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Pharmacological analysis of dopamine stimulation of [^{35}S]-GTPγS binding via human D_{2short} and D_{2long} dopamine receptors expressed in recombinant cells

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1 The activation of G-proteins by agonist-occupied D_2 or D_3 dopamine receptors in membranes from recombinant cells expressing the cloned receptors has been analysed by a [^{35}S]-guanosine 5'-[γ-thio] triphosphate ([^{35}S]-GTPγS) binding assay.

2 The rate of [^{35}S]-GTPγS binding was increased by dopamine in a dose-dependent manner in membranes from CHO cells stably expressing either the D_{2short} or D_{2long} dopamine receptor.

3 The dopamine-induced stimulation of [^{35}S]-GTPγS binding could be inhibited by a range of antagonists. Affinities for antagonists derived from the inhibition of the dopamine stimulation of [^{35}S]-GTPγS binding correlated very well with affinities derived from radioligand binding studies.

4 When the maximum [^{35}S]-GTPγS binding responses stimulated by dopamine acting at different receptor subtypes were compared, there was a tendency for the stimulation via the D_{2short} receptor to be greater than via the D_{2long} receptor and for the stimulation via the D_3 dopamine receptor to be less than for either D_2 receptor. These differences in maximal response were also seen when the inhibitory effects of dopamine on adenylyl cyclase via the three receptor subtypes were compared.

5 The stimulation of [^{35}S]-GTPγS binding by dopamine in membranes from recombinant cells therefore provides an excellent system for studying the molecular nature of agonism and the receptor/G-protein interactions for these receptors.

Keywords: Dopamine receptors; D_2; D_3; G-protein; [^{35}S]-GTPγS binding; dopamine antagonists

Introduction

Dopamine receptors are members of the G-protein coupled receptor superfamily and at least five subtypes of dopamine receptors have been identified (D_1, D_2, D_3, D_4, D_5, Sibley & Monsma, 1992; Civelli *et al.*, 1993). These can be divided into two groups on the basis of their amino acid sequence and their pharmacological properties. The first group comprises the D_1 and D_5 dopamine receptors which are termed D_1-like and when expressed in mammalian cells stimulate adenylyl cyclase. The second group comprises the D_2, D_3 and D_4 dopamine receptors which are termed D_2-like. The D_2-like subgroup is unusual in that its members exist in variant forms e.g. D_{2short} and D_{2long}. All the D_2-like dopamine receptors have been shown to inhibit adenylyl cyclase activity when expressed in CHO cells (Chio *et al.*, 1994a,b; Hall & Strange, 1994). It has also been shown that, like other receptors that inhibit adenylyl cyclase (Limbird, 1988), the D_2-like dopamine receptors influence additional signalling mechanisms including the modulation of potassium and calcium channels (Vallar & Meldolesi, 1989).

While a number of different responses are mediated by D_2-like dopamine receptors they have a common step which is activation of pertussis toxin-sensitive G-proteins. The mechanism of G-protein activation has been extensively reviewed (Freissmuth *et al.*, 1989; Ross, 1989; Conklin & Bourne, 1993). The role of the receptor is to catalyse the exchange of GDP for GTP at the α-subunit of the interacting G-protein. The binding of antagonists to the receptor will not affect the rate of GDP/GTP exchange but the binding of agonists causes an increase in the rate of GDP/GTP exchange, resulting in an increase in the concentration of active G-protein. The exchange of GDP for GTP is the primary response in the signalling pathway that leads to the final tissue response and is the only step in the response pathway that is directly regulated by ligands. The exchange of GDP for GTP at the α-subunit can be observed by following the high affinity binding of a non-hydrolysable analogue of GTP such as [^{35}S]-guanosine 5'-[γ-thio] triphosphate ([^{35}S]-GTPγS). It has been shown that activation of dopamine receptors increases the binding of [^{35}S]-GTPγS in reconstituted systems (Elazar *et al.*, 1989; Senogles *et al.*, 1990) and increased [^{35}S]-GTPγS binding in response to agonists has also been observed in membrane preparations containing muscarinic acetylcholine receptors (Hilf *et al.*, 1989; Lazareno *et al.*, 1993), α_2 adrenoceptors (Tian *et al.*, 1994) and 5-HT_{1D} receptors (Thomas *et al.*, 1995).

The aim of this study was to develop an assay for the primary response in the signalling pathway of the D_2 dopamine receptor. An assay of this kind should allow the study of agonist action without the 'masking effects' that are produced by the saturable responses that may occur between receptor and final tissue response, as discussed by Black & Shankley (1990). We show that in washed membranes from CHO-K1 cells expressing human D_{2short} or D_{2long} dopamine receptors that the rate of [^{35}S]-GTPγS binding is increased in a dose-dependent manner by dopamine and can be inhibited in a dose-dependent manner by dopamine antagonists. We demonstrate the validity of this assay by comparing the potencies of a range of antagonists for inhibiting this response with their potencies determined by radioligand binding assay.

