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Comparison of the Ability of Dopamine Receptor Agonists to Inhibit Forskolin-stimulated Adenosine 3′5′-cyclic Monophosphate (cAMP) Accumulation via D_{2L} (Long Isoform) and D_3 Receptors Expressed in Chinese Hamster Ovary (CHO) Cells

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ABSTRACT. The pharmacological properties of the human D_{2L} (long isoform) and rat D_3 dopamine receptors in functional assays were examined. A range of dopamine agonists were assessed for their ability to inhibit adenosine 3′5′-cyclic monophosphate (cAMP) accumulation via the two receptors expressed stably in Chinese hamster ovary cells. Dopamine caused a significantly greater maximal inhibition ($P < 0.05$) of cAMP accumulation via the D_{2L} receptor (~70%) as compared to the D_3 receptor (~50%). The pattern of agonist effects was different at the two receptors. The absolute and relative potencies for inhibition of cAMP accumulation were different for a range of agonists acting at the two receptors. Similarly, the maximal inhibitions achieved by a range of agonists were different for the two receptors. BIOCHEM PHARMACOL **58**;2:285–289, 1999.

KEY WORDS. dopamine; D_3 receptor; D_{2L} receptor; adenylyl cyclase

The D_2 dopamine receptor is a major therapeutic target for the treatment of schizophrenia and Parkinson's disease [1]. However, molecular biology has now revealed that the pharmacologically defined D_2 receptor in fact corresponds to three distinct subtypes, D_2, D_3, and D_4 (for reviews see [2, 3]). Moreover, the D_2 receptor has been shown to exist as two functional splice variants (D_{2L}§ and D_{2S}) [2, 3], variants of the D_3 receptor have been reported in some species [4], and the D_4 receptor has a number of allelic variants [5].

D_2 dopamine receptors are known to couple via G_i/G_o proteins to a number of functional responses, including inhibition of adenylyl cyclase and increases in cytosolic calcium concentration [6], and competition binding studies with agonists show marked sensitivity to guanine nucleotides (see for example [7]). The reports for the D_3 receptor are, however, less clear-cut. Some groups have reported that agonist binding to D_3 receptors is insensitive to guanine nucleotides [8–12], whilst others have shown nucleotide sensitivity [13–16], although the shifts were relatively small suggesting poor coupling to G-protein.

Studies on functional responses elicited by D_3 receptors have been similarly contradictory. Several laboratories have reported a lack of coupling of D_3 receptors to a number of functional responses (e.g. inhibition of adenylyl cyclase, calcium mobilisation, stimulation of arachidonic acid release, and induction of potassium currents) when transfected into a variety of cell lines (e.g. CHO, HEK 293, NG 108-15, and GH_4C_1 cells) [8, 10–13]. Others have, however, been successful in measuring D_3-mediated responses in heterologous cell lines. These include stimulation of [^{3}H]thymidine incorporation in CHO or NG 108-15 cells [15, 16], increased extracellular acidification in CHO or C6 glioma cells [16, 17], which was in the latter case pertussis toxin-insensitive, and inhibition of adenylyl cyclase in CHO cells [16]. Interestingly, although only a small number of compounds were tested, a comparison of the relative potencies for inhibition of cAMP accumulation and stimulation of [^{3}H]thymidine incorporation in [16] reveals a rather different profile. This may provide some evidence that different G-proteins are involved in the two responses and that the pharmacological profile is dependent on the particular receptor/G-protein combination.

We have previously reported that in the DUK25 cell line, a CHO cell line which expresses the rat D_3 receptor, dopamine inhibits forskolin-stimulated cAMP accumula-

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§ *Abbreviations:* 7-OH DPAT, 7-hydroxy, 2-*N*,*N*′-dipropylaminotetralin; cAMP, adenosine 3′5′-cyclic monophosphate; CHO, Chinese hamster ovary; DHEC, dihydroergocristine; 3-PPP, S(-)-3-(3-hydroxyphenyl)-*N*-propylpiperidine; NPA, *N*-propylnorapomorphine; D_{2L} and D_{2S}, long and short isoforms of the D_2 receptor.

