Coupling of a Cloned Rat Dopamine-D₂ Receptor to Inhibition of Adenylyl Cyclase and Prolactin Secretion*

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We have previously described a cDNA which encodes a binding site with the pharmacology of the D₂-dopamine receptor (Bunzow, J. R., VanTol, H. H. M., Grandy, D. K., Albert, P., Salun, J., Christie, M., Machida, C., Neve, K. A., and Civelli, O. (1988) Nature 336, 783–787). We demonstrate here that this protein is a functional receptor, i.e. it couples to G-proteins to inhibit cAMP generation and hormone secretion. The cDNA was expressed in GH₄C₁ cells, a rat somatotrophinoma cell strain which lacks dopamine receptors. Stable transfectants were isolated and one clone, GH₄Z₁, which had the highest levels of D₂-dopamine receptor mRNA on Northern blot, was studied in detail. Binding of D₂-dopamine antagonist [³¹P]piperoxane to membranes isolated from GH₄Z₁, cells was saturable, with Kᵣ = 96 pm, and Bₘₐₓ = 2300 fmol/mg protein. Addition of GTP/NaCl increased the IC₅₀ value for dopamine competition for [³¹P]piperoxane binding by 2-fold, indicating that the D₂-dopamine receptor interacts with one or more G-proteins. To assess the function of the dopamine-binding site, acute biological actions of dopamine were characterized in GH₄Z₁, cells. Dopamine, at concentrations found in vivo, decreased resting intra- and extracellular cAMP levels (EC₅₀ = 8 ± 2 nM) by 50–70% and blocked completely vasopressin-induced intestinal peptide (VIP) induced enhancement of cAMP levels (EC₅₀ = 6 ± 1 nM). Antagonism of dopamine-induced inhibition of VIP-enhanced CAMP levels by piperoxane, (+)-butaclamol, (-)-sulpiride, and SCH23390 occurred at concentrations expected from Kᵣ values for these antagonists at the D₂-receptor and was stereoselective. Dopamine (as well as several D₂-selective agonists) inhibited forskolin-stimulated adenylate cyclase activity by 45 ± 0%, with EC₅₀ of 500–800 nM in GH₄Z₁, membranes. Dopaminergic inhibition of cellular cAMP levels and of adenyl cyclase activity in membrane preparations was abolished by pretreatment with pertussis toxin (50 ng/ml, 16 h). Dopamine (200 nM) abolished VIP- and thyrotropin-releasing hormone-induced acute prolactin release. These data show conclusively that the cDNA clone encodes a functional dopamine-D₂ receptor which couples to G-proteins to inhibit adenyl cyclase and both cAMP-dependent and cAMP-independent hormone secretion.

The major element controlling PRL secretion from the pituitary is the concentration of dopamine in the hypophyseal portal bloodstream (1). Dopamine acts via dopamine-D₂ receptors on pituitary lactotrophs to inhibit basal and hormone-stimulated secretion of PRL (1–5). The dopamine-D₂ receptor interacts with pertussis toxin-sensitive, inhibitory G-proteins (6–9) to reduce adenylyl cyclase activity, and to block enhancement of CAMP levels by other agents (6, 10–12). Dopamine also decreases [Ca²⁺] in lactotrophs and partially inhibits elevation of [Ca²⁺] by other agents, such as TRH (13–15). Both dopaminergic inhibition of CAMP and of [Ca²⁺] are mediated through coupling to one or more pertussis toxin-sensitive G-proteins and appear to contribute to dopamine inhibition of PRL secretion (15). The precise relation between these components of dopamine action has been difficult to study (15, 16) due to the presence of heterogeneous cell types, limitations of cell number, and variations in responsiveness of diverse lactotroph preparations.

