0026-895X/94/010051-10$03.00/0

MOLECULAR PHARMACOLOGY, 45:51-60

Activation of Heterologously Expressed D3 Dopamine Receptors: Comparison with D2 Dopamine Receptors

CHRIS L. CHIO, MARY E. LAJINESS, and RITA M. HUFF
Cell Biology, The Upjohn Company, Kalamazoo, Michigan 49001
Received April 12, 1993; Accepted October 12, 1993

SUMMARY

Recombinant rat D3 dopamine receptors heterologously expressed in Chinese hamster ovary (CHO) cells are functionally coupled to endogenous G proteins. The affinity of the receptors for agonists is regulated by guanine nucleotides in the same manner as that of other G protein-linked receptors. The magnitude of the change in affinity induced by GTP is much less, however, than what is observed for recombinant rat D2 receptors expressed in CHO cells at similar densities. The striking difference is that the low affinity state (uncoupled D3 receptors) has a much higher affinity for agonists than does the low affinity state (uncoupled) of D2 receptors. Both receptors in the high affinity state (G protein coupled) have similar affinities for dopamine. Three functional responses result from activation of D3 or D2 receptors expressed in CHO cells. Both receptor subtypes mediate inhibition of adenylyl cyclase activity, increases in extracellular acidification rates that are prevented by removal of external Na^+ and by amiloride analogs, and stimulation of cell division. However, these three functional results of D3 and D2 receptor activation are both quantitatively and qualitatively different. Dopamine activation of D3 receptors is always 2–5-fold less efficacious than dopamine activation of D2 receptors, despite similar densities of receptors. Both D3 and D2 receptor-mediated increases in extracellular acidification rates are blocked by pertussis toxin; however, the D3 response and not the D2 response is partially attenuated by membrane-soluble cAMP analogs. D3 and D2 receptor-mediated stimulation of mitogenesis is blocked by pertussis toxin and unaffected by cAMP analogs. The results show that D2 and D3 dopamine receptors mediate similar signaling events and are additional examples of G protein-linked receptors that can activate more than one pathway. Having functionally coupled D2 and D3 receptors expressed in the same cell type enabled determinations of agonist potencies at both D2 and D3 receptors. Comparison of the potencies at the two receptors reveals that none of the agonists is as selective for D3 receptors as was previously thought based on radioligand binding data.

The gene for a new subtype of dopamine receptors, D3 receptors, was recently discovered (1, 2). Characterization of this dopamine receptor subtype is important for several reasons. D3 receptors are structurally homologous to D2 dopamine receptors, which have long been considered as targets for treatment of disorders such as schizophrenia and Parkinson's disease. The anatomical distribution of mRNA for D3 receptors indicates that they are expressed in limbic regions of the brain, areas that are innervated by dopamine nerve terminals and that are suspected to be dysfunctional in schizophrenia (3). With the realization that there are additional dopamine receptor subtypes, the possibility of developing therapeutic agents with greater specificity is raised. Indeed, the D3 receptor has been claimed to be an appropriate target for antipsychotic agents (4).

Due to the lower abundance of D3 receptors, compared with D2 receptors, and the lack of highly selective drugs, it is difficult to study native D3 receptors. Sokoloff *et al* (1) expressed the cDNA for the rat D3 receptor in CHO dhfr$^-$ cells and determined that the pharmacological profile of the receptor was similar to that of the D2 receptor. Classical D2 antagonists were found to bind to D3 receptors with high affinity, although some were 20–30-fold less potent. Nevertheless, it is clear that in many cases antipsychotic treatment of schizophrenia leads to blockade of D3 dopamine receptors as well as D2 receptors (4). The more striking pharmacological differences between D2 and D3 receptors came from comparisons of agonist binding constants, with D3 receptors apparently having higher affinities than D2 receptors for most agonists. In the original description of D3 receptor expression in CHO cells, however, the receptors did not appear to couple to endogenous G proteins, because the affinity of the receptors for dopamine was not regulated by guanine nucleotides.

We have obtained lines of CHO cells expressing recombinant rat D3 receptors in which activation of the receptors by dopamine and other agonists leads to a functional response. Studies

ABBREVIATIONS: CHO, Chinese hamster ovary; PCR, polymerase chain reaction; αMEM, α minimum essential medium; MEM, minimum essential medium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMA, 5-*N*,*N*-dimethylamiloride; MIA, 5-(*N*-methyl-*N*-isobutyl)amiloride; 7-OHDPAT, 7-hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin; 3-PPP, 3-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP.

in these cells have enabled us to determine the signaling events resulting from D3 receptor activation. In addition, comparisons can be made of the potencies of dopaminergic agonists at both functionally active D2 and D3 receptors.

Materials and Methods

Rat D3 receptor cDNA cloning. The rat D3 receptor was cloned using the PCR on template poly(A)$^+$ RNA isolated from rat whole brain. First-strand cDNA was synthesized using oligothymidylic acid and SuperScript reverse transcriptase from BRL, in a final volume of 100 μl with a final composition of 50 mM Tris·HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM levels each of dATP, dCTP, dGTP, and dTTP, 5 μg of oligothymidylic acid, 10 μg of rat brain mRNA, and 200 units of SuperScript reverse transcriptase. After 1 hr at 42°, the reaction was heated at 94° for 10 min and cooled on ice. Primers for amplification were derived from published sequence (1) (5′ sense primer, 5′-CGCTTTGTCGACCATGGCACCTCTGAGC-CAGATAAG-3′; 3′ antisense primer, 5′-CCACGTCGACAGATCTCG-AAGTGGGTAAAGGGAGTG-3′. Amplifications were performed with the Perkin Elmer Cetus GeneAmp kit and DNA thermal cycler. Ten microliters of the first-strand reaction were used as the substrate with 100 nM concentrations of each primer and 2.5 units of Amplitaq polymerase. The reaction was carried out for 30 cycles of 1 min at 60°, 2 min at 72°, and 1 min at 94°. After digestion with *Sal*I (10 units/1 hr), 10-μl samples of the reactions were separated on a 1% agarose gel. The PCR products were subcloned into the *Sal*I site of Bluescript SK(+) (Stratagene) for sequencing and mapping. Five cloned PCR-generated D3 dopamine receptor candidates were subjected to nucleotide sequence analysis by the enzymatic sequence method, using T7 polymerase (Sequenase) purchased from United States Biochemicals. A clone encoding a D3 receptor with the same amino acid sequence as that published by Sokoloff *et al* (1) was ligated into the *Sal*I site of the cytomegalovirus promoter-driven expression vector p3CLneo. This vector has an SV40 promoter-driven *neo*^{R} gene, which confers resistance to the antibiotic G418. Polyadenylation signals are provided by bovine growth hormone. Insert orientation was determined by *Hind*III digestion and agarose gel electrophoresis.

Rat D2 dopamine receptor (D2A) cDNA, cloned as described previously (5), was subcloned into p3Cneo using *Bam*HI/*Hind*III adaptors. This vector is identical to p3CLneo, except that the cytomegalovirus natural leader sequence has been replaced with the HSV late gene leader sequence.

Cell cultures and transfection. CHO 10001 (6) cells were grown in αMEM supplemented with 10% fetal bovine serum and 2 mM glutamine. Transfections were carried out using the calcium phosphate transfection system from BRL. Selection and cell maintenance were carried out with 1 mg/ml G418 (BRL) in the growth medium. After 10–14 days, G418-resistant colonies were picked and maintained in individual wells. Clonal lines of CHO cells transfected with D2 receptors were obtained after limiting dilution of isolated colonies. Clonal lines of CHO cells transfected with D3 receptors were obtained by selecting well isolated G418-resistant colonies. The remaining unpicked colonies were pooled and harvested for some of the radioligand binding assays. CHO cells were also transfected with the p3C vector without a dopamine receptor DNA insert. G418-resistant colonies from this transfection have been maintained as a control cell line, CHO-3C cells.

Radioligand binding assays. CHO cells from 100-mm dishes were rinsed, scraped into 5 ml of phosphate-buffered saline, pelleted, resuspended in 0.5 ml of 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, and homogenized. The supernatant resulting from centrifugation at 1000 × *g* was recentrifuged at 47,000 × *g*. The pellet was washed once with the Tris/EDTA/EGTA buffer, and the final pellet was resuspended in 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, at 0.2 ml/plate. Membrane aliquots were stored at −70° until use in radioligand binding assays.