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tion via the transfected receptor [7, 18]. Here, we report the ability of a number of other dopamine receptor agonists to inhibit forskolin-stimulated cAMP accumulation in DUK25 cells. These agonists include 7-OH-DPAT and PD128907, which have been claimed to be strongly selective for D_3 receptors in binding assays [19] although they have been reported to be much less selective in functional assays (e.g. [20]). These data are compared with the abilities of these compounds to inhibit cAMP accumulation in CHO cells expressing the human D_{2L} receptor.

MATERIALS AND METHODS

Cell Culture

CHO-D2L cells expressing recombinant human D_{2L} dopamine receptors [21] were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5% fetal bovine serum, and 200 μg/mL active geneticin. DUK25 cells expressing recombinant rat D_3 dopamine receptors [14] were grown in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% dialysed foetal bovine serum, and 50 nM methotrexate. Cells were maintained at 37° in an atmosphere of 5% CO_2 and were passaged every 4–5 days.

Measurement of cAMP Accumulation

For the measurement of cAMP accumulation, cells were seeded at either 35,000 (CHO-D2L) or 70,000 (Duk25) cells per well in 24-well plates and grown for a further 3 days until confluent. The medium was then replaced with fresh medium, 300 μL per well, containing 1 μCi/well of [^{3}H]adenine. After 2 hr, this medium was removed and the cells were washed with 1 mL of serum-free RPMI 1640 containing 20 mM HEPES, pH 7.5 (HEPES-RPMI). The cells were then incubated at 37° for 40 min in 1 mL of HEPES-RPMI containing 1 mM isobutylmethylxanthine. Forskolin (10 μM) and appropriate concentrations of agonists were then added in 20 μL of 50% DMSO (the original solvent of the forskolin) and the cells were incubated for a further 10 min. The assay was terminated by the addition of 0.5 mL of ice-cold perchloric acid (0.5 M, containing ~2500 dpm of [^{14}C]cAMP to act as a recovery standard). After 40 min on ice, the cAMP in the perchloric acid extracts was separated from the other labelled nucleotides by sequential chromatography on Dowex and alumina as described in [22]. 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 1% DMSO and 0.05% ascorbic acid. When concentration–response curves were determined for agonists other than dopamine, the effect of a maximally active concentration of dopamine (10 μM for CHO-D2L or 100 nM for DUK25 cells) was also determined as an internal reference for the calculation of relative maximal activities (intrinsic activities).

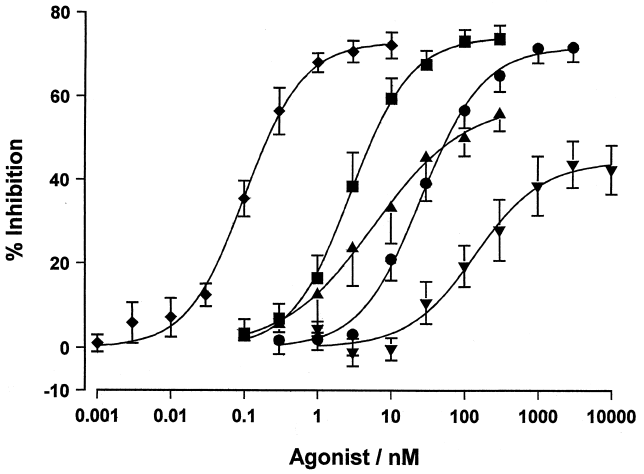

FIG. 1. Inhibition of forskolin-stimulated cAMP accumulation in CHO-D2L cells by bromocriptine (squares), dopamine (circles), DHEC (triangles), NPA (diamonds), and 3-PPP (inverted triangles) acting via D_{2L} receptors. Values are the mean of 4 separate experiments in duplicate; vertical bars show the SEM.

Data Analysis

To determine EC_{50} values, pseudo-Hill coefficients, and maximal effects, the recovery-corrected ^{3}H dpm data were fitted to a four parameter logistic equation using Fig. P. When concentration–response curves were pooled, the data were calculated as % inhibitions of the effect of forskolin after correction for cAMP accumulated in the presence of 3-isobutyl-l-methylxanthine alone.

Materials

[2,8-^{3}H]Adenine (20–40 Ci/mmol) and [8-^{14}C]cyclic AMP (40–60 mCi/mmol) were obtained from New England Nuclear. Dopamine agonists and (+)-butaclamol were obtained from Research Biochemicals Incorporated. Geneticin was from GIBCO. Tris base was obtained from Boehringer Mannheim. Perchloric acid and inorganic buffer salts were from Fisons Scientific Equipment. All other chemicals were from Sigma.