The recent cloning of the dopamine-D₂ receptor cDNA (17) provides a useful tool to examine the intracellular actions and regulation of the receptor. To examine whether the D₂ receptor directs synthesis of a functional receptor, and to define the pathway between dopamine-D₂ receptor activation and biological effect, we have transfected the dopamine-D₂ receptor cDNA into a pituitary-derived cell strain, GH₄C₁ cells. GH₄C₁ cells are rat pituitary cells which synthesize and secrete PRL and GH, and possess a variety of hormone, growth factor, and neurotransmitter receptors, second messenger systems, and ion channels and have provided an accessible model of lactotroph function (18, 19). However, these cells lack dopamine-D₂ receptors, which are present on normal lactotrophs, and thus provide an ideal host for studying the function of the dopamine-D₂ receptor. This report demonstrates that the gene product of the cDNA clone functions as a dopamine-D₂ receptor and couples to inhibitory G-proteins to decrease CAMP accumulation and PRL release. The GH₄C₁, transfectants characterized herein should provide a useful cell system in which the mechanisms of dopamine action at D₂ receptors may be studied further.

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† The abbreviations used are: PRL, prolactin; GH, growth hormone; [Ca²⁺], cytosolic free calcium concentration; VIP, vasoactive intestinal peptide; TRH, thyrotropin-releasing hormone; BBM, 3-isobutyl-1-methyl xanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

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EXPERIMENTAL PROCEDURES

Materials

Dopamine agonists and antagonists were from Research Biochemicals Incorporated (Waltham, MA), except quinpirole (Lilly), bromocriptine (Sandoz Research Institute), and (+)- and (-)-5-PPP (As- tral). EC50 values (for CA-3) to 2-Cl, 4-OMe-CAMP-binding site in serum albumin was obtained from ICN (Irvine, CA). rPRL standard and anti-rPRL antibody were from Dr. Salvatore Raiti, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD. Peptides were from Peninsula (CA) or Sigma. [3H]Spiperone ([3H]Sp) (29 Ci/mmol), [3H]dihydroergocristine ([3H]DHE, 200 Ci/mmol), [3H]GTP (250 Ci/mmol), [3H]GTP ([3H]GTP (250 Ci/mmol), [3H]ATP ([3H]ATP (10 5 Ci/ mmol), [3H]cAMP (31.9 Ci/mmol) were from Du Pont-New England Nuclear. All other chemicals were reagent grade, obtained primarily from Sigma.

Methods

Construction of pZEM-D2-cDNA—The pZEM-3 plasmid (20) was cut at the BglII site between the metallothionein promoter and hGH g'-linking sequence. Full-length dopamine D2-cDNA (17) was excised from XGT10 with SalI and was ligated to the cut pZEM-3 plasmid in the presence of dATP and dGTP (250 μM) and transformed into Escherichia coli strain XL-1 (Stratagene). Recombinants were characterized by their hybridization to [32P]labeled D2-cDNA, followed by specific radioimmunoassay using Staphylococcus A lysate (IgSorb, Enzfitter program (Elsevier Biosoft). Average affinity, E&0, and I&0 were obtained by nonlinear regression analysis using the method of Cheng and Prusoff

RESULTS

Characterization of Stable Transfectants—GH3 cells were cotransfected with pZEM-D2-cDNA and prSV-neo, and colonies resistant to the antibiotic G418 were isolated and initially characterized by Northern blot analysis. One clone, GH3ZR, had higher levels of 2.5-kilobase D2-mRNA than...
other clones (Fig. 1A). Wild-type (untransfected) GH,C, cells, as well as a GH,C, cell transfectant (GHdZDlo) expressing the rat 5-HT1A receptor gene in the pZEM-3 vector, showed no hybridization to the D2 receptor probe. Pretreatment of GHdZR, cells with 100 μM ZnSO4 for 16 h induced a marked enhancement of D2 receptor mRNA, indicating that the transcribed mRNA is regulated by the zinc-sensitive metallothionein promoter (20). The GHdZR, clone was used for further analysis (below) because of the high levels of dopamine-D2 receptor expression in this clone.