The radioligand binding assays were carried out in a volume of 1 ml containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM EDTA, 200–600 pM [^{3}H]spiroperidol (90–120 Ci/mmol), and vehicle, competing drug, or 2 μM (+)-butaclamol. The binding reaction was initiated by the addition of 10–20 μg of membrane protein from CHO cells transfected with either D2 cDNA, D3 cDNA, or the vector without receptor DNA (CHO-3C cells). Incubations were carried out for 1 hr at 30°. The reactions were stopped by the addition of 3.5 ml of ice-cold 50 mM Tris, pH 7.5, and were filtered over Whatman GF/B filters, followed by two 10-ml washes with ice-cold 50 mM Tris, pH 7.5. The filters were counted by liquid scintillation counting.

cAMP assays. cAMP accumulation was measured in intact cells that had been plated at a density of 70,000 cells/well in a six-well plate or at 15,000 cells/well in a 24-well plate 48 hr before the experiment. The cells were incubated in serum-free medium for 60 min before the beginning of the assay. Two milliliters of fresh medium containing forskolin (100 μM), isobutylmethylxanthine (100 μM), and varying concentrations of drugs were added to each well and cAMP was allowed to accumulate for 15 min at 37°. The reactions were terminated by the addition of 1 ml/well (for six-well plates) or 0.5 ml/well (for 24-well plates) of cold 25% trichloroacetic acid, and the cAMP was assayed by radioimmunoassay using a cAMP radioimmunoassay kit (Biomedical Technologies Inc.).

Measurement of the rate of extracellular acidification. Extracellular acidification rates were measured using a Cytosensor microphysiometer (Molecular Devices Corp., Menlo Park, CA). CHO cells were seeded into 12-mm capsule cups (Molecular Devices Corp.) at 4 × 10^5 cells/cup, in αMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin. The cells were incubated at 37° in 5% CO_2 for 24 hr. The capsule cups were loaded into the sensor chambers of the microphysiometer and the chambers were perfused with running buffer (bicarbonate-free αMEM supplemented with 4 mM L-glutamine, 10 units/ml penicillin, 10 μg/ml streptomycin, and 26 mM NaCl), at a flow rate of 100 μl/min. In some experiments the running buffer was prepared with either 145 mM choline chloride or 145 mM NaCl and the following: 1.8 mM $CaCl_2$, 5.3 mM KCl, 0.8 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM glucose, 1 mM sodium pyruvate, 2 mM glutamine, 0.6 mM cysteine, 0.3 mM ascorbic acid, 0.4 μM biotin, and 1 μM vitamin B_{12}; the remaining vitamins and amino acids were provided by MEM essential amino acid solution, MEM nonessential amino acid solution, and MEM vitamin solution (GIBCO/BRL), pH 7.4. The final concentration of NaCl in the choline chloride buffer was 2.5 mM. Dopamine was diluted into running buffer and perfused through a second fluid path. Electronic operation of valves directed the flow from either fluid path to the sensor chamber. The pump cycle was controlled by the Cytosoft program running on a Macintosh IIsi computer. During each 1-min pump cycle, the pump was on for 38 sec and was then switched off for the remaining 22 sec. The pH of the running buffer was recorded from 43 to 58 sec, and the pump was started at 60 sec to start the next cycle. The rate of acidification of the running buffer during the recording time was calculated by the Cytosoft program. All of the eight chambers of the instrument were determined to have identical values for the change in mV/change in pH units, i.e., 61 mV/pH unit. Changes in the rates of acidification were calculated as the difference between the maximum effect after agonist addition and the average of four measurements taken immediately before agonist addition.

Mitogenesis assays. CHO cells were seeded into 96-well plates at a density of 5000 cells/well and were grown at 37° in αMEM, with 10% fetal calf serum, for 48 hr. The wells were rinsed three times with serum-free αMEM. Ninety microliters of fresh αMEM were added along with 10 μl of drug (diluted in sterile water and filtered through 0.2-μm filters) or sterile water alone. Eight wells of every plate received 100 μl of αMEM with 10% fetal calf serum. After culture for 16–17 hr, [^{3}H]thymidine (1 μCi/well) was added for 2 hr. The cells were trypsinized and harvested onto filter mats with a Skatron cell harvester. The filters were counted in a Betaplate counter.

Analysis of dose-response curves. Dose-response curves for competition with [^{3}H]spiroperidol binding at D2 or D3 receptors were analyzed by a nonlinear least-squares fit program provided by F. Kezdy (The Upjohn Co.), in which the curves were fitted to the equations $A = B \times [C/(D + C)]$ or $A = [B \times [C/(D + C)]] + [E \times [F/(D + F)]]$, where A is specifically bound radioligand, B is B_{max1}, C is IC_{501}, D is the concentration of drug, E is B_{max2}, and F is IC_{502}. A is experimentally determined, D is fixed, and B, C, E, and F are determined by Simplex optimization. Agonist competition curves in the absence of GTP were fitted to the second equation, whereas all other dose-response curves were fitted to the first equation. IC_{50} values were corrected to K_i values by the method of Cheng and Prusoff (7).

Dose-response curves for inhibition of forskolin-stimulated cAMP and agonist-stimulated mitogenesis were analyzed by the same program using the equation $A = B \times [C/(D + C)] + G$, where A is the percentage of forskolin-stimulated cAMP or [^{3}H]thymidine cpm, B is the maximal effect, C is the IC_{50} or EC_{50}, D is the concentration of agonist, and G is the percentage of forskolin-stimulated cAMP not inhibited by agonist or [^{3}H]thymidine cpm in the absence of agonist. Parameters B, C, and G were determined by Simplex optimization.

Results

Radioligand binding studies of D3 receptors. Saturation analysis of [^{3}H]spiroperidol binding to *neo*^{R} CHO cell membranes after transfection with rat D3 cDNA showed that a protein was expressed that had high affinity for the dopamine receptor radioligand. Among 24 isolated colonies, receptor expression levels ranged from 0.050 pmol/mg of protein to 3.5 pmol/mg of protein. Four lines were studied, i.e., D3–3 with 2.5 pmol/mg of protein, D3–8 with 0.8 pmol/mg of protein, D3–11 with 3.5 pmol/mg of protein, and D3–22 with 2.4 pmol/mg of protein. Analysis of the binding in membranes from isolated colonies was carried out. Fig. 1 shows the Scatchard transformation of the binding data from a clonal line (D3–8), and linear regression indicates that the D3 receptors have a K_d of 289 pM for [^{3}H]spiroperidol and the cells have a receptor density of 0.83 pmol/mg of protein. The D3 receptor affinity for [^{3}H]spiroperidol is 10-fold lower than the D2 receptor affinity for [^{3}H]spiroperidol (20 pM). A CHO cell line (L6) expressing the D2 receptor (D2A, or $D2_{in}$) at 3.5 pmol/mg of protein was also included in many of the studies. In the D3–3 cell line the K_i values for UH232 and AJ76 were 3.5 ± 0.48 nM (three experiments) and 81 ± 17 nM (three experiments), respectively, 6.3- and 2.1-fold higher affinity than at the D2 receptors. These values are similar to the values reported by Sokoloff *et al.* (1) for UH232 and AJ76. Metoclopramide was 5.1-fold more selective for the D2 receptor (D2 K_i = 21 nM, D3 K_i = 107 nM).

We used guanine nucleotide modulation of agonist binding to D3 and D2 receptors as a primary indication of functional coupling of the receptors to G proteins. Washed membrane preparations from our CHO cell lines had a small but reproducible shift in the dose-response curve for agonist competition with radioligand binding (shown in Fig. 2, *top*, is the analysis of CHO D3–3 cells). Analysis of the competition curve in the absence of GTP indicates receptors in a high affinity state (representing 18% of the receptors) with a K_i of 0.9 nM and receptors in a lower affinity state with a K_i of 22 nM. Analysis of the competition curve in the presence of GTP, which is clearly steepened, indicates a K_i of 24 nM. The K_{iL} and the K_i in the presence of GTP are quite similar to the K_i values reported by Sokoloff *et al.* (1) in the presence or absence of GTP (25 and 27 nM, respectively). The difference in our studies

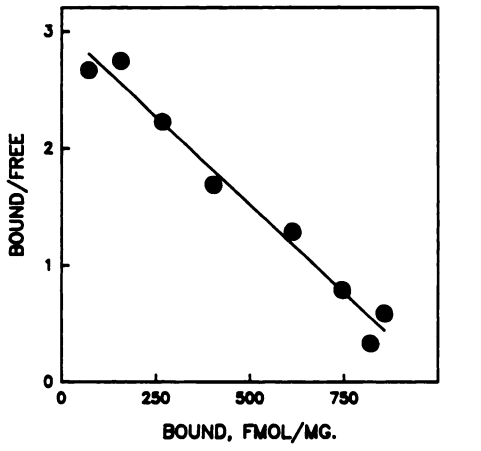

Fig. 1. Scatchard plot of [^{3}H]spiroperidol binding to D3 receptors. Membranes were prepared from CHO D3–8 cells and saturation binding analysis was performed as described in Materials and Methods. Nine concentrations of [^{3}H]spiroperidol, from 80 to 7500 pM, were incubated with 40 μg of membrane protein in the absence or presence of 2 μM (+)-butaclamol. Scatchard transformation of the specific binding at each concentration is shown (means of triplicates). The K_d was 289 pM and the B_{max} was 833 fmol/mg of protein.

