with antiserum 2B were performed with bovine brain, but when membranes of whole rat brain (minus cerebellum) were analysed major labelled bands of 95 kDa and 38 kDa and a minor band of 65 kDa (results not shown) were detected.

Antiserum 2B was also tested against membranes from a

Figure 4 Immunofluorescent localization of D_2 dopamine receptors

Antiserum 1B or 2B was used to localize D_2 dopamine receptors, as described in the Experimental section, on recombinant CHO cells expressing the gene for the $D_{2(\text{long})}$ receptor. Antiserum 1B (undiluted): (a) untransfected CHO cells; (b) recombinant CHO- D_2 cells. Antiserum 2B (diluted 1/10): (c) untransfected CHO cells; (d) recombinant CHO- D_2 cells. There was no fluorescence if either antiserum was omitted. Magnification $\times 28$.

recombinant CHO cell line expressing the long form of the D_2 dopamine receptor (Castro and Strange, 1992). These cells express D_2 dopamine receptors at a level of about 1 pmol/mg of protein, about 10^5 receptors/cell. Antiserum 2B detected several bands in Western blots of membranes from these cells (Figure 2), but except for a clear band at 80 kDa, these were also present in membranes from untransfected CHO cells.

Immunoprecipitation of D_2 dopamine receptors

Antisera 1A and 2B effected immunoprecipitation of the D_2 dopamine receptors from soluble preparations of bovine caudate nucleus. The immunoprecipitation was dose-dependent for different amounts of antiserum (Figure 3), and the maximum amount of receptor that could be precipitated was about 20% of the receptors in a soluble preparation for antiserum 2B and about 10% for antiserum 1A. This was not limited by the incubation times with either antiserum or Protein A. A lower concentration of antiserum 2B was required to effect the same amount of immunoprecipitation as compared with antiserum 1A (Figure 3). In control experiments it was shown that neither antiserum directly affected [^3H]spiperone binding to D_2 dopamine receptors.

Immunofluorescent localization of D_2 dopamine receptors

Using the antiserum 1B or 2B and a fluorescent-labelled second antibody, it was possible to detect fluorescence on recombinant CHO cells expressing the gene for the long form of the D_2 dopamine receptor (Castro and Strange, 1992) (Figure 4). This was seen at a much lower level in parent untransfected CHO cells, so it is likely to correspond to the localization of the D_2 dopamine receptor. The different patterns observed with the two antisera probably reflect different concentrations of the antibodies present. The fixation method permeabilizes the cells, so that antibodies directed against the presumed extracellular (1B) or intracellular (2B) faces of the receptor should have equal access.

Effects of antisera on receptor/G-protein coupling

D_2 dopamine receptors from bovine caudate nucleus were solubilized with cholate/NaCl and treated with preimmune serum or the antiserum 1A or 2B. The soluble preparation was then diluted 5-fold as in Chazot and Strange (1992a), which yields a preparation in which agonist binding is sensitive to guanine nucleotides, i.e. receptor/G-protein coupling is present. Receptor/G-protein coupling was assayed from the effect of

Figure 5 Receptor/G-protein coupling: effect of antiserum

D₂ dopamine receptors in bovine caudate nucleus were solubilized by using 0.3% cholate as described in the Experimental section. The soluble preparation was treated with preimmune serum (1/50 dilution) (▲, △) or antiserum 2B (1/50 dilution) (●, ○) and then diluted 5-fold. Dopamine/[³H]spiperone competition experiments were then carried out in the presence (△, ○) and absence (▲, ●) of 0.1 mM GTP. The data points are the means of [³H]spiperone binding in triplicate determinations, and the data shown are the best-fit curves to two-binding-site models. ▲, 43% 3 nM, 57% 300 nM; △, 32% 21 nM, 68% 2.7 μM; ●, 43% 44 nM, 57% 4.8 μM; ○, 32% 43 nM, 68% 6.6 μM. The experiment has been repeated three times with similar results.

GTP (100 μM) on dopamine/[³H]spiperone competition curves (Figure 5).

None of the treatments (preimmune serum or antiserum) altered [³H]spiperone binding to the preparations. In the presence of preimmune serum, the dopamine/[³H]spiperone competition curve was characterized by a Hill coefficient less than 1, and a two-binding-site model was the best fit to the data. Addition of GTP shifted the competition curve to higher concentrations. In the presence of GTP, the dopamine competition curve was still best fitted by a two-binding-site model and there were changes in both the affinities and proportions of the two binding sites.

The results obtained with antiserum 1A (not shown) were essentially identical with those obtained with the preimmune serum. When antiserum 2B was tested, however, the effects of GTP on dopamine competition were largely eliminated. The antiserum induced a shift in the dopamine competition curve to higher concentrations. This effect can be seen as a change in the affinities of both the higher- and lower-affinity dopamine-binding sites. In the presence of antiserum 2B, dopamine competition curves (with or without GTP) fitted best to two-site models.