Specific binding of the selective dopamine-D2 receptor antagonist, [3H]spiperone was assayed in crude membranes prepared from GHdZR, cells (Fig. 1B). The GHdZR, membranes showed a saturable component of [3H]spiperone binding which was displaced by 2 μM (+)-butaclamol, whereas membranes from wild-type GH,C, cells showed no specific [3H] spiperone binding (data not shown). In five experiments, the GHdZR, cell membranes showed maximal specific [3H]spiperone binding of 2046 ± 315 fmol/mg of protein, and a mean Ka value of 96 ± 1 μM. These values demonstrate robust expression of dopamine-D2-binding site receptors in these cells with affinity for [3H]spiperone comparable to that obtained in rat striatal membranes and in Ltk- cells transfectcd with pZEM-D2-cDNA (17).

To ascertain whether the expressed dopamine-binding site interacted with a G-protein, inhibition of [3H]spiperone binding by dopamine was assayed in GHdZR, cell membranes, in the absence or presence of 100 μM GTP and 120 mM NaCl (Fig. 1C). Assays were carried out in the presence of 4 mM MgCl2 to promote high affinity binding of dopamine. In the absence of added GTP and NaCl, two classes of binding sites were present (p < 0.001), such that 57 ± 3% of the receptors had a high affinity (Ka = 0.05 μM) for dopamine and the remaining receptors had low affinity (Ka = 5.4 μM) for the agonist. In the presence of 120 mM NaCl, the goodness-of-fit of the inhibition curves was improved by assuming the existence of two classes of binding sites (p < 0.001 in each of three experiments), but the potency of dopamine at each class of sites was decreased. Thus, the mean Ka value for binding to the high affinity component was 0.2 μM, whereas the value for binding to the low affinity component was 19 μM. When assays were carried out using 100 μM GTP but no NaCl, the mean Hill coefficient was increased from 0.67 in the absence of GTP to 0.70 in the presence of GTP. The Ka value for dopamine was 4.4 μM in the presence of GTP, a value close to that for low affinity binding of dopamine in the absence of GTP. In the presence of both GTP and NaCl, competition with dopamine indicated the presence of only the low affinity component of binding (Ka = 7 μM), with Hill coefficient close to unity (0.99). Thus, the presence of GTP/NaCl converts the dopamine receptors from a heterogeneous population of high and low affinity receptors to a nearly homogeneous population.

**Fig. 1. Expression of specific dopamine-D2 receptor mRNA and specific binding in GHdZR, transfectant cells.** A, Northern blot analysis of GH,C, cell total RNA (20 μg/lane). Y axes indicate the migration of RNA molecular mass standards (kb). B, specific binding of [3H]spiperone to membranes prepared from GHdZR, cells was characterized by saturation analysis ("Experimental Procedures"). Data from one of five independent experiments are plotted as specifically bound radioligand (ordinate) versus corrected free radioligand concentration (total added minus total bound). Calculated Ka and Bmax values for this experiment were 60 pM and 1165 fmol/mg protein. Inset, transformation of the data by the method of Scatchard which are plotted as specific bound/free (Y-axis) versus specific bound concentrations of [3H]spiperone (X axis). C, displacement of specific [3H]spiperone binding by dopamine: effect of GTP/NaCl. GHdZR, cell membranes were incubated with [3H]spiperone (0.38 nM) and indicated concentrations of dopamine (X axis) with no additions (●), in the presence of 100 μM GTP (□), or in the presence of 120 mM NaCl and 100 μM GTP (▲). Results are shown for one of three independent experiments. Calculated IC50 and Hill coefficient values for dopamine in the experiment shown were 4 μM and 0.60 in the absence of GTP/NaCl, 20 μM and 0.67 in the presence of GTP, and 50 μM and 1.18 in the presence of GTP/NaCl.
CAMP levels reflect changes in the rate of synthesis of CAMP over 90% in these cells (22). Thus, the observed changes in bition of VIP-enhanced levels of CAMP. GH,C, cells respond to VIP with an enhancement of CAMP accumulation (Fig. 1). GH,ZR, cells, dopamine inhibited both basal CAMP levels (by 50-70%), and abolished enhancement of CAMP levels by VIP. Dopamine was equally effective in lowering intracellular CAMP levels (Fig. 2B), and these actions of dopamine were observed in all experiments. Inhibition of CAMP accumulation in GH,ZR, cells by activation of endogenous somatostatin or muscarinic receptors was 50-75% as effective as dopamine action (data not shown). Dopamine actions on CAMP accumulation were blocked by (-)-sulpiride, a highly selective dopamine-D<sub>2</sub> antagonist, whereas the inactive stereoisomer, (+)-sulpiride, did not block dopaminergic inhibition of CAMP accumulation. Stereoselective blockade by sulpiride suggested that inhibition of CAMP levels in GH,ZR, by dopamine was mediated by activation of a dopamine-D<sub>2</sub> receptor not present in wild-type GH,C, cells.