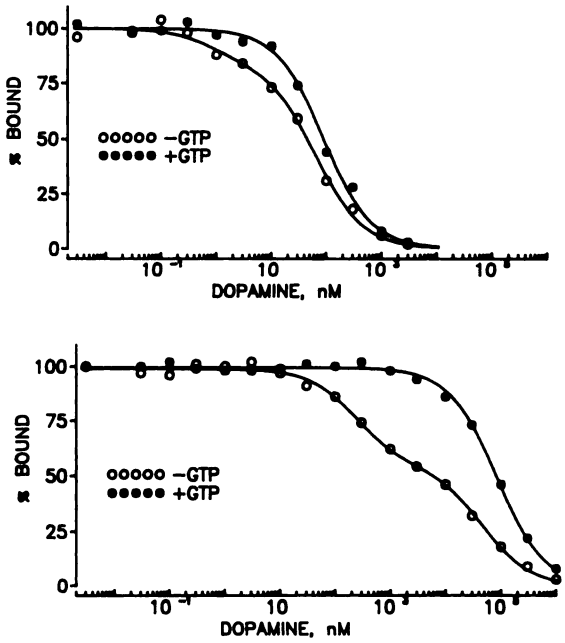

Fig. 2. Dopamine competition for radioligand binding at D3 and D2 receptors heterologously expressed in CHO cells. Washed membranes from the CHO D3–3 cell line (*top*) or the CHO-L6 cell line (*bottom*) were incubated with [^{3}H]spiroperidol (500 pM) and varying concentrations of dopamine in the absence (○) or presence (●) of 300 μM GTP. The percent inhibition of specific binding [radioligand binding blocked by 2 μM (+)-butaclamol] is plotted. The *data points* are the means of triplicates at each concentration of dopamine. The data were analyzed as described in Materials and Methods (two sites for the curves in the absence of GTP and one site for the curve in the presence of GTP). *Solid lines*, theoretical inhibition curves using the IC_{50} values and B_{max} values determined by the analysis. For dopamine inhibition of radioligand binding to D3 receptors, analysis of this curve determined an IC_{50} value that was converted to a K_{iH} value of 0.9 nM for 18% of the sites and a second IC_{50} value that was converted to a K_{iL} value of 22 nM for 72% of the sites. The dopamine competition curve in the presence of GTP had a corrected K_i value of 24 nM. For dopamine inhibition of radioligand binding to D2 receptors, the corrected K_{iH} value was 8 nM for 45% of the sites and the K_{iL} value was 1600 nM for 54% of the sites in the absence of GTP, with a K_i value of 2700 nM in the presence of GTP.

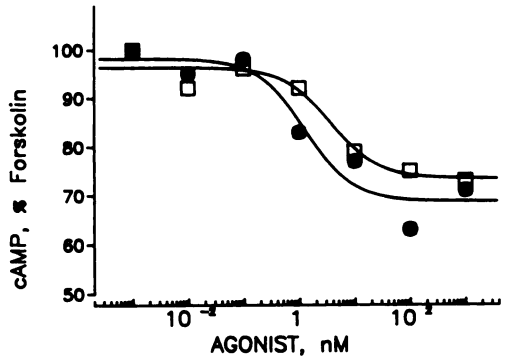

Fig. 3. Inhibition of forskolin-stimulated cAMP accumulation in CHO cells expressing D3 receptors. CHO D3–3 cells were plated and analyzed for agonist-dependent inhibition of forskolin-stimulated cAMP accumulation as described in Materials and Methods. Each *point* is the mean of triplicates determined at each concentration of agonist. ●, Dopamine; □, quinpirole.

is the resolution of a high affinity component of the dopamine binding. The magnitude of the GTP shift in D3 receptor affinity for dopamine is much smaller than what is observed for D2 receptors expressed in CHO cells (Fig. 2, *bottom*). There is both a greater percentage of D2 receptors in the high affinity state (38 ± 5%, seven experiments, compared with 20 ± 1.7%, eight experiments) and a greater difference between high and low affinity (300-fold, compared with 25-fold). The differences in GTP shifts between the two receptor subtypes are not due to differences in expression levels. A CHO cell line that expresses 10-fold fewer D2 receptors than does CHO-L6 also has a 100-fold difference between high and low K_i values for dopamine, and the four CHO lines that have a 4-fold range of D3 receptor expression all have similar ratios of K_{iL}/K_{iH} and percentages of R_H (from a single experiment, the K_{iL}/K_{iH} ratios and percentage of R_H, respectively, were as follows: D3–3, 29 and 24%; D3–8, 36 and 18%; D3–11, 83 and 13%; D3–22, 30 and 19%).

cAMP inhibition mediated by D3 receptors in CHO cells. Dopamine and other agonists inhibit cAMP accumulation in CHO cells expressing D3 receptors. Shown in Fig. 3 are the dose-response curves for dopamine and quinpirole inhibition of forskolin-stimulated cAMP accumulation in CHO D3–3 cells. In D3–3 cells the average maximal inhibition by dopamine in five of eight experiments (in three experiments inhibition was inexplicably not observed) was 26 ± 3.1%. In D3–8 cells the maximal inhibition was 31 ± 3.9% (four experiments). No agonist-induced inhibition of cAMP accumulation was observed in CHO-3C cells, which lack dopamine receptors; thus, the response requires D3 receptors.

D3 and D2 receptor-mediated changes in rates of extracellular acidification. The application of dopamine to CHO D3–3 cells causes a dose-dependent increase in the rate of extracellular acidification measured with a microphysiometer (Fig. 4). The acidification rate peaked at 7 min; longer pulses of dopamine did not produce larger changes in acidification rates. One of the lines in Fig. 4 shows data obtained from a chamber plated with CHO-3C cells, to demonstrate that the dopamine-mediated response requires the expression of D3 receptors. Two other sets of data in Fig. 4 were obtained from D3–3 cells that had been pretreated with 100 ng/ml pertussis toxin; thus, the response requires a D3 receptor and a pertussis toxin-sensitive G protein. Cells in all of the chambers responded to thrombin (10 units/ml) with a large increase in acidification rate (D3–3 cells, thrombin effect = 82.9 ± 3.3 μV/sec, four experiments; CHO-3C cells, thrombin effect = 78.7 μV/sec, one experiment; and pertussis toxin-treated D3–3 cells, thrombin effect = 69 ± 4.1 μV/sec, two experiments).

D2 receptor activation in CHO-L6 cells also leads to an increase in extracellular acidification rates, in a dose-dependent fashion (Fig. 5). A similar dopamine-mediated increase in extracellular acidification was recently reported by Neve *et al.* (8) for D2 receptor-transfected C_6 glioma and L cells. Unlike the D2-mediated acidification observed in C_6 and L cells, the D2-mediated acidification in CHO-L6 cells was entirely blocked by overnight pretreatment with 100 ng/ml pertussis toxin (Table 1).