Methods

Cell culture

The CHO-D2S (D_{2short}) and CHO-D2L (D_{2long}) cells expressing the recombinant human gene (Hayes *et al.*, 1992) were grown

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in 175 cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 2 mM glutamine, 5% foetal bovine serum and 200 $\mu g\ ml^{-1}$ active geneticin in an atmosphere of 5% CO_2 at 37°C. Cells were passaged every 4–5 days. The CHO $dhfr^-$ cells expressing the rat D_3 dopamine receptor (DUK25) were grown as described in Castro & Strange (1993b).

Membranes from CHO cells expressing the rat D_{2long} dopamine receptor (CHO6) and Ltk^- cells expressing the rat D_{2short} (LZR1) or D_{2long} (Ltk59) dopamine receptors were obtained as described in Castro & Strange (1993a,b).

Preparation of cell membranes

The cells were grown to confluency and the medium removed. The cells were washed with 10 ml of Buffer A (20 mM HEPES, 6 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA, pH 7.4) at 4°C. The wash buffer was removed and replaced with a further 5 ml of Buffer A. The cells were then scraped from the flasks and homogenized with 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 1,700 *g* for 10 min at 4°C and the resulting supernatant was centrifuged at 48,000 *g* for 1 h at 4°C. The pellet from this centrifugation was resuspended in Buffer A and again centrifuged at 48,000 *g* for 1 h at 4°C. The resulting pellet was resuspended in Buffer A at a concentration of 2–3 mg of protein ml^{-1} and stored at −80°C before use.

Radioligand binding assays

Washed cell membranes (25–75 μg) were incubated with [^{3}H]-spiperone (0.3 nM for competition experiments, 40 pM to 2 nM in saturation experiments) and competing drugs in Buffer B (20 mM HEPES, 10 mM $MgCl_2$ and 100 mM NaCl, pH 7.4) in a final volume of 1 ml for 45 min at 25°C. The assay was terminated by rapid filtration using a Brandel cell harvester with four washes of 4 ml of ice-cold phosphate buffered saline (0.14 M NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 5 mM Na_2HPO_4, pH 7.4). Filters were soaked for at least 6 h in 2 ml LKB optiphase 'Hisafe' 3 scintillation fluid before determination of radioactivity by liquid scintillation counting. Non-specific binding was defined in saturation and competition experiments in the presence of 3 μM (+)-butaclamol. In saturation experiments the total binding was determined in the presence of 3 μM (−)-butaclamol.

[^{35}S]-GTPγS binding assay

The [^{35}S]-GTPγS binding assay was carried out essentially as described by Lazareno *et al.* (1993). Washed membrane protein (25–75 μg) was incubated in Buffer B with 0.1 mM dithiothreitol (DTT) and 1 μM GDP (unless specified elsewhere) and drugs in a volume of 0.9 ml for 30 min at 30°C. This preincubation ensured that dopamine and the antagonists tested were at equilibrium when the [^{35}S]-GTPγS was added. [^{35}S]-GTPγS (50–150 pM) was added in 100 μl of Buffer B to initiate the reaction and the assay mixture was incubated for a further 20 min, unless stated otherwise. The assays were terminated and bound radioactivity determined as described under radioligand binding assays above. The total binding of [^{35}S]-GTPγS was less than 20% of that added.

Adenylyl cyclase assay

For the measurement of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation, cells were seeded at 30–35,000 (CHO-D2S or CHO-D2L) or 70,000 (DUK25) per well in 24 well plates and grown until ~80% confluent. The medium was then replaced with fresh medium, 300 μl per well, containing 1 μCi of [^{3}H]-adenine. After a further 2 h, this medium was removed and cells were washed with 1 ml of RPMI 1640 containing 20 mM HEPES (pH 7.5) (Buffer C). The cells were then incubated at 37°C for 40 min in 1 ml of Buffer C containing 1 mM isobutylmethylxanthine. Forskolin (10 μM) and appropriate concentrations of dopamine were then added in 20 μl of 50% dimethylsulphoxide (DMSO) and the cells were incubated for a further 10 min. The assay was terminated by aspiration of the medium and addition of 0.5 ml of ice-cold perchloric acid (0.5 M, containing ~2500 d.p.m. of [^{14}C]-cyclic AMP to act as a recovery standard). The cyclic AMP was separated from the other labelled nucleotides by sequential chromatography on Dowex and alumina as described by Salomon *et al.* (1974). The ^{3}H and ^{14}C were quantified by liquid scintillation counting and the ^{3}H present was corrected for the recovery of ^{14}C. All assays were performed in the presence of DMSO (1%, to control for the solvent used to dissolve the forskolin) and 0.05% ascorbic acid.