The physiological outcome of dopamine action is inhibition of secretion, which was assayed by measuring acute (30 min) PRL release in GH,ZR, cells in the absence of IBMX (Fig. 2C). VIP and TRH enhanced PRL secretion 1.5- and 3-fold, respectively. As observed for somatostatin (27), dopamine did not inhibit basal PRL release, but both VIP- and TRH-induced enhancement of PRL secretion were blocked by dopamine. Inhibition of stimulated secretion induced by dopamine in GH,ZR, cells was nearly complete, whereas somatostatin inhibits prolactin release by 50% at most (23, 27). These actions of dopamine were reversed by (-)-sulpiride but not by (+)-sulpiride. In untransfected GH,C, cells, dopamine had no effect on basal, VIP-stimulated, or TRH-stimulated secretion of PRL (data not shown). VIP is thought to enhance PRL release by a CAMP-dependent mechanism (22, 23, 26) while TRH acts by a CAMP-independent mechanism linked to calcium mobilization (19, 28). Thus, dopamine blocked both CAMP-dependent on CAMP-independent enhancement of secretion in GH,ZR, cells.

Dose-response relations were examined for dopamine actions on CAMP levels (Fig. 3). Dopamine inhibited intracellular and extracellular levels of CAMP with similar IC<sub>50</sub> values. Furthermore, dopamine inhibited both basal and VIP-enhanced CAMP accumulation with IC<sub>50</sub> values of 8 ± 2 and 6 ± 1 nM, respectively. The high potency of these inhibitory actions of dopamine supports the assertion that GH,ZR, cells express a functional dopamine-D<sub>2</sub> receptor.

The pharmacological specificity of dopaminergic inhibition of VIP-enhanced levels of CAMP in GH,ZR, was examined further using specific receptor antagonists (Fig. 4). The data show that certain receptor blockers reverse dopamine-induced inhibition of VIP-enhanced levels of extracellular CAMP. Maximal CAMP (100%) corresponded to CAMP levels in the presence of VIP alone. Low concentrations of dopamine-D<sub>2</sub> antagonists (pipероне, (+)-butaclamol, (-)-sulpiride) blocked dopamine action, whereas SCH23390, a specific dopamine-D<sub>2</sub> antagonist, was active only at very high concentrations. Inactive stereoisomers of D<sub>2</sub>-antagonists ((-)-butaclamol, (+)-sulpiride (Fig. 2C)) had little or no effect on dopamine action. Antagonists added in the absence of dopamine did not alter CAMP concentrations. Estimated IC<sub>50</sub> values obtained from IC<sub>50</sub> values for the antagonists (legend, Fig. 4) were similar to values determined from binding competition studies of the dopamine-D<sub>2</sub> receptor (17), showing that inhibition of CAMP levels by dopamine in GH,ZR, cells is me-