Fig. 6 shows the dose-response curves for dopamine-stimulated extracellular acidification mediated by D3 and D2 receptors. The data are taken from the experiments shown in Figs. 4 and 5. The EC_{50} for the D3 receptor response was 1.2 nM, whereas the EC_{50} for the D2 receptor response was 10 nM. The

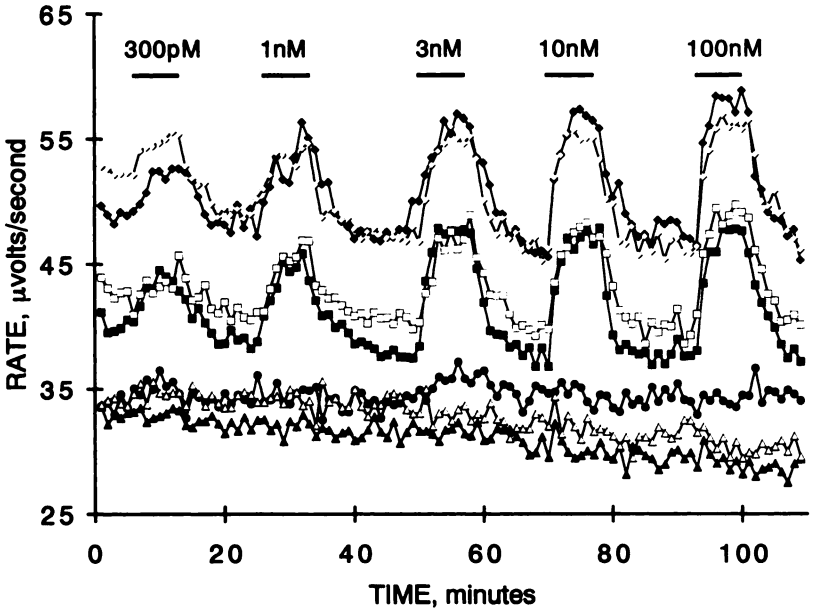

Fig. 4. Dopamine-dependent increase in extracellular acidification in CHO D3–3 cells. CHO D3–3 or CHO-3C cells were seeded into capsule cups, and the next day the cups were loaded into a Cytosensor microphysiometer as described in Materials and Methods. Each *point* represents the measured rate of acidification for one of seven chambers of cells. ◇, ◆, □, ■, Chambers seeded with CHO D3–3 cells; △, ▲, chambers seeded with CHO D3–3 cells treated overnight and perfused with 100 ng/ml pertussis toxin; ●, chambers seeded with CHO-3C cells. Successive applications of dopamine at the indicated concentrations were present in the second fluid path and the cells were perfused with this for the time indicated by the *bar* (7 min).

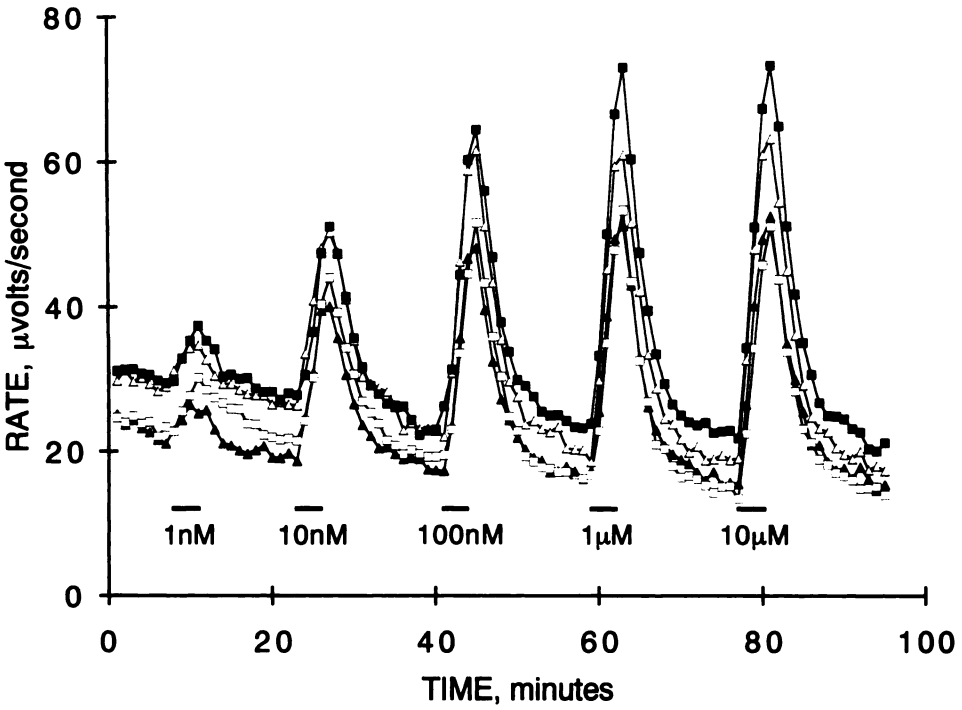

Fig. 5. Dopamine-dependent increase in extracellular acidification in CHO-L6 cells. CHO-L6 cells were seeded into capsule cups, and the next day the cups were loaded into a Cytosensor microphysiometer as described in Materials and Methods. Each *symbol* represents the measured rate of acidification for one of four chambers treated identically. Dopamine was present in the second fluid path at the indicated concentrations and the cells were perfused with this fluid for the time indicated by the *bar* (3 min).

TABLE 1

D2 and D3 receptor-mediated changes in extracellular acidification rates induced by dopamine

Maximal increases in the rates of extracellular acidification were measured after perfusion of CHO-L6 or CHO D3-3 cells with buffer containing dopamine, as described in Materials and Methods. Dopamine was used at 100 nM with D2 receptors and at 10 nM with D3 receptors.

Treatment	Acidification rate	
	D2[a]	D3
	μV/sec	
Control	102 ± 4 (2)	9.9 ± 0.5 (2)
Pertussis toxin[b]	−0.1 ± 1 (2)[c]	−0.9 ± 0.5 (2)[c]
Control	79 ± 11 (4)	20 ± 0.5 (2)
Low $[Na^+]_o$[d]	16 ± 3.5 (4)	−2.3 ± 0.7 (3)[c]

[a] D2 or D3 receptors expressed in CHO-L6 or CHO D3-3 cells.
[b] Pertussis toxin, 100 ng/ml overnight.
[c] These measurements were made at a time equivalent to the peak effect in the control. There were no increases in acidification rates.
[d] Low $[Na^+]_o$ medium is defined in Materials and Methods as running buffer that contains choline chloride.

maximal responses elicited by the two types of receptor activations were also different. The maximal change in acidification rate elicited by D3 receptor activation was 21 ± 2.9 μV/sec (31 experiments) and that elicited by D2 receptor activation was 75 ± 6.4 μV/sec (29 experiments).

Dopamine-stimulated increases in extracellular acidification rates in the CHO-L6 cells or D3–3 cells were markedly reduced (by 80%) or not detected when extracellular sodium was replaced by choline (Table 1). In CHO-L6 cells amiloride and two amiloride analogs, DMA and MIA, decreased dopamine-stimulated increases in extracellular acidification, with IC_{50} values of 29 μM, 1.4 μM, and 0.9 μM, respectively. Even with the highest concentrations of amiloride and DMA, a small D2-mediated effect was observed, i.e., 10–20% of the full response. In D3–3 cells dopamine-stimulated increases in extracellular acidification rates were completely blocked by MIA, with an IC_{50} value of 1.8 μM. (Amiloride, DMA, and MIA dose-response curves were measured in medium containing sodium.)

Fig. 7 shows the effects of membrane-soluble cAMP analogs on the maximal acidification rate changes resulting from D3 or D2 receptor activation. In these experiments each chamber of cells received two perfusions of dopamine, separated by 60 min. For some of the chambers the second perfusion was preceded by a 30-min perfusion with CPT-cAMP or 8-bromo-cAMP. Both D2- and D3-mediated increases in acidification rates could be repeated at 60-min intervals without significant changes in the response. With D3 receptors the addition of the membrane-soluble cAMP analogs, however, markedly reduced the acidification enhancement produced by a second pulse of dopamine. The addition of the cAMP analogs had no effect on the ability of the D2 receptors to respond to a second pulse of dopamine. The cAMP analogs alone caused a small gradual increase in extracellular acidification rates (ranging from 5 to 10 μV/sec).