DISCUSSION

In this report we have described the production of antisera directed against two peptides derived from the sequence of D₂ dopamine receptors and the characterization of their anti-receptor activity. Others have reported the production of analogous antisera (David and Fuchs, 1991; McVittie et al., 1991; Plug et al., 1992), but this is the first full structural and functional characterization.

Two peptides were chosen for evaluation, peptide-1, a 11-mer from the putative second extracellular loop, and peptide-2, a 13-mer from the putative third intracellular loop. Both peptides are common to the short and long versions of the D₂ dopamine

receptor (Strange, 1990) and should not show cross-reactivity with other receptors. In each case, high-titre anti-peptide antisera were obtained in rabbits with titres comparable with those reported in other systems (e.g. Duggan and Stephenson, 1989).

Western blot analysis was used to determine the reactivity of the anti-peptide antisera with denatured D₂ dopamine receptor. Tissues rich in D₂ dopamine receptors, e.g. caudate nucleus, and tissues with few or no D₂ receptors, e.g. cerebellum, were compared to establish specificity. One antiserum against peptide-1 (1A) labelled a wide range of species in bovine caudate nucleus. Although these were mostly specific to the caudate nucleus and not seen in cerebellum, the multiplicity of the species rendered this antiserum less useful for use in Western blot analysis.

By contrast, a second antiserum against peptide-1 (1B) and an antiserum against peptide-2 (2B) showed strong labelling of a band of 95 kDa in caudate nucleus, which was either not seen in cerebellum (1B) or present at much decreased levels (2B). The size of this species corresponds to the principal species seen in photoaffinity labelling and in purification studies on D₂ dopamine receptors from bovine caudate nucleus (Williamson et al., 1988), so we presume this corresponds to the D₂ dopamine receptor. A similar band was seen in Western blots of bovine olfactory tubercle and putamen, two other tissues rich in D₂ dopamine receptors. Similarly, in rat brain the same species was observed. Labelling of the 95 kDa species by antiserum 2B in caudate nucleus could be blocked by the corresponding peptide, supporting the specificity of the interaction. The labelling of a faint band at 95 kDa in cerebellum by antiserum 2B was unexpected, as this tissue contains no D₂-like receptors, as judged by ligand-binding experiments with ³H-labelled ligands (Chazot and Strange, 1992b). Using ¹²⁵I-labelled ligands, however, Martres et al. (1985a,b) have shown that D₂-like receptors are present in cerebellum, but at a level 1% of that in caudate nucleus. Molecular-biology studies have suggested somewhat larger relative amounts of mRNA for D₂-like receptors in cerebellum (O'Malley et al., 1990; Gandleman et al., 1991).

In recombinant CHO cells expressing D₂ dopamine receptors a band of 80 kDa was labelled by antiserum 2B, and this band was absent from untransfected CHO cells. The different size of the receptor expressed in CHO cells and in brain presumably reflects differences in glycosylation.

In immunoprecipitation studies, antisera against both peptides were found to be able to deplete soluble D₂ dopamine receptors from a preparation of bovine caudate nucleus, showing that they react with the native receptor. The degree of immunoprecipitation compares favourably with that seen in other studies on related systems, e.g. for 5HT_{1A} 5-hydroxytryptamine receptors (El Mestikawy et al., 1990). The inability to precipitate more than 20% of the soluble receptors may reflect constraints of the assay system, but could also (for peptide-2) reflect modification of the peptide sequence in the native state, e.g. by phosphorylation.

Antisera 1B and 2B were used to localize D₂ dopamine receptors on recombinant CHO cells bearing the gene for the long form of the D₂ dopamine receptor and expressing the protein at high levels. The fluorescence was seen to be distributed uniformly over the cell, and there was little or no fluorescence seen with the parent untransfected CHO cell line. The antisera raised here may be of use in localizing receptors in tissues such as brain.

Peptide-2 used in this study is derived from the putative third intracellular loop of the D₂ dopamine receptor (Bunzow et al., 1988). For other G-protein-linked receptors, the putative third intracellular loop is thought to be a major site of receptor/G-protein interaction (see, e.g., Liggett et al., 1991). Therefore the antisera against peptide-2 might interfere with receptor/

G-protein coupling at the D₂ dopamine receptor. Secondary-structure predictions on the putative third intracellular loop of the D₂ dopamine receptor (results not shown) suggest that the only region of this loop likely to form α -helical structure corresponds to peptide-2. It is believed for the β -adrenergic receptor that receptor/G-protein interaction depends in part on the contact between an α -helical region of the receptor in the third intracellular loop and a predicted α -helix in the C-terminal region of the G-protein (Strader et al., 1989). Thus the region of the D₂ dopamine receptor corresponding to peptide-2 may be of particular significance in receptor/G-protein coupling.