RESULTS

All of the agonists tested caused concentration-dependent inhibition of forskolin-stimulated cAMP accumulation in both CHO-D2L and DUK25(D3) cells (Figs. 1 and 2). Dopamine caused a significantly greater maximal level of inhibition in the CHO-D2L cells (71.3 ± 3.7%, N = 4) than in the DUK25 cells (49.5 ± 3.0%, N = 3) ($P < 0.05$, Student's *t*-test). The data are summarised in Tables 1 and 2. Dopamine had no effect on forskolin-stimulated cAMP accumulation in either CHO-K1 cells or DUK (CHO-dhfr$^-$) cells, the cell lines from which the CHO-D2L and DUK25(D3) cells were derived (data not shown).

In CHO-D2L cells, bromocriptine, NPA, quinpirole, 7-OH-DPAT, and PD128907 caused maximal levels of

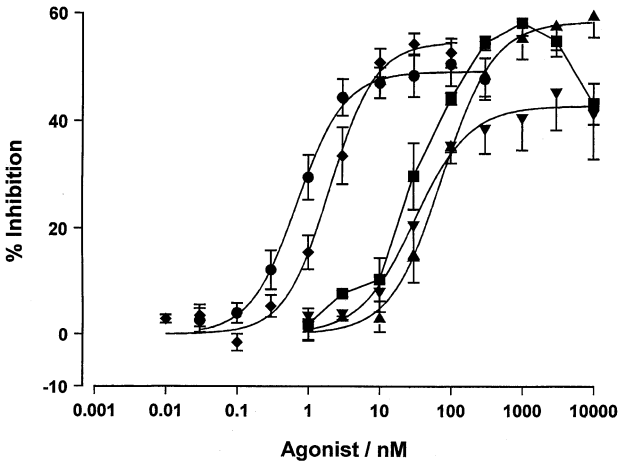

FIG. 2. Inhibition of forskolin-stimulated cAMP accumulation in DUK25 cells (D_3 dopamine receptor) by bromocriptine (squares), dopamine (circles), DHEC (triangles), NPA (diamonds), and 3-PPP (inverted triangles). Values are the mean of 3 separate experiments in duplicate; vertical bars show the SEM.

inhibition which were not significantly different from the dopamine control ($P > 0.05$, paired *t*-test). However, the maximal effects of apomorphine, DHEC, and 3-PPP were significantly lower than that of dopamine ($P < 0.05$, paired *t*-test), indicating that these compounds were partial agonists at this receptor under these conditions.

In DUK25(D_3) cells, however, the maximal inhibition induced by all of the agonists was at least as great as that induced by dopamine. Indeed, the maximal inhibition induced by DHEC was significantly greater than that induced by dopamine ($P < 0.05$, paired *t*-test). The concentration–response curve produced by bromocriptine was markedly bell-shaped, the peak occurring at 0.3–1 μM. In the three experiments performed with bromocriptine, its peak effect was markedly greater than that of the 1 μM dopamine control (59.4 ± 1.2% compared to 47.0 ± 5.1%, N = 3 in both cases). However, this difference did not reach significance at the 5% level.

DISCUSSION

In this study, we have determined the maximal effects and potencies of a series of agonists for inhibition of adenylyl cyclase at D_{2L} and D_3 dopamine receptors expressed in CHO cells. Although agonist responses at recombinant receptors may be dependent on the cell system used, the patterns of agonist activity seen in the present study are different for the two receptors. This is reflected in the absolute and relative potencies for inhibition of cAMP accumulation being different for a range of agonists acting at the two receptors. Moreover, the absolute and relative maximal inhibitions achieved for a range of agonists acting at the two receptors were different.

Agonists elicit greater inhibitions (maximal effects) of adenylyl cyclase via the D_{2L} receptor than via the D_3 receptor, and this is in agreement with other work which has shown that functional responses, e.g. stimulation of [^{35}S]GTPγS binding, inhibition of cAMP accumulation [7], stimulation of extracellular acidification, and stimulation of mitogenesis [16], are smaller via the D_3 receptor than via D_2 receptors. The cell lines used here express either D_{2L} receptors at levels of 1.3 pmol/mg [7] or D_3 receptors at levels of 2.7 ± 0.2 pmol/mg (the present study, mean ± SEM, 6 experiments); thus, the relative size of the response via the D_3 receptors in the present experiments cannot be due to relatively lower receptor expression, although we cannot rule out a lack of suitable G-proteins.