D3 receptor-mediated stimulation of mitogenesis in CHO cells. Agonist activation of D3 receptors expressed in CHO cells leads to a stimulation of mitogenesis in serum-free medium. Fig. 8 shows the dose-dependent effects of dopaminergic agents on [^{3}H]thymidine incorporation in D3–3 cells. The agonists dopamine, apomorphine, and quinpirole elicited 2–3-fold increases in incorporation of radioactivity, whereas the antagonist (+)-butaclamol had no effect. Incubation of CHO-3C cells, which lack D3 receptors, with these agonists led to no change in [^{3}H]thymidine incorporation over the same concentration range (data not shown). Dopamine receptor antagonists inhibited agonist-stimulated mitogenesis at D3 receptors with the following rank order of potency and IC_{50} values: spiroperidol (0.58 nM) > UH232 (1.0 nM) > chlorpromazine (3.2 nM) > (+)-butaclamol (5 nM) > domperidone (16 nM) > sulpiride (88 nM). This is the same rank order of potency for these agents to compete for radioligand binding at D3 receptors (1). We have shown that agonist activation of D2 receptors in CHO-L6 cells also leads to an increase in mitogenesis (9). The maximal stimulation of mitogenesis induced by dopamine at D3 receptors (2.4 ± 0.12-fold stimulation, six experiments) is

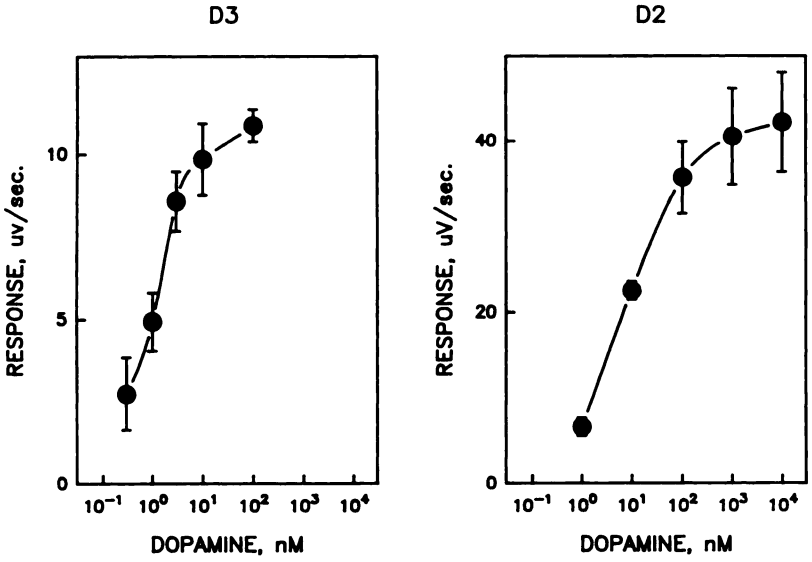

Fig. 6. Dose-response curves for dopamine changes in extracellular acidification measured at D3 and D2 receptors. The dose-response *curves* are taken from the data shown in Figs. 4 and 5. The peak change in acidification rate was measured at each concentration of dopamine. Each *point* is the mean ± standard error for four chambers.

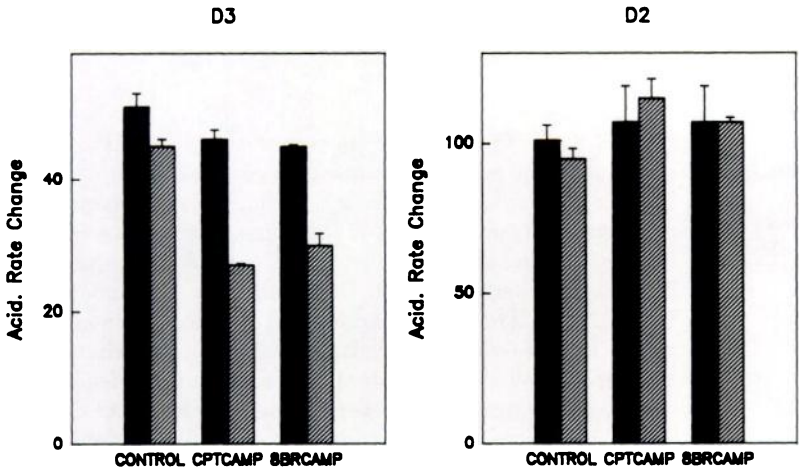

Fig. 7. Effects of membrane-soluble cAMP analogs on dopamine-stimulated extracellular acidification rates at D3 and D2 receptors. CHO D3–3 cells or CHO-L6 cells were seeded into capsule cups, and the next day the cups were loaded into a Cytosensor microphysiometer as described in Materials and Methods. *Bars*, changes in acidification rate elicited by two 7-min perfusions with 10 nM dopamine (CHO D3–3 cells) or two 3-min perfusions with 100 nM dopamine (CHO-L6 cells), separated by 60 min. ■, Mean changes resulting from the first perfusion with dopamine in a pair of chambers. Thirty minutes after dopamine, CPT-cAMP or 8-bromo-cAMP was added and perfused over the cells in two pairs of chambers for an additional 30 min. ■, Mean changes from each pair of chambers resulting from the second perfusion with dopamine. These are representative results from two similar experiments.

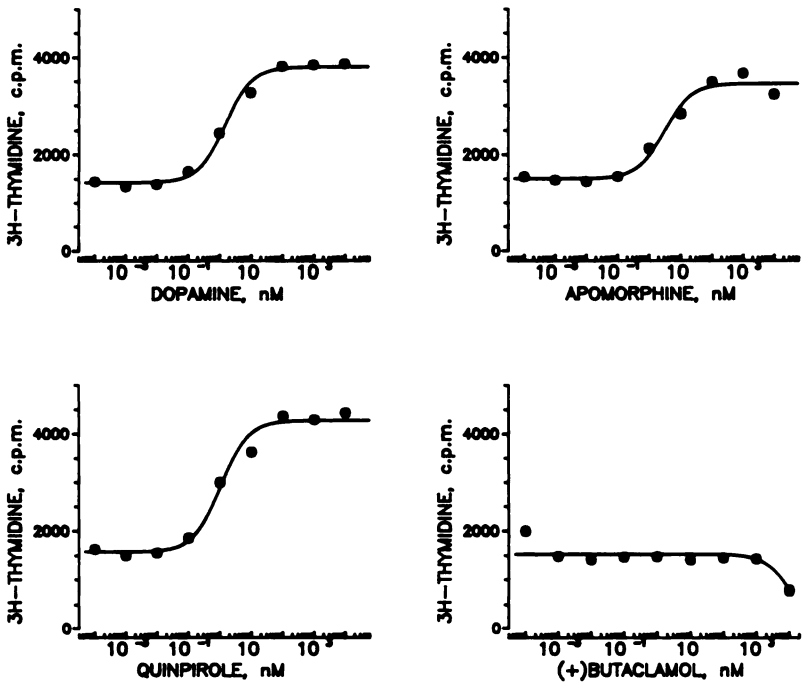

Fig. 8. D3 receptor-mediated stimulation of mitogenesis in CHO D3–3 cells. CHO D3–3 cells were seeded into 96-well plates and allowed to grow for 48 hr before the addition of dopamine agonists as described in Materials and Methods. After 16 hr of agonist exposure [^{3}H]thymidine was added for 2 hr and the incorporation was measured as described. Shown are the dose-dependent changes in [^{3}H]thymidine incorporation in CHO cells with dopamine, apomorphine, quinpirole, and (+)-butaclamol. Each *point* is the mean of quadruplicates.

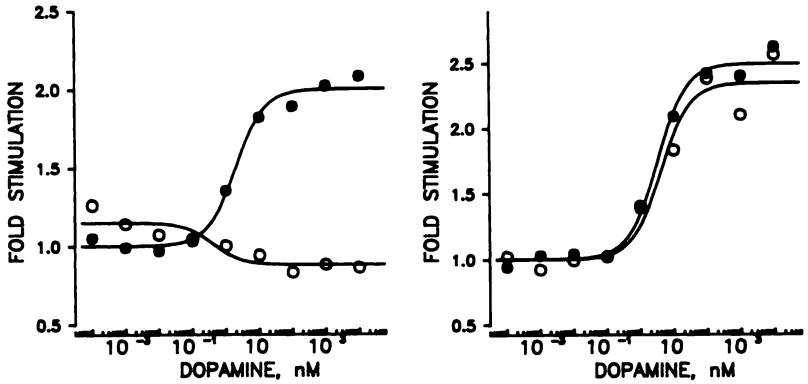

Fig. 9. Effects of pertussis toxin and a cAMP analog on D3 receptor-mediated mitogenesis. CHO D3–3 cells were seeded into 96-well plates and assayed for dopamine-stimulated mitogenesis as described in Materials and Methods. *Left*, effects of pertussis toxin treatment on dopamine-stimulated mitogenesis in CHO D3–3 cells. Half of each 96-well plate was pretreated, 6 hr before the addition of dopamine, with 100 ng/ml pertussis toxin in serum-containing medium. The other half of the plate received vehicle and served as a control. Varying concentrations of dopamine were added along with fresh pertussis toxin (100 ng/ml) to the wells that had been previously exposed to the toxin. The data were calculated as fold stimulation of [^{3}H]thymidine incorporation, relative to cells that were incubated without serum or dopamine. In the absence of pertussis toxin, the no-serum/no-dopamine value was 1380 cpm. In the presence of pertussis toxin, the no-serum/no-dopamine value was 419 cpm. Serum stimulated growth in both cases (16-fold in the absence of pertussis toxin and 25-fold in the pertussis toxin-treated cells). *Right*, effects of treatment with a membrane-soluble cAMP analog (CPT-cAMP) on dopamine-stimulated mitogenesis in CHO D3–3 cells. Increasing concentrations of dopamine were added to wells containing CHO D3–3 cells. Half of the plate received 100 μM CPT-cAMP, whereas the other half served as a control and received vehicle. The data are expressed as fold stimulation, relative to no-serum/no-dopamine values. The control no-serum/no-dopamine cells incorporated 2468 cpm and the treated no-serum/no-dopamine cells incorporated 1527 cpm. ●, Control values; ○, CPT-cAMP values.