Data analysis

Data from the [^{35}S]-GTPγS binding assays were analysed by non-linear regression analysis using the 'Inplot' curve fitting program (Graphpad). The time course data were fitted to the first order rate equation to derive values for the apparent first order rate constant, k_{app} and the maximal number of [^{35}S]-GTPγS binding sites, B_t. The initial rates of [^{35}S]-GTPγS binding were calculated as $k_{app} \times B_t$ as in Asano & Ross (1984). The percentage stimulation of [^{35}S]-GTPγS binding by dopamine was calculated by dividing the total binding of [^{35}S]-GTPγS observed in the presence of 100 μM dopamine by the total binding observed in the absence of dopamine. Radioligand binding experiments were analysed with 'LIGAND' (Elsevier-BIOSOFT). Data were assumed to conform to a one site binding model unless a statistically significant improvement was obtained with a two site fit.

K_b and K_i values for antagonists were obtained from [^{35}S]-GTPγS and [^{3}H]-spiperone binding experiments respectively, assuming competitive interactions between antagonist and dopamine or [^{3}H]-spiperone. This assumption is justified for the [^{35}S]-GTPγS binding experiments as agonist stimulation and antagonist inhibition curves all fit best to single binding site models (see below) and high agonist concentrations were used compared to the EC_{50} value (Lazareno & Birdsall, 1993a).

Materials

[^{35}S]-GTPγS, [^{3}H]-adenine and [^{14}C]-cyclic AMP were purchased from Du Pont, [^{3}H]-spiperone was purchased from Amersham. Dopamine, spiperone, (+)- and (−)-butaclamol and haloperidol were purchased from RBI. (−)-Sulpiride was obtained from Ravizza Laboratories. *N*-[(1-propyl-2-pyrrolidinyl)-methyl],2-methoxy, 5-methylsulphanoyl beuzamide (DO710) was generously donated by Dr A. Mann, Strasbourg. All other materials were of the highest commercial purity available.

Results

Radioligand binding studies on D_{2short} and D_{2long} dopamine receptors

Membranes from CHO-D2S or CHO-D2L cells showed saturable binding of [^{3}H]-spiperone. The binding data conformed to a one binding site model (Figure 1) with an expression level (B_{max}) of 2.7 ± 0.4 pmol mg^{-1} and 1.3 ± 0.1 pmol mg^{-1} (mean ± s.e.mean, 7 observations) for D_{2short} and D_{2long} expressing cell lines respectively. The dissociation constants of the receptors for [^{3}H]-spiperone binding were not significantly different (Student's *t* test, $P < 0.05$) (Table 1).

Competition experiments were performed with a range of antagonists, and in all cases these competed in a manner consistent with interaction at a single class of binding sites (Figure 2). The dissociation constants for the classical antagonists tested (Table 1) were similar to those reported for the rat D_{2short} and D_{2long} dopamine receptor expressed in other cell lines (Castro & Strange, 1993a). In contrast to the observations of Castro & Strange (1993a) and Malmberg *et al.* (1993) the

substituted benzamides tested did not show any significant differences in affinity for the two subtypes (Student's t test, $P>0.05$).

Regulation of [^{35}S]-GTPγS binding by D_{2short} and D_{2long} dopamine receptors

In other systems the extent of agonist stimulated [^{35}S]-GTPγS binding has been shown to be dependent upon the GDP concentration present (Hilf *et al.*, 1989; Lazareno *et al.*, 1993; Tian *et al.*, 1994) and this was investigated in the present study for the D_2 receptor using stimulation by 100 μM dopamine (Figure 3). The maximal dopamine-stimulated [^{35}S]-GTPγS binding, for both CHO-D2S and CHO-D2L membranes, was observed at 1 μM GDP. At 1 μM GDP, dopamine (100 μM) stimulated [^{35}S]-GTPγS binding in membrane preparations of CHO-D2S and CHO-D2L cells by about 62% and 44% of basal respectively (Table 2). The stimulation of [^{35}S]-GTPγS binding by dopamine was not observed in membrane preparations of untransfected control CHO-K1 cells (data not shown).

The time course of [^{35}S]-GTPγS binding in membrane preparations of CHO-D2S cells, in response to different concentrations of dopamine (0.1–100 μM), was followed over a period of 60 min (Figure 4). The initial rate of dopamine stimulated [^{35}S]-GTPγS binding was calculated for each concentration of dopamine, as described in Methods, and was seen to increase in a dose-dependent manner (Figure 4) with a pEC_{50} for dopamine of 6.07±0.02 (mean±range, $n=2$) and a Hill coefficient of 0.99±0.05 (mean±range, $n=2$).