At the D_{2L} receptor, agonists exhibited a spectrum of maximal effects (intrinsic activities), many being full agonists but DHEC and 3-PPP being partial; no agonist exceeded the maximal inhibition observed with dopamine at this receptor. The pattern of maximal effects for the agonists tested is very similar to that for the D_{2S} dopamine receptor assayed under similar conditions [7]. The rank order of potency of the agonists is also very similar for the two receptors, although agonists appear more potent (by a factor of two) at the D_{2S} receptor in these experiments; this may reflect the higher level of expression in the CHO-D2S cells used (2.7 pmol/mg [7]). The receptor/G-protein ratio is, therefore, higher for D_{2S} than for D_{2L}, leading to greater

TABLE 1. Functional parameters of dopamine receptor agonists at recombinant D_{2L} receptors

Drug	pEC_{50}*	EC_{50} (nM)	Relative maximal activity*†	Relative potency‡	N*
Dopamine	7.59 ± 0.09	25.9	1.00 (71.3 ± 3.7%)	1.0	4
Apomorphine	8.51 ± 0.12	3.1	0.95 ± 0.02	8.4	4
Bromocriptine	8.54 ± 0.12	2.9	0.97 ± 0.03	8.9	4
DHEC	8.21 ± 0.21	6.2	0.71 ± 0.3	4.2	4
7-OH-DPAT	8.02 ± 0.03	9.6	0.90 ± 0.06	2.7	3
NPA	9.99 ± 0.07	0.1	1.03 ± 0.04	259	4
PD128907	7.55 ± 0.02	28.0	1.02 ± 0.01	0.9	3
3-PPP	6.81 ± 0.14	156	0.57 ± 0.07	0.2	4
Quinpirole	7.85 ± 0.15	14.3	0.99 ± 0.01	1.8	4

* Values are the mean ± SEM of N determinations.

† The relative maximal activity (intrinsic activity) was calculated relative to the effect of a maximally active concentration of dopamine (10 μM) which was determined with each concentration–response curve. The maximal inhibition caused by dopamine (from full concentration–response curves) is shown in parentheses.

‡ The relative potency of each agonist is defined as the reciprocal of the ratio of its EC_{50} to that of dopamine.

TABLE 2. Functional parameters of dopamine receptor agonists at recombinant D_3 receptors

Drug	pEC_{50}*	EC_{50} (nM)	Relative maximal activity*†	Relative potency‡	N*
Dopamine	8.99 ± 0.05	1.0	1.00 (49.5 ± 3.0%)	1.0	3
Apomorphine	8.41 ± 0.08	3.9	0.95 ± 0.07	0.25	3
Bromocriptine	7.60 ± 0.11	25.1	1.30 ± 0.16§	0.04	3
DHEC	7.13 ± 0.02	74.1	1.09 ± 0.04	0.013	3
7-OH-DPAT	8.52 ± 0.06	3.0	1.18 ± 0.13	0.33	3
NPA	8.69 ± 0.09	2.0	1.02 ± 0.05	0.50	3
PD128907	8.14 ± 0.06	7.3	1.00 ± 0.03	0.14	4
3-PPP	7.42 ± 0.23	37.7	0.92 ± 0.11	0.027	3
Quinpirole	8.60 ± 0.12	2.5	1.06 ± 0.04	0.40	3

* Values are the mean ± SEM of N determinations.

† The relative maximal activity (intrinsic activity was calculated relative to the effect of a maximally active concentration of dopamine [100 nM] which was determined with each concentration–response curve. The maximal inhibition caused by dopamine (from full concentration–response curves) is shown in parentheses.

‡ The relative potency of each agonist is defined as the reciprocal of the ratio of its EC_{50} to that of dopamine.