TABLE 2

Agonist potencies at D3 and D2 receptors

Potencies or affinities of dopamine agonists were determined as indicated in Materials and Methods.

Drug	Radioligand competition parameters		cAMP inhibition		Mitogenesis	
	D2[a]	D3	D2	D3	D2	D3
	nM					
Dopamine	4.7 ± 1.2[b] (7) 1459 ± 260[f] (7) 2569 ± 604[h] (7)	0.83 ± 0.19 (8) 22.0 ± 1.6 (8) 29.0 ± 4.1 (8)	13.0 nM[c,d] 100%[g]	16 ± 16 nM (4) 100%	5.4 nM[c,e] 100%[g]	2.2 ± 0.4 nM (6) 100%
Quinpirole	6.4 (1) 2500 (1) 4730 (1)	0.43 ± 0.03 (2) 16.0 ± 1.5 (2) 25.0 ± 3.9 (2)	6.0 nM[c] 93 ± 17% (2)	3.7 nM (1) 88% (1)	2.2 nM[c] 96 ± 10% (4)	1.7 ± 0.43 nM (5) 111 ± 12% (5)
U-86170	1.3 ± 0.6 (4) 300 ± 30 (4) 563 ± 142 (4)	0.97 ± 0.33 (2) 18.0 ± 1.0 (2) 22.0 ± 3.4 (2)	0.60 nM[c] 103 ± 9% (4)	1.2 ± 0.7 nM (2) 81 ± 7% (2)	0.20 nM[c] 90 ± 5% (15)	1.6 ± 0.17 nM (12) 99 ± 5.6% (12)
Apomorphine	1.7 (1) 81 (1) 106 (1)	0.42 ± 0.05 (3) 20.0 ± 8 (3) 35.0 ± 17 (3)	46 nM[c] 100 ± 6% (2)	79 nM (1) 63% (1)	0.40 nM[c] 91 ± 2% (3)	1.1 ± 0.39 nM (4) 96 ± 8% (4)
(−)-3-PPP	19 ± 13 (2) 256 ± 79 (2) 404 ± 94 (2)	18 ± 5 (2) 241 ± 95 (2) 330 ± 137 (2)	9.7 nM[c] 58 ± 3% (3)	3.9 ± 3 nM (2) 44% (2)	9.5 nM[c] 79 ± 7% (5)	23 ± 1.9 nM (3) 116 ± 25% (3)
(+)-7-OHDPAT	1.4 ± 0.9 (2) 31 ± 0.5 (2) 66 ± 16 (2)	0.29 ± 0.14 (2) 2.3 ± 1.2 (2) 4.5 ± 3.0 (2)	1.1 nM[c] 129%	0.08 ± 0.03 nM (3) 103 ± 21% (3)	1.1 nM[c] 77 ± 14% (4)	0.30 ± 0.005 nM (3) 77 ± 13% (3)

[a] D2 or D3 receptors heterologously expressed in CHO-L6 or CHO D3-3 cells.
[b] K_{hi}.
[c] Values are from Ref. 9.
[d] IC_{50}.
[e] EC_{50}.
[f] K_{lo}.
[g] Intrinsic activity; maximal effect as percentage of dopamine effect.
[h] K_{iGTP}.

comparable to thrombin-stimulated mitogenesis in CHO cells (2.5 ± 0.13-fold stimulation, seven experiments) but is less than that induced by dopamine at D2 receptors in CHO cells (8.4 ± 0.5-fold stimulation, seven experiments).

Pretreatment of the cells with pertussis toxin abolished the D3 receptor-mediated stimulation of mitogenesis (Fig. 9, *left*). Pertussis toxin also reduced the incorporation of [^{3}H]thymidine in unstimulated cells; however, the pertussis toxin-treated cells still responded to the addition of serum (data not shown). These data indicate that D3 receptor-mediated mitogenesis requires the activity of a pertussis toxin-sensitive G protein.

We explored the possibility that the D3-stimulated mitogenesis results from lowering of intracellular cAMP. Elevation of intracellular cAMP levels or activation of the cAMP-dependent protein kinase in CHO cells inhibits the growth of these cells (6, 10). Thus, it follows that D3 receptor activation may stimulate mitogenesis by reducing cellular cAMP levels. However, in the presence of the membrane-soluble cAMP analog CPT-

cAMP dopamine elicited the same fold stimulation of mitogenesis as in untreated cells (Fig. 9, *right*). This treatment reduced the [^{3}H]thymidine incorporation in serum-stimulated cells by 50%. Thus, D3-stimulated mitogenesis is independent of cAMP changes. D2 receptor-mediated stimulation of mitogenesis in CHO-L6 cells is also independent of cAMP changes, as evidenced by an insensitivity of the response to CPT-cAMP and the finding that dopamine stimulates mitogenesis in D2 receptor-transfected CHO cells that are mutant in cAMP-dependent protein kinase activity (9).

With functionally coupled D2 and D3 receptors, comparisons can be made of the potencies and efficacies of various agonists at the two receptor subtypes. Table 2 shows the K_i values, EC_{50} values, IC_{50} values, and intrinsic activities determined for six agonists at D2 and D3 receptors, as determined by competition for radioligand binding, inhibition of cAMP accumulation, and stimulation of mitogenesis. At both D2 and D3 receptors agonist potencies in either inhibition of cAMP accumulation or stimulation of mitogenesis were similar, with the exception of apomorphine, which was markedly more potent in stimulating mitogenesis by either receptor than in inhibiting cAMP accumulation. None of the six agonists studied had a high degree of selectivity for either D2 or D3 receptors, when K_{iH} values, IC_{50} values, or EC_{50} values were compared. Only when the K_{iL} or K_{iGTP} values for agonists at either receptor were compared was there a remarkable difference for some of the agonists, such as dopamine, quinpirole, and U-86170. Because K_{iL} or K_{iGTP} values reflect binding to the uncoupled receptors, uncoupled D3 receptors have higher affinities for these agonists than do uncoupled D2 receptors. A noteworthy finding is that (+)-7-OHDPAT, used as a radioligand for selectively labeling D3 receptors (11), was quite potent in activating D2 receptors and had a low K_{iH} value in binding assays.

The functional assays also yielded measurements of intrinsic activities for the agonists at both D2 and D3 receptors, although the precision of the measurement was greater for D2 receptor activation than for D3 receptor activation, because of the larger magnitude of the effect. Most of the agonists had nearly the same efficacy as dopamine, with the exception of (−)-3-PPP, which is a partial agonist mediating inhibition of cAMP accumulation by both D2 and D3 receptors.

Discussion

We have determined that recombinant rat D3 receptors heterologously expressed in CHO cells functionally interact with endogenous G proteins. Evidence for this comes from four types of measurements, i.e., guanine nucleotide effects on agonist affinity at D3 receptors, D3 receptor-mediated inhibition of cAMP accumulation, D3 receptor-mediated stimulation of extracellular acidification rates, and D3 receptor-mediated stimulation of mitogenesis; the latter two responses were blocked by pertussis toxin. This is the first description of D3 receptor activation.

Guanine nucleotide effects on agonist binding are a characteristic of G protein-linked receptors. In washed membrane preparations it is possible to alter the affinity of dopamine receptors for agonists by the addition of GTP or nonhydrolyzable analogs of GTP (12–14). The dose-response curves, generally shallow with small Hill coefficients, are markedly steepened and agonist affinity is lowered by GTP. The shallow curves are thought to reflect the accumulation of a high affinity ternary complex of agonist, receptor, and G protein (ARG) and the remaining low affinity uncoupled receptor (AR). Guanine nucleotides promote the dissociation of the ternary complex, and all of the receptors appear in the low affinity uncoupled state (15, 16).