The stimulation of [^{35}S]-GTPγS binding by different concentrations of dopamine via D_{2short} or D_{2long} dopamine receptors in membrane preparations was compared by use of a fixed period of incubation (20 min) following addition of [^{35}S]-GTPγS. Dopamine stimulated [^{35}S]-GTPγS binding in a dose-dependent manner via both the D_{2short} and D_{2long} dopamine receptors with pEC_{50} values of 5.88±0.09 and 6.05±0.09 (mean±s.e.mean, $n=6$) respectively. The pEC_{50} values were not significantly different (Student's t test, $P>0.05$) and the Hill coefficients of the dose-response curves were not significantly different from 1 (Student's t test, $P>0.05$) and so data were fitted with Hill coefficients constrained to 1. The pEC_{50} value for dopamine to stimulate [^{35}S]-GTPγS binding via the D_{2short} dopamine receptor determined by this approach was similar to that obtained in the time course experiments (Fig. 4).

The antagonists tested caused a dose-dependent inhibition of [^{35}S]-GTPγS binding stimulated by 30 μM dopamine (Figure 5). In all cases, antagonist inhibition curves had Hill coeffi-

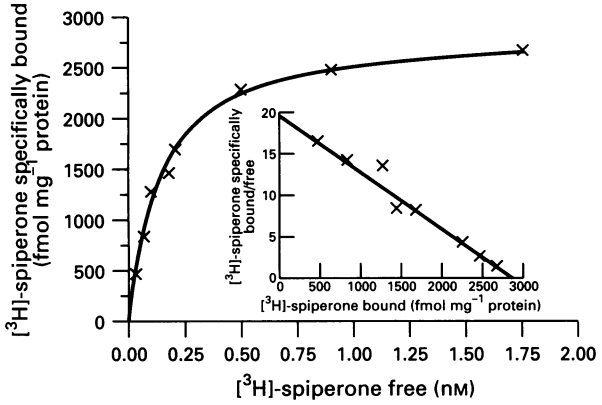

Figure 1 Saturation analysis of [^{3}H]-spiperone binding to D_{2short} dopamine receptors expressed in CHO-K1 cells. [^{3}H]-spiperone binding was determined as described in Methods and the data are displayed as a saturation curve with the best fit to a single binding site model. The inset is the Scatchard plot of the data from the saturation analysis shown. The data are representative of 7 similar experiments performed in triplicate and replicated as in Table 1.

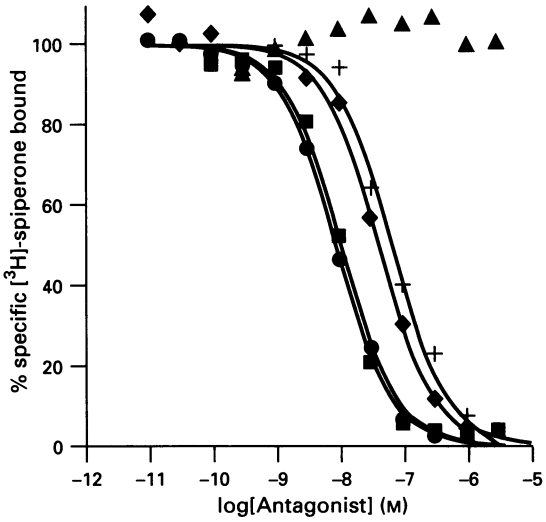

Figure 2 Pharmacological characterization of D_{2short} dopamine receptors expressed in CHO-K1 cells: antagonist competition assays. [^{3}H]-spiperone binding to membrane preparations was determined in the presence of increasing concentrations of (+)-butaclamol (■), haloperidol (●), DO710 (◆), (−)-sulpiride (+) and (−)-butaclamol (▲) as described in Methods. The data are representative curves for single binding site models replicated as in Table 1.

Table 1 Binding of antagonists to D_{2short} and D_{2long} dopamine receptors

		Spiperone	*Haloperidol*	*(+)-Butaclamol*	*DO710*	*(−)-Sulpiride*	*(−)-Butaclamol*
D_{2short} dopamine receptors							
[^{3}H]-spiperone	pK_i	9.86±0.09	8.62±0.05	8.47±0.05	7.84±0.06	7.63±0.05	ND
binding assay	K_i (nM)	0.14	2.4	3.4	14.5	23.4	>10000
[^{35}S]-GTPγS	pK_b	9.38±0.11	8.68±0.05	8.41±0.05	8.09±0.21	7.39±0.08	ND
binding assay	K_b (nM)	0.42	2.1	3.9	8.1	40.7	>10000
D_{2long} dopamine receptors							
[^{3}H]-spiperone	pK_i	10.15±0.08	8.67±0.06	8.43±0.06	7.98±0.05	7.69±0.02	ND
binding assay	K_i (nM)	0.07	2.1	3.7	10.5	20.4	>10000
[^{35}S]-GTPγS	pK_b	10.20±0.13	8.41±0.08	8.57±0.24	8.33±0.19	7.80±0.04	ND
binding assay	K_b (nM)	0.06	3.9	2.7	4.7	15.8	>10000

The pK_i values were derived from competition experiments versus [^{3}H]-spiperone binding for all antagonists except spiperone as described in Methods. The value quoted for spiperone is a pK_d determined by saturation binding experiment as described in Methods. The pK_b values were derived from [^{35}S]-GTPγS binding experiments as described in Methods. All experiments were carried out using membrane preparations of CHO-K1 cells stably expressing human D_{2short} or D_{2long} dopamine receptors. Data are mean±s.e.mean values from 3 or more experiments. ND, not determined.