§ The 'intrinsic activity' of bromocriptine was calculated from the level of inhibition at the peak of the concentration–response curve.

amplification of signal and higher agonist potency. The efficacies of a range of agonists are therefore quite similar for the two D_2 receptor isoforms when assayed for inhibition of adenylyl cyclase. Very similar maximal effects were reported for a limited range of agonists for inhibition of adenylyl cyclase via the D_{2L} dopamine receptor [16], but the potencies differ from those reported here for some compounds. For the D_3 receptor in the present study, all of the agonists gave maximal responses that were at least as great as that of dopamine, and one agonist, DHEC, gave a maximal response that was significantly greater than that of dopamine. The peak response of bromocriptine (a structural analogue of DHEC) was also greater than that of dopamine, although in this case the difference was not significant at the 5% level. Partial agonism was reported for apomorphine and 3-PPP at the D_3 receptor in adenylyl cyclase assays [16]. This may reflect differences in coupling efficiency between the two cell lines used for the two studies, particularly as they are derived from distinct CHO cell lineages.

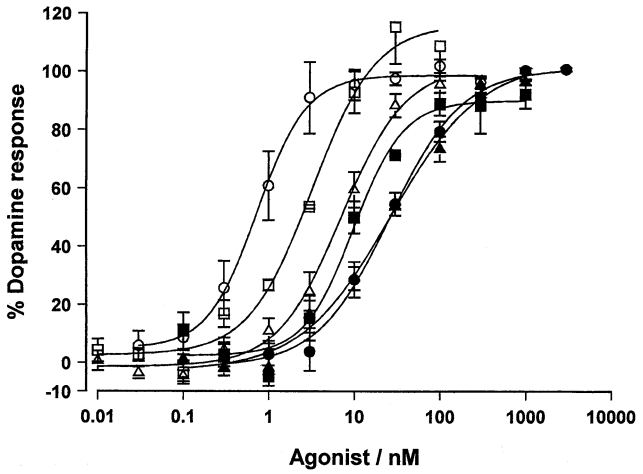

FIG. 3. A comparison of the potencies of dopamine (circles), 7-OH-DPAT (squares), and PD128907 (triangles) at D_{2L} (filled symbols) and D_3 (open symbols) receptors. The data for 7-OH-DPAT and PD128907 have been normalised to the effect of a maximally active concentration of dopamine at each receptor (see Materials and Methods section). The data for dopamine are normalised to the maximal effect of the fitted curve. Values are the mean of 3 or 4 separate experiments in duplicate; vertical bars show the SEM.

The profile of agonist selectivity for the D_2 versus the D_3 receptor that emerges from this study is illustrated in Fig. 3 in terms of dose–response curves and in Table 3 in terms of the ratio of EC_{50} values for inhibition of adenylyl cyclase via the two receptors. Based on these data, it is clear that the two receptors exhibit quite different pharmacological profiles and a distinct selectivity profile emerges. The rank order of selectivity in the present study is quite similar to that described using ligand binding (see for example [8]). Although exact numerical agreement between the two sets of data in terms of binding and functional data would not be expected, there is, nevertheless, some agreement. The data also show that, as previously reported [16, 20], 7-OH DPAT and PD 128907 are only weakly selective for D_3 receptors when assayed functionally.

There is also remarkably good agreement between the present results for inhibition of adenylyl cyclase by D_2 and D_3 receptors and the data of [20] on D_2 and D_3 receptor-mediated mitogenesis, especially given that the present responses were measured after 10-min exposure to agonist,

TABLE 3. Selectivity of agonists for D_{2L} versus D_3 dopamine receptors for inhibition of adenylyl cyclase

Agonist	D_3/D_{2L} selectivity ratio
Dopamine	26
Apomorphine	0.8
Bromocriptine	0.1
DHEC	0.08
7-OH DPAT	3.2
NPA	0.05
PD128907	3.8
3-PPP	4.2
Quinpirole	5.8

The table gives the D_3/D_{2L} selectivity ratio, which is defined as the ratio of EC_{50} values (D_{2L}/D_3) for inhibition of adenylyl cyclase.

whereas those of [20] were determined after 16 hr in the presence of agonist, which could allow considerable receptor desensitisation/degradation. There is also reasonable agreement between the present data on inhibition of adenylyl cyclase and the mitogenesis data reported in [16]. Adenylyl cyclase data reported in [16] are, however, markedly different from the present data and indeed the mitogenesis data of the same group. This may be a reflection of the different CHO cell lineages used in the two studies and the putative differential G-protein complements which might affect potency series for different responses.

In conclusion, from the present study of the inhibition of adenylyl cyclase by a range of agonists acting at D_2 and D_3 dopamine receptors it is clear that the two receptor isoforms have different agonist selectivities. These different selectivities may be important in the design of agonists selective for the different receptor subtypes.

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