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**Fig. 2.** Inhibition of CAMP accumulation and PRL release by dopamine in GH,ZR<sub>7</sub> cells. Incubations were performed in triplicate as described under "Experimental Procedures" and results are expressed as mean ± S.E. of a typical experiment. A, inhibition of extracellular CAMP accumulation by dopamine. Parallel dishes of GH,C, and GH,ZR, cells were incubated with concentrations of VIP, dopamine (D), and (-)-sulpiride (-S) of 250 nM, 10 μM, and 5 μM, respectively. Untreated controls are denoted as C. Media were collected and assayed for CAMP (ordinate) expressed as pmol/dish. B, inhibition of intracellular CAMP accumulation by dopamine in GH,ZR, cells. Cell extracts were assayed for CAMP, expressed on the ordinate. Drug concentrations were as in (A), except (+)-sulpiride (+S), 5 μM. C, inhibition of stimulated PRL release by dopamine in GH,ZR, cells. Media samples were assayed for PRL (ordinate) after the indicated treatments. The concentrations of VIP, TRH, dopamine (D), and (-)-sulpiride (-S) were 200 nM, 200 nM, 100 nM, and 2 μM, respectively.
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FIG. 3. Dose-response relations for dopamine inhibition of basal and VIP-enhanced cAMP accumulation in $G_{H,2R_7}$ cells. Results are mean values of triplicate determinations, with S.E. as indicated in Fig. 2. Basal intracellular (C) and extracellular (O) cAMP accumulation in the presence of indicated concentrations of dopamine. Basal cAMP levels in the absence of dopamine were $22 \pm 6$ pmol/dish (intracellular) and $12.4 \pm 0.6$ pmol/dish (extracellular). EC$_{50}$ values for dopamine actions were $4.9$ nM (intracellular) and $8.5$ nM (extracellular). B, VIP-enhanced intracellular (C) and extracellular (O) cAMP accumulation in the presence of indicated dopamine concentrations. VIP (250 nM)-enhanced levels of intracellular and extracellular cAMP (in the absence of dopamine) were $145 \pm 1.2$ and $146 \pm 2.8$ pmol/dish, and basal cAMP levels were $35 \pm 1.6$ and $15 \pm 0.2$ pmol/dish, respectively. EC$_{50}$ values for dopamine inhibition were $5.5$ (intracellular) and $5.8$ nM (extracellular).

Inhibition of Adenylyl Cyclase—To assess directly inhibition of adenylyl cyclase activity by dopamine receptor agonists, the conversion of $[^{32}P]ATP$ to $[^{32}P]cAMP$ was measured in membranes prepared from $G_{H,2R_7}$ cells (Fig. 5). Dopamine inhibited total forskolin (10 nM)-stimulated activity by 45% with an average EC$_{50}$ value of $0.36$ nM ($n = 8$). As observed in pituitary (11) and striatal (29) membranes, bromocryptine behaved as a partial agonist, maximally inhibiting enzyme activity by 23% (EC$_{50} = 6$ nM). Inhibition of adenylyl cyclase activity by selective $D_2$-agonists was stereoselective. Quinpirole inhibited forskolin-stimulated cyclase activity by 41% (EC$_{50} = 0.32$ nM), whereas LY181990, the inactive (+)-enantiomer of quinpirole, caused no consistent reduction in enzyme activity. Similarly, (+)-3-PPP (EC$_{50} = 0.86$ nM) was as efficacious as dopamine, whereas the enantiomer (-)-3-PPP did not consistently reduce adenylyl cyclase activity. VIP also stimulated adenylyl cyclase activity in $G_{H,2R_7}$ cell membranes, as reported for wild-type $G_{H,C}$ cell membranes (30). Total activity stimulated by 200 nM VIP was $22 \pm 7$ pmol/
Data presented for membrane adenylyl cyclase activity represent means (± S.E.) and % inhibition (% Inh.) below. % Inhibition was calculated from the equation 100 × [1 - (S - B)/ (I - B)], where B, S, and I are values of basal activity, activity in the presence of stimulator (S) or inhibitor (I), respectively and B is basal activity in the presence of inhibitor. Results were obtained from parallel assays in controls and cells pretreated (16 h, 50 ng/ml) with pertussis toxin (indicated as + P.T.). Adenylyl cyclase (A). Membranes for cyclase assay were exposed acutely to 10 μM forskolin (FSK) or 100 μM dopamine (DA), and adenylyl cyclase activity expressed as pmol/mg protein/min. Intracellular cAMP (B). Cells were treated acutely with VIP (200 nM) and dopamine (1 μM) and cAMP accumulation (expressed as pmol/mg) was measured in cell extracts. Extracellular cAMP (C). Media samples from the same dishes of cells were assayed for cAMP accumulation, expressed as pmol/dish.