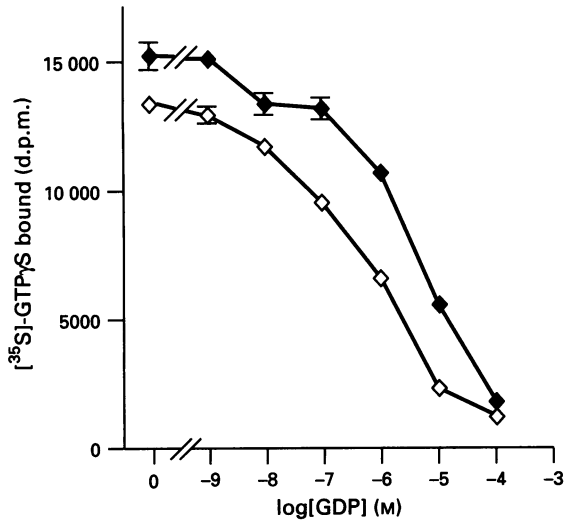

Figure 3 Influence of GDP on the binding of [^{35}S]-GTPγS to CHO-D2S membranes. Binding of [^{35}S]-GTPγS was determined in the absence (◇) and presence (◆) of 100 μM dopamine at increasing concentrations of GDP as described in Methods. The data are mean determinations (± s.e.mean, triplicate determinations) from a single assay and are representative of 3 similar experiments.

Table 2 Comparison of the percentage stimulation of [^{35}S]-GTPγS binding in membrane preparations from various cell lines

	% stimulation	n	B_{max} (pmol mg^{-1})
CHO-K1 (D_{2short})	62±5	33	2.7
CHO-K1 (D_{2long})	44±4	35	1.3
CHO6 (D_{2long})	39±5	3	0.9
Ltk59 (D_{2long})[a]	9±3	3	1.2
LZR1 (D_{2short})[a]	31±4	2	0.9
DUK25 (D_3)	17±1	10	1.5

The percentage stimulation, over background, of [^{35}S]-GTPγS binding produced by 100 μM dopamine was determined as described in Methods. The B_{max} values were determined by saturation analysis using [^{3}H]-spiperone as described in Methods. The data are mean values ± s.e.mean ($n \geq 3$) or range ($n = 2$). [a] Membranes were kindly donated by S. Hoare.

cients that were not significantly different from 1 (Student's t test, $P > 0.05$) so data were fitted with the Hill coefficient constrained to 1 and pK_b values (Table 1) were calculated from the observed IC_{50} values. The antagonist affinity estimates derived from this assay were in excellent agreement with those obtained in radioligand binding assays (Table 1). There was a correlation of 0.93 and 0.95 between the affinity estimates of the five antagonists derived from the two assays for the short and long iso-forms of the D_2 receptor respectively (Figure 7). The antagonists had little or no effect on basal levels of [^{35}S]-GTPγS binding (data not shown) and as such no inverse agonist activity was observed under the standard assay conditions. This was expected as basal [^{35}S]-GTPγS binding had been greatly suppressed (Figure 3) rendering the assay insensitive to the detection of inverse agonist activity.

The maximal stimulated rate of [^{35}S]-GTPγS binding as produced by 100 μM dopamine was studied in membrane preparations from various cell lines expressing D_2-like dopamine receptors (Table 2). It was observed that in membranes from both CHO and L cells expressing D_{2short} dopamine receptors, 100 μM dopamine produced a larger stimulation of [^{35}S]-GTPγS binding than in membranes from cell lines expressing D_{2long} dopamine receptors. In addition the stimulation observed for membranes of DUK25 cells, a CHO dhfr$^-$ cell line that stably expresses D_3 dopamine receptors, was less than that observed for either iso-form of the D_2 dopamine receptor in CHO membrane preparations. The differences in the percentage stimulation of [^{35}S]-GTPγS binding could not be accounted for by differences in the level of basal binding (binding