We show here that recombinant rat D3 receptors expressed in CHO cells are regulated by guanine nucleotides in the same way that D2 receptors are regulated. Another group recently described similar results with rat D3 receptors expressed in CHO dhfr$^-$ cells (17). The magnitude of the GTP-induced decrease in the affinity of D3 receptors for dopamine and other agonists is much less than the magnitude of the GTP shift in agonist affinity at D2 receptors. The differences are reflected in both a smaller K_{iH}/K_{iL} ratio and a smaller percentage of receptors measured at K_{iH}. It has been predicted that the ratio of K_{iH}/K_{iL} is an estimate of the ability of the agonist (A) to stabilize the association of the receptor (R) and the G protein (G), or the ternary complex (ARG) (15). These data suggest that dopamine binding to D3 receptors stabilizes less ternary complex formation than does dopamine binding to D2 receptors.

Not only have we detected D3 interactions with G proteins but we can measure D3 receptor activation. cAMP inhibition is a response associated with G_i-linked receptors, and both D2 and D3 receptors have structural features that encourage predictions of G_i linkage. Dopamine inhibits adenylyl cyclase activity in the caudate nucleus (18, 19) and pituitary (20, 21) through "D2-like" receptors, and recombinant D2 receptors in heterologous expression systems have been shown to inhibit adenylyl cyclase activity or forskolin-stimulated cAMP accumulation (22, 23). We have shown that activation of either D3 or D2 receptors inhibits cAMP accumulation in CHO cells. Receptors that inhibit cAMP accumulation often have additional signaling mechanisms (see Ref. 24), and we found that both D3 and D2 receptors are not exceptions to this generalization.

Another measurement of receptor activation for both D3 and D2 receptors is dopamine-stimulated increase in the rates of extracellular acidification. Increases in extracellular acidification rates can be attributed to many factors, including increases in glycolysis, ATP hydrolysis occurring during restoration of transmembrane ion gradients after opening of ion channels, and changes in the activity of Na^+/H^+ exchange proteins (25). Many receptors mediate changes in the activity of an amiloride-sensitive Na^+/H^+ exchanger (see Ref. 26), including those linked to G_i proteins (24, 26). D2 receptors in anterior pituitary cells have previously been found to inhibit Na^+/H^+ exchange, leading to intracellular acidification (27). Recently, the opposite effect was described for D2 receptor activation in mouse L cells and C_6 glioma cells, where dopamine stimulated the rates of extracellular acidification measured with a microphysiometer and this was attributed to activation of Na^+/H^+ exchange (8). These two observations represent another case in which the types of signaling mediated by D2 receptors are cell type dependent (22). Like what was observed for D2 receptors in C_6 glioma and L cells, D2- and D3-mediated increases in extracellular acidification rates require an inward concentration gradient of sodium and can be blocked by amiloride and/or amiloride analogs, suggesting that a Na^+/H^+ exchanger is required for the dopamine-stimulated outward transport of protons. There is a residual component of D2-mediated extracel-

lular acidification that is insensitive to the amiloride analogs and occurs in the absence of an inward Na^+ gradient, suggesting that D2 receptors in CHO cells have an additional mechanism for acidification. Unlike what was observed for D2 receptors in C_6 glioma and L cells (8), however, both D3- and D2-mediated acidifications in CHO cells are blocked by pertussis toxin. Thus, the signaling pathways that result in increased Na^+/H^+ exchange must be different in the two cell types.

There are many signaling pathways that can lead to activation of Na^+/H^+ exchangers, in particular the more widely expressed NaH-1 exchange protein, although activation is generally independent of changes in cAMP (26). D2-mediated acidification was unaffected by membrane-soluble cAMP analogs, but dopamine-stimulated acidification via D3 receptors was attenuated by pretreatment with cAMP analogs. We do not interpret this result to mean that D3 receptor-mediated acidification is dependent on lowering of cAMP levels, because the cAMP analogs alone also increased acidification. The results could indicate a cAMP-mediated modification in the response pathway elicited by D3 receptors. The selective cAMP effect on D3- and not D2-mediated acidification suggests that the modification is on the receptors, but it could also be on the G proteins or some other component of a pathway that is unique for D3 activation of the exchanger. Alternatively, cAMP could induce a modification of the same G proteins used by both D2 and D3 receptors but the impact might be greater on D3 signaling because of the reduced efficiency of D3 receptor-G protein coupling. Nevertheless, these experiments have revealed a difference in the regulation of D2- and D3-mediated responses.

The third measurement of D3 receptor activation is agonist-induced stimulation of DNA synthesis and cell division. Activation of several G protein-linked receptors has been shown to stimulate mitogenesis in various cell types (28–30), including D2 receptors in CHO cells (9). Stimulation of mitogenesis cannot be a consequence of D2 or D3 receptor activation in nonmitotic neurons; however, the initial signaling events that cause this response in fibroblasts may well occur in neuronal cells with a different outcome. For this reason we have attempted to determine which, if any, of the already described D2- and D3-mediated signaling events contribute to the mitogenic response. Both receptors require a pertussis toxin-sensitive G protein for the effect; however, stimulated mitogenesis is independent of cAMP levels. Although many growth factors stimulate amiloride-sensitive Na^+/H^+ exchange (31), the activity of this exchanger is not thought to be required for growth in HCO^{-3}-containing medium (32). Likewise, at least for D2 receptor-stimulated mitogenesis, accelerated Na^+/H^+ exchange is not obligatory because amiloride blocks the latter and not the former response (9). It is interesting that there are many examples now showing that the effects of dopamine on pituitary cells are opposite to the effects of dopamine on fibroblast-like cells (8, 22, 27). In pituitary cells and transfected GH_4C_1 cells D2 receptor activation leads to an inhibition of cell proliferation (33, 34), which is accompanied by an increase in tyrosine phosphatase activity (34). In contrast, we have found that D2 and D3 receptor-mediated stimulation of mitogenesis in CHO cells is accompanied by increases in tyrosine phosphorylations (9).[1]

[1] R. M. Huff and M. E. Lajiness, unpublished observations.

For five agonists the potencies in the two functional assays are similar for either D2 or D3 receptors. Apomorphine is an exception; the reason for the 100-fold discrepancy between apomorphine potency in inhibiting cAMP and in stimulating mitogenesis at both receptors is unclear. Previously it was shown that K_{iH} values for agonists at D2 receptors correlate well with the potency of the agonists for inhibition of cAMP accumulation in anterior pituitary membranes (35). Our data also show a good agreement between the K_{iH} values determined in radioligand binding assays and the EC_{50} values for mitogenesis, particularly at the D2 receptor. A comparison of the potencies of agonists for D2- and D3-mediated responses in the same cell type shows that the dopaminergic agonists do not have the high degree of selectivity for D3 receptors that was estimated based on radioligand binding assays (1).

It remains to be determined which kinds of signaling pathways D3 receptors utilize *in vivo*, but this will no doubt require more selective D2 or D3 receptor agonists and antagonists than are currently available. Clearly, signaling by D2 receptors varies in different cell types, probably due to the availability of different G proteins with which they can interact (22). Our studies reveal that D3 receptors, like D2 receptors, belong to the family of G_i-linked receptors, for which multiple effectors are activated. It is interesting that the D3 receptors mimic the signaling output of D2 receptors in the same cell, but with lower efficacy. If D3 receptors and D2 receptors interact with the same G proteins in CHO cells with equivalent receptor densities, then the reduced efficacy of the D3 receptors implies a less efficient coupling of D3 receptors. If D2 and D3 receptors interact with multiple G proteins, then the D3 interaction is consistently less efficient. Because the same signaling events are triggered by both receptors, it is probable (although not necessary) that D2 and D3 receptors interact with the same G_α subunits. The weaker D3 receptor-G protein coupling may be inherent to the receptor or may result from a shortage of other components necessary for receptor-G protein coupling, such as $G_{\beta\gamma}$ subunits. The importance of G_β subunits in the specificity of receptor-G protein coupling has recently been demonstrated for somatostatin and muscarinic M_4 receptors, which both inhibit Ca^{2+} currents in the same cell (36). The different efficacies of D2 and D3 receptor activation may be an important means for varying the information resulting from dopamine neurotransmission.