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Pertussis Toxin Sensitivity—Sensitivity to pertussis toxin is a hallmark of receptors, such as the dopamine-D₂ receptor, which couple to inhibitory G proteins (e.g. G_i or G_o) to induce responses (6-12, 15). Pretreatment of GH₃ZR cells with diadenylyl cyclase inhibition of forskolin-stimulated membrane adenylyl cyclase activity, abolishes inhibition of cAMP accumulation by dopamine. By contrast, basal and VIP-stimulated cAMP accumulation, as well as basal and forskolin-stimulated cyclase activity, were not significantly altered by pertussis toxin pretreatment, as seen previously (27). The concentration of pertussis toxin and incubation time used produced maximal blockade of somatostatin responses in wild-type cells (27), and dopamine responses were almost completely inhibited under these conditions. These data support the assertion that the expressed cDNA clone codes for a dopamine-D₂-binding site which is functionally coupled to inhibitory G proteins present in GH₃C cells, and thus represents a bona fide receptor.

**DISCUSSION**

The cDNA clone coding for a dopamine-D₂-binding site (17) was expressed in GH₃C cells to determine whether the clone expresses a functional D₂ receptor. The presence of dopamine-D₂ binding in the GH₃ZR transfectant correlated with potent and powerful inhibition of cAMP accumulation and PRL release, as well as inhibition of forskolin-stimulated adenylyl cyclase activity, actions of dopamine not observed in untransfected GH₃C cells. These inhibitory actions of dopamine match exactly the known physiological actions of dopamine in pituitary lactotrophs (1, 2). In both normal lactotrophs (1, 3-5) and GH₃ZR cells (Fig. 2), the presence of maximal concentrations of dopamine blocks enhancement of cAMP levels and PRL secretion by secretagogues such as VIP. The potency of dopamine for inhibition of basal and VIP-enhanced cAMP accumulation in GH₃ZR cells (Fig. 3) was in the range of concentration expected for lactotrophs, given that dopamine concentrations in hypophysal portal blood vary from 7 nM in female rats during proestrus, to 20 nM during estrus, and are 3 nM in male rats (31). The similarity between dopamine potency for inhibition of cAMP accumulation and PRL secretion is consistent with a causal relation between the two processes. Detailed analysis of the pharmacology of dopamine-induced inhibition of adenylyl cyclase and cAMP accumulation using specific agonists and antagonists are fully consistent with the conclusion that dopaminergic actions in GH₃ZR cells are mediated by a receptor indistinguishable from the dopamine-D₂ receptor. Thus, the expressed clone meets five basic criteria for classification as a functional dopamine-D₂ receptor: 1) the cDNA clone possesses the archetypical structure of G-protein-coupled receptors (17); 2) the clone directs expression of a protein with saturable and specific dopamine-D₂ binding properties (17); 3) agonist affinity for the expressed binding site is decreased in the presence of GTP and Na⁺ (Fig. 1C), as observed in rat brain (29); 4) the expressed receptor is coupled to functions (e.g. inhibition of adenylyl cyclase) with the pharmacology known for dopamine-D₂ receptors in vivo (Figs. 2 and 3); 5) agents (e.g. pertussis toxin) which uncouple G-protein function uncouple activation of the expressed receptor from generation of the appropriate response (Table I).

**References**

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Coupling of a cloned rat dopamine-D2 receptor to inhibition of adenylyl cyclase and prolactin secretion.

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