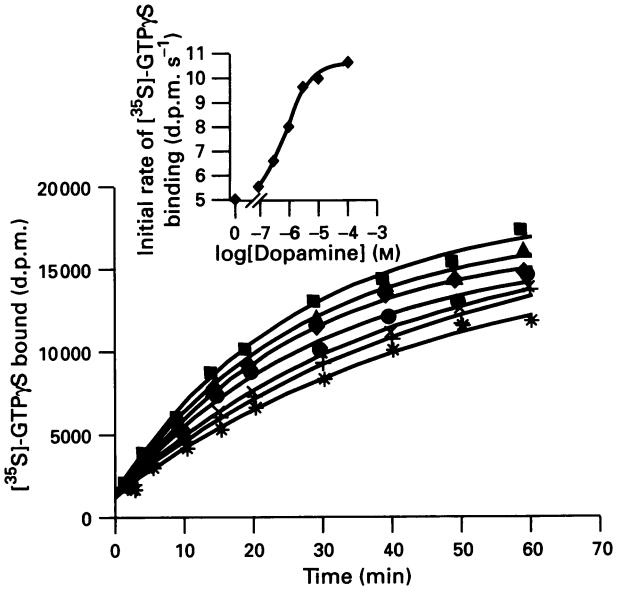

Figure 4 Time courses of dopamine-stimulated [^{35}S]-GTPγS binding to membrane preparations of CHO-D2S cells. The time course of dopamine stimulated [^{35}S]-GTPγS binding was investigated over a range of dopamine concentrations: 0 μM (*), 0.1 μM (+), 0.3 μM (×), 1.0 μM (●), 3.0 μM (◆), 10 μM (▲) and 100 μM (■) as described in Methods. The inset is the dose-response curve for the data presented based on the initial rate of [^{35}S]-GTPγS binding calculated as in Methods. The data are mean determinations from a single assay and are representative of 2 similar experiments performed in triplicate.

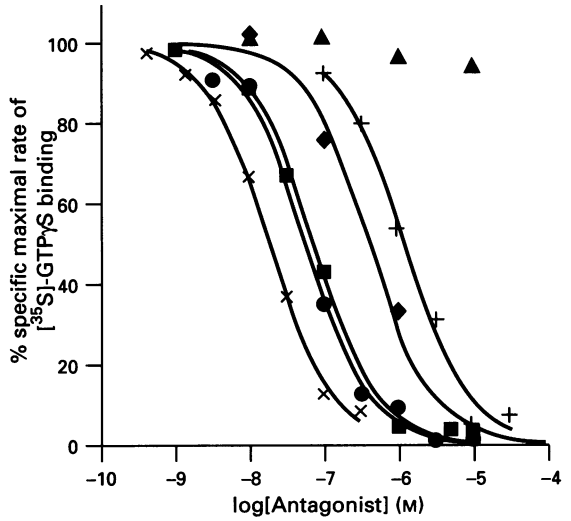

Figure 5 Pharmacological characterization of D_{2short} dopamine receptors expressed in CHO-K1 cells: antagonist inhibition of 30 μM dopamine stimulated [^{35}S]-GTPγS binding. Inhibition of dopamine stimulated [^{35}S]-GTPγS binding to membrane preparations was determined in the presence of different concentrations of spiperone (×), (+)-butaclamol (■), haloperidol (●), DO710 (◆), (−)-sulpiride (+) and (−)-butaclamol (▲) as described in Methods. The data are representative curves for single site models and the experiments have been replicated as in Table 1.

seen in the absence of agonist stimulation) as the level of basal [^{35}S]-GTPγS binding in membranes from CHO-D2S cells was 1.6 fold greater than in membranes from CHO-D2L cells. The levels of basal binding in membranes from L cells expressing either receptor were similar and in the case of the DUK25 cell line, the basal level was consistently less than that observed when either iso-form of the D_2 dopamine receptor was expressed in the parental CHO cell line. Differences in non-specific [^{35}S]-GTPγS binding also could not account for the different percentage stimulation as non-specific binding (in the presence of 100 μM GTP) was very similar in the CHO-D2S and CHO-D2L cell lines.

Inhibition of forskolin-stimulated cyclic AMP accumulation by D_{2short}, D_{2long} and D_3 dopamine receptors

The inhibition of forskolin-stimulated cyclic AMP accumulation by dopamine was investigated in CHO-D2S, CHO-D2L and DUK25 cells. In these cell lines dopamine inhibited cyclic AMP accumulation in a dose-dependent manner via D_{2short}, D_{2long} and D_3 dopamine receptors with pEC_{50} values of 7.78±0.18 ($n=4$), 7.82±0.24 ($n=3$) and 8.54±0.08 ($n=8$) (mean±s.e.mean) respectively (Figure 6). While the pEC_{50} values for dopamine acting via D_{2short} and D_{2long} dopamine receptors were not significantly different (Student's t-test, $P>0.05$), dopamine was significantly (~60 fold) more potent at inhibiting cyclic AMP accumulation than in stimulating [^{35}S]-GTPγS binding. A comparison of the maximal percentage inhibition of cyclic AMP accumulation elicited by dopamine in the three cell lines showed the same pattern as was observed for the stimulation of [^{35}S]-GTPγS binding. Dopamine produced a significantly greater percentage inhibition via D_{2short} dopamine receptors (87±3%) than D_{2long} dopamine receptors (65±3%) (Student's t test, $P<0.05$) and the inhibition produced via both these receptors was significantly greater than that produced via D_3 dopamine receptors (44±3%) (Student's t test, $P<0.05$) (Figure 6). No effect of dopamine was seen on forskolin stimulated cyclic AMP accumulation in untransfected cells (data not shown).