Receptor/G-protein interaction was assessed from the ability of GTP to decrease the affinity of dopamine for binding to the receptor. When introduced into this system, antisera against peptide-1 (1A) behaved similarly to preimmune-serum controls, both having no effect on receptor/G-protein coupling. This is as expected, as peptide-1 is from the second extracellular loop of the receptor, a region thought not to be important for receptor/G-protein interaction. In contrast, an antiserum against peptide-2 (2B) substantially affected agonist binding to the receptor. The affinity of the agonist dopamine was substantially decreased, and superficially at least the antiserum behaved like GTP itself. Given that it is thought that the effects of GTP are to dissociate receptor/G-protein interaction (Gilman, 1987), then the antiserum can be seen to be achieving the same. This could then be via the binding of the antiserum to peptide-2 in the third intracellular loop of the receptor, which sterically prevents receptor/G-protein interaction. An antibody is a rather large molecule, so that its effects may be exerted over a long distance, but the observation is at least consistent with the third intracellular loop of the D₂ dopamine receptor being important for receptor/G-protein interaction. Analogous observations have been made for opiate receptors, but using anti-G-protein antibodies (McKenzie and Milligan, 1990). The present results suggest that antisera directed against the third intracellular loop of the receptor may be useful for identifying dopamine-induced responses linked to D₂ receptors.

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REFERENCES

- Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A. and Civelli, O. (1988) *Nature (London)* **336**, 783–787
- Castro, S. and Strange, P. G. (1992) *J. Neurochem.*, in the press
- Chazot, J. A. and Strange, P. G. (1992a) *Biochem. J.* **281**, 369–375
- Chazot, P. L. and Strange, P. G. (1992b) *Neurochem. Int.* **21**, 159–169
- Creese, I. and Fraser, C. M. (eds.) (1987) *Dopamine Receptors*, A. R. Liss, New York
- Dal Toso, R., Sommer, B., Ewart, M., Herb, A., Pritchett, D. B., Bach, A., Stevens, B. D. and Seeburg, P. (1989) *EMBO J.* **8**, 4025–4034
- David, C. and Fuchs, S. (1991) *Mol. Pharmacol.* **40**, 712–716
- Duggan, M. J. and Stephenson, F. A. (1989) *J. Neurochem.* **53**, 132–139
- El Mestikawy, S., Riad, M., Laporte, A. M., Verge, D., Daval, G., Gozlan, H. and Hamon, M. (1990) *Neurosci. Lett.* **118**, 189–192
- Gandleman, K., Harmon, S., Todd, R. D. and O'Malley, K. L. (1991) *J. Neurochem.* **56**, 1024–1029
- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649
- Giros, B., Sokoloff, P., Martres, M. P., Rios, J. F., Emorine, L. J. and Schwartz, J. C. (1989) *Nature (London)* **342**, 923–926
- Kebabian, J. W. and Calne, D. B. (1979) *Nature (London)* **277**, 93–96
- Leonard, M. N., Williamson, R. A. and Strange, P. G. (1988) *Biochem. J.* **255**, 877–883
- Liggett, S. B., Caron, M. G., Lefkowitz, R. J. and Hnatowich, M. (1991) *J. Biol. Chem.* **266**, 4816–4821
- Mansour, A., Meador-Woodruff, J. H., Bunzow, J. R., Civelli, O., Akil, H. and Watson, S. J. (1990) *J. Neurosci.* **10**, 2587–2600
- Martres, M. P., Sales, N., Bouthenet, M. L. and Schwartz, J. C. (1985a) *Eur. J. Pharmacol.* **118**, 211–219
- Martres, M. P., Bouthenet, M. L., Sales, N., Sokoloff, P. and Schwartz, J. C. (1985b) *Science* **228**, 752–755
- McKenzie, F. and Milligan, G. (1990) *Biochem. J.* **267**, 391–398
- McVittie, L. D., Ariano, M. A. and Sibley, D. R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1441–1445
- O'Malley, K. L., Mack, K. J., Gandleman, K. and Todd, R. D. (1990) *Biochemistry* **29**, 1367–1371
- Plug, M. J., Dijk, J., Maassen, J. A. and Moller, W. (1992) *Eur. J. Biochem.* **206**, 123–130
- Snyder, L. A., Roberts, J. L. and Sealfon, S. C. (1991) *Neurosci. Lett.* **122**, 37–40
- Sokoloff, P., Giros, B., Martres, M. P., Bouthenet, M. L. and Schwartz, J. C. (1990) *Nature (London)* **347**, 146–151
- Strader, C. D., Sigal, I. S. and Dixon, R. A. F. (1989) *FASEB J.* **3**, 1825–1832
- Strange, P. G. (1990) *Trends Neurosci.* **13**, 373–378
- Strange, P. G. (1991a) *Trends Neurosci.* **14**, 43–45
- Strange, P. G. (1991b) *Curr. Opin. Biotechnol.* **2**, 269–277
- Strange, P. G. (1992) *Biochem. Soc. Trans.* **20**, 126–129
- Van Tol, H. H. M., Bunzow, J. R., Guan, H. C., Sunahara, R. K., Seeman, P., Niznik, H. B. and Civelli, O. (1991) *Nature (London)* **350**, 610–614
- Williamson, R. A., Worrall, S., Chazot, P. L. and Strange, P. G. (1988) *EMBO J.* **7**, 4129–4133