References

1. Sokoloff, P., B. Giros, M.-P. Martres, M.-L. Bouthenet, and J.-C. Schwartz. Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. *Nature (Lond.)* **347**:146–151 (1990).
2. Giros, B., M.-P. Martres, P. Sokoloff, and J.-C. Schwartz. cDNA cloning of the human dopaminergic D3 receptor and chromosome identification. *C. R. Hebd. Seances Acad. Sci. Ser. C Sci. Chim.* **311**:501–508 (1990).
3. Bouthenet, M.-L., E. Souil, M.-P. Martres, P. Sokoloff, B. Giros, and J.-C. Schwartz. Localization of dopamine D_3 receptor RNA in the rat brain using *in situ* hybridization histochemistry: comparisons with dopamine D_2 receptor mRNA. *Brain Res.* **564**:203–219 (1991).
4. Sokoloff, P., M.-P. Martres, B. Giros, M.-L. Bouthenet, and J.-C. Schwartz. The third dopamine receptor (D_3) as a novel target for antipsychotics. *Biochem. Pharmacol.* **43**:659–666 (1992).
5. Chio, C. L., G. F. Hess, R. S. Graham, and R. M. Huff. A second molecular form of D_2 dopamine receptor in rat and bovine caudate nucleus. *Nature (Lond.)* **343**:266–269 (1990).
6. Gottesman, M. M., A. LeCam, M. Bukowski, and I. Pastan. Isolation of multiple classes of mutants of CHO cells resistant to cyclic AMP. *Somat. Cell Genet.* **6**:45–61 (1980).
7. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).

8. Neve, K. A., M. R. Kozlowski, and M. P. Rosser. Dopamine D2 receptor stimulation of Na^+/H^+ exchange assessed by quantification of extracellular acidification. *J. Biol. Chem.* **267**:25748–25753 (1992).
9. Lajiness, M. E., C. L. Chio, and R. M. Huff. D2 dopamine receptor stimulation of mitogenesis in transfected Chinese hamster ovary cells: relationship to dopamine stimulation of tyrosine phosphorylations. *J. Pharmacol. Exp. Ther.* **267**:1573–1581 (1993).
10. Le Cam, A., M. M. Gottesman, and I. Pastan. Mechanism of cyclic AMP effect on nutrient transport in Chinese hamster ovary cells: a genetic approach. *J. Biol. Chem.* **255**:8103–8108 (1980).
11. Levesque, D., J. Diaz, C. Pilon, M.-P. Martres, B. Giros, E. Couil, D. Schott, J.-L. Morgat, J.-C. Schwartz, and P. Sokoloff. Identification, characterization, and localization of the dopamine D_3 receptor in rat brain using 7-[^{3}H] hydroxy-*N*,*N*-di-*n*-propyl-2-aminotetralin. *Proc. Natl. Acad. Sci. USA* **89**:8155–8159 (1992).
12. Zahniser, N. R., and P. B. Molinoff. Effects of guanine nucleotides on striatal dopamine receptors. *Nature (Lond.)* **275**:453–455 (1978).
13. Creese, I., T. B. Usdin, and S. H. Snyder. Dopamine receptor binding regulated by guanine nucleotides. *Mol. Pharmacol.* **16**:69–71 (1979).
14. Hamblin, M. W., S. L. Leff, and I. Creese. Interactions of agonists with D-2 dopamine receptors: evidence for a single receptor population existing in multiple agonist affinity states in rat striatal membranes. *Biochem. Pharmacol.* **33**:877–887 (1984).
15. DeLean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
16. Wregget, K. A., and A. DeLean. The ternary complex model: its properties and application to ligand interactions with the D_2-dopamine receptor of the anterior pituitary gland. *Mol. Pharmacol.* **26**:214–227 (1984).
17. Castro, S. W., and P. G. Strange. Coupling of D2 and D3 dopamine receptors to G-proteins. *FEBS Lett.* **315**:223–226 (1993).
18. Onali, P., M. C. Olianas, and G. L. Gessa. Characterization of dopamine receptors mediating inhibition of forskolin-stimulated adenylate cyclase activity in rat striatum. *Mol. Pharmacol.* **28**:138–145 (1985).
19. Battaglia, G., G. A. Norman, E. J. Hess, and I. Creese. D_2 dopamine receptor-mediated inhibition of forskolin-stimulated adenylate cyclase activity in rat striatum. *Neurosci. Lett.* **59**:177–182 (1985).
20. De Camilli, P., D. Macconi, and A. Spada. Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. *Nature (Lond.)* **278**:252–254 (1979).
21. Cote, T. E., E. A. Frey, C. W. Grewe, and J. W. Kebabian. Evidence that the dopamine receptor in the intermediate lobe of the rat pituitary gland is associated with an inhibitory guanyl nucleotide component. *J. Neural Transm. Suppl.* **18**:129–147 (1983).
22. Vallar, L., C. Muca, M. Magni, P. Albert, J. Bunzow, J. Meldolesi, and O. Civelli. Differential coupling of dopaminergic D_2 receptors expressed in different cell types: stimulation of phosphatidyl inositol 4,5-bisphosphate hydrolysis in Ltk^- fibroblasts, hyperpolarization and cytosolic-free Ca^{2+} concentration decrease in GH_4C_1 cells. *J. Biol. Chem.* **265**:10320–10326 (1990).
23. Albert, P. R., K. A. Neve, J. R. Bunzow, and O. Civelli. Coupling of a cloned rat dopamine-D_2 receptor to inhibition of adenylate cyclase and prolactin secretion. *J. Biol. Chem.* **265**:2089–2104 (1990).
24. Limbird, L. Receptors linked to inhibition of adenylate cyclase: additional signalling mechanisms. *FASEB J.* **2**:2686–2695 (1988).
25. Owicki, J. C., and J. W. Parce. Biosensors based on the energy metabolism of living cells: the physical chemistry and cell biology of extracellular acidification. *Biosensors Bioelectronics* **7**:255–272 (1992).
26. Barber, D. L. Mechanisms of receptor-mediated regulation of Na-H exchange. *Cell. Signalling* **3**:387–397 (1991).
27. Ganz, M. B., J. A. Pachter, and D. L. Barber. Multiple receptors coupled to adenylate cyclase regulate Na-H exchange independent of cAMP. *J. Biol. Chem.* **265**:8989–8992 (1990).
28. Ashkenazi, A., J. Ramachandran, and D. J. Capon. Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. *Nature (Lond.)* **340**:146–150 (1989).
29. Seuwen, K., I. Magnaldo, and J. Pouyssegur. Serotonin stimulates DNA synthesis in fibroblasts acting through 5-HT_{1B} receptors coupled to a G_i-protein. *Nature (Lond.)* **335**:254–256 (1988).
30. Julius, D., T. J. Livelli, T. M. Jessell, and R. Axel. Ectopic expression of the serotonin 1C receptor and the triggering of malignant transformation. *Science (Washington D. C.)* **244**:1057–1062 (1989).
31. Rozengurt, E. Early signals in the mitogenic response. *Science (Washington D. C.)* **234**:161–166 (1986).
32. Grinstein, S., D. Rotin, and M. J. Mason. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes: role in cellular proliferation. *Biochim. Biophys. Acta* **988**:73–89 (1989).
33. Lloyd, H. M., J. D. Meares, and J. Jacobi. Effects of oestrogen and bromocryptine on *in vivo* secretion and mitosis in prolactin cells. *Nature (Lond.)* **255**:497–498 (1975).
34. Florio, T., M.-G. Pan, B. Newman, R. E. Hershberger, O. Civelli, and P. J. S. Stork. Dopaminergic inhibition of DNA synthesis in pituitary tumor cells is associated with phosphotyrosine phosphatase activity. *J. Biol. Chem.* **267**:24169–24172 (1992).
35. McDonald, W. M., D. R. Sibley, B. F. Kilpatrick, and M. G. Caron. Dopaminergic inhibition of adenylate cyclase correlates with high affinity agonist binding to anterior pituitary D_2 dopamine receptors. *Mol. Cell. Endocrinol.* **36**:201–209 (1984).
36. Kleuss, C., H. Scherubl, J. Hescheler, G. Schultz, and B. Wittig. Different β-subunits determine G-protein interaction with transmembrane receptors. *Nature (Lond.)* **358**:424–426 (1992).

Send reprint requests to: Rita M. Huff, Cell Biology (7239–267–311), The Upjohn Company, Kalamazoo, MI 49001.