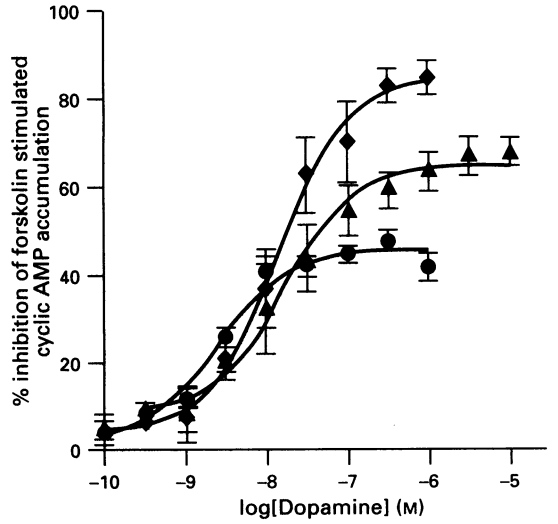

Figure 6 Effect of dopamine on forskolin stimulated cyclic AMP accumulation mediated via D_{2short}, D_{2long} and D_3 dopamine receptors expressed in CHO cells. Inhibition of forskolin-stimulated cyclic AMP accumulation was determined over a range of dopamine concentrations for CHO-D2S (◆), CHO-D2L (▲) and DUK25 (●) cells as described in Methods. The data are mean determinations (± s.e.mean) from 3 or more experiments performed in triplicate.

Discussion

The data presented in this study show that dopamine stimulates the rate of [^{35}S]-GTPγS binding in membrane preparations of CHO-K1 cells stably expressing either D_{2short} or D_{2long} dopamine receptors. This stimulation was dose-dependent for agonists and was inhibited in a dose-dependent manner by all dopamine antagonists tested. No stimulation of [^{35}S]-GTPγS binding was observed in membrane preparations from untransfected CHO-K1 cells.

The time course of dopamine stimulated [^{35}S]-GTPγS binding (Figure 4) shows clearly that the initial rate of [^{35}S]-GTPγS binding is dependent upon the concentration of dopamine and the effects of dopamine are saturable as would be expected for a receptor-mediated event. This is consistent with the receptor acting as a catalyst for the exchange of GDP for GTP and occupancy of the receptor increases the rate of GDP/GTP exchange. It is currently widely believed that agonist occupancy of the receptor increases the rate of GDP/GTP exchange by increasing the affinity of the receptor for G-protein (De Lean *et al.*, 1980; Wregget & De Lean, 1984; Costa *et al.*, 1992) and this concept is central to the operational model of agonism when applied to G-protein linked receptors (Black & Leff, 1983). In this study the Hill coefficients for the dopamine concentration-effect curves were not different from unity. This might indicate that when D_2 dopamine receptors are expressed in CHO-K1 cells they interact only with a single G-protein or that there is no significant difference in the affinity of the receptor for the different G-proteins with which it interacts, as Hill coefficients of less than unity might be expected if the receptor interacted with a number of different G-proteins with different affinities (Lazareno *et al.*, 1993).

A comparison of dopamine stimulated [^{35}S]-GTPγS binding in membrane preparations of CHO-K1 cells stably expressing either D_{2short} or D_{2long} dopamine receptors was performed using a fixed incubation period of 20 min following addition of [^{35}S]-GTPγS, when the nucleotide exchange rate is still essentially linear. The percentage stimulations of [^{35}S]-GTPγS binding achieved by 100 μM dopamine were greater for D_{2short} dopamine receptors and a similar difference was observed for the two receptors when expressed in L cells (Table 2). A greater percentage inhibition of adenylyl cyclase was also seen in the present experiments for dopamine acting via the D_{2short} dopamine receptor. These differences in agonist responses were also seen for other agonists e.g. N-propylnorapomorphine (Gardner, Hall & Strange, unpublished observations). Similar observations have been reported by others indicating a greater efficiency of coupling to signalling systems for D_{2short} dopamine receptors (Montmayeur & Borrelli, 1991; Hayes *et al.*, 1992) or the coupling of the D_{2short} and D_{2long} iso-forms with different efficiencies to different G-proteins (Montmayeur *et al.*, 1992; Senogles, 1994; Fang Liu *et al.*, 1994). From the present data the possibility cannot, however, be ruled out that the differential effects are due to differences in expression levels of receptors or G-proteins in the cell lines.

It was also shown that the percentage stimulation of [^{35}S]-GTPγS binding produced by 100 μM dopamine, in membranes from CHO dhfr$^-$ cells expressing D_3 dopamine receptors was significantly lower than that observed for either iso-form of the D_2 dopamine receptor expressed in the parental cell type, CHO cells, (Table 2). Since the D_3 dopamine receptor is expressed at comparable levels to those for the D_2 dopamine receptors this could indicate that the efficiency of G-protein activation is lower for the D_3 dopamine receptor than either iso-form of the D_2 dopamine receptor. This hypothesis is supported by the observation that dopamine also produced a lower percentage inhibition of forskolin-stimulated cyclic AMP accumulation via D_3 dopamine receptors than either iso-form of the D_2 dopamine receptor (Figure 6). These observations are consistent with the work of Chio *et al.* (1994b) who showed that D_3 dopamine receptors coupled to the same functional responses in CHO cells as D_{2long} dopamine receptors but with lower efficiency.

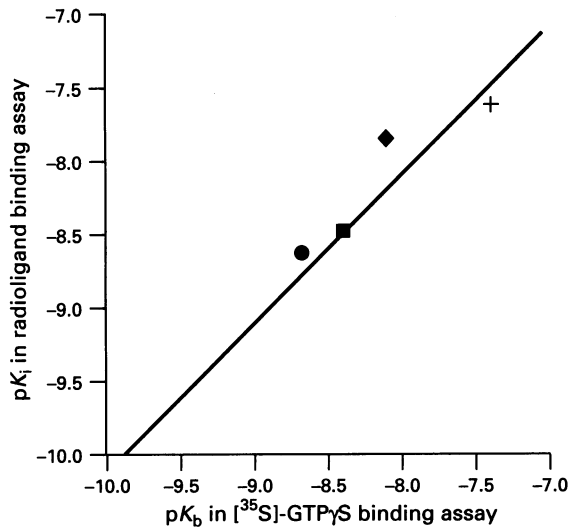

Figure 7 Comparison of antagonist affinities at D_{2short} dopamine receptors derived by radioligand and [^{35}S]-GTPγS binding assays. The pK_i and pK_b values for the antagonists spiperone (×), haloperidol (●), (+)-butaclamol (■), DO710 (◆) and (−)-sulpiride (+) at the D_{2short} dopamine receptor are taken from Table 1. The line has a slope of 1.01 and intercept of 0.

The pEC_{50} values of dopamine acting via D_{2short} and D_{2long} dopamine receptors for the responses of stimulated [^{35}S]-GTPγS binding and inhibition of forskolin-stimulated cyclic AMP accumulation are different and this may reflect the degree of amplification that is occurring (Ross, 1989). One mechanism that can explain this amplification is if the response pathway from receptor to G-protein to adenylyl cyclase is made up of a series of saturable responses (Black & Leff, 1983; Kenakin, 1992).

All antagonists tested inhibited the stimulation of [^{35}S]-GTPγS binding produced by 30 μM dopamine in a dose-dependent manner. The affinities obtained by this approach and using radioligand binding showed a high degree of correlation for both receptor iso-forms (Figure 7). The correlation coefficients for these two estimates of antagonist affinity for D_{2short} and D_{2long} dopamine receptors were 0.93 and 0.95 respectively. These data show clearly that the dopamine stimulation of [^{35}S]-GTPγS binding is modulated via the D_2 dopamine receptor expressed in these cells and is inhibited by antagonists in a manner consistent with data obtained from radioligand binding assays.

The classical antagonists, spiperone, haloperidol and (+)- or (−)-butaclamol were found to bind to the D_2 receptor with similar potencies to those reported by Castro & Strange (1993a) as determined by radioligand binding assay. The substituted benzamides were found to be less potent in the present study and differences in affinity for the short and long iso-forms of the D_2 receptor were not observed in either assay system. Substituted benzamides have previously been shown to display a 2–5 fold selectivity for D_{2short} dopamine receptors expressed in Ltk$^-$ cells (Castro & Strange, 1993a; Malmberg *et al.*, 1993). It is not clear why this subtype selectivity was not observed although it could be due to differences in buffer conditions.

The data presented demonstrate that the increased binding of [^{35}S]-GTPγS caused by dopamine is mediated via the expressed dopamine receptors in the cell lines used. They also show that this functional assay can be used to characterize antagonist binding as previously described for muscarinic acetylcholine receptors by Lazareno & Birdsall (1993b). The assay can also be used to demonstrate quickly the functional coupling of expressed receptor to endogenous G-proteins. The use of this assay to study the functional activation of the G-proteins which interact with the cloned D_{2short} and D_{2long} dopamine receptors will lead to the elucidation of some of the molecular processes that underlie agonist action.

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