Activation of Type II Adenylate Cyclase by D\textsubscript{2} and D\textsubscript{4} but Not D\textsubscript{3} Dopamine Receptors

VAL J. WATTS and KIM A. NEVE

Medical Research Service, Veterans Affairs Medical Center and Department of Behavioral Neuroscience, Oregon Health Sciences University, Portland, Oregon 97201

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SUMMARY

The D\textsubscript{2}-like dopamine receptors couple to a variety of signal transduction pathways, including inhibition of adenylate cyclase, mitogenesis, and activation of potassium channels. Although these effects are mediated via pertussis toxin-sensitive G proteins, G\textsubscript{i/o}, it is likely that some of these effects are influenced by the release of G protein \( \beta\gamma \) subunits. Type II adenylate cyclase (ACII) is highly regulated by multiple biochemical stimuli, including protein kinase C, forskolin, G protein \( \alpha \) subunits, and G protein \( \beta\gamma \) subunits. The ability of \( \beta\gamma \) subunits to activate this enzyme in the presence of activated \( \alpha \) has been particularly well characterized. Although stimulation by \( \beta\gamma \) subunits has been described as conditional on the presence of activated \( \alpha \), with the addition of \( \beta\gamma \) subunits also potentiate ACII activity after activation of protein kinase C. We created stable cell lines expressing ACII and the D\textsubscript{2L} receptor, the D\textsubscript{3} receptor, or the D\textsubscript{4.4} receptor. Activation of D\textsubscript{2L} or D\textsubscript{4.4} receptors, but not D\textsubscript{3} receptors, potentiated \( \beta\)-adrenergic receptor/G\textsubscript{s}-stimulated activity of ACII, as measured by the intracellular accumulation of cAMP. Similarly, stimulation of D\textsubscript{2L} or D\textsubscript{4.4} receptors potentiated phorbol-12-myristate-13-acetate-stimulated ACII activity in the absence of activated \( \alpha \), whereas stimulation of D\textsubscript{3} receptors did not. The effect of D\textsubscript{2-like} receptor stimulation was blocked by pretreatment with Pertussis toxin and by inhibition of protein kinase C. We propose that activation of both D\textsubscript{2} and D\textsubscript{4.4} dopamine receptors potentiated phorbol-12-myristate-13-acetate-stimulated ACII activity through the release of \( \beta\gamma \) subunits from pertussis toxin-sensitive G proteins. In contrast, the lack of D\textsubscript{3} receptor-mediated effects suggests that stimulation of D\textsubscript{3} receptors does not result in an appreciable release of \( \beta\gamma \) subunits.

The D\textsubscript{2}-like receptor family is composed of D\textsubscript{2}, D\textsubscript{3}, and D\textsubscript{4} dopamine receptors, which share considerable amino acid homology and generally have high affinity for butyrophenone and benzamide ligands. The most striking differences observed among these receptors are found in their ability to activate pertussis toxin-sensitive signaling events. For example, D\textsubscript{2} and D\textsubscript{3} receptors inhibit dopamine synthesis in a dopamine-producing cell line, whereas D\textsubscript{4} receptors do not (1, 2), and D\textsubscript{2} and D\textsubscript{4} receptors mediate robust inhibition of cAMP accumulation in a variety of cell lines, whereas inhibition of cAMP accumulation by the D\textsubscript{3} receptor is modest or absent (2–5).\textsuperscript{1} On the other hand, D\textsubscript{2}, D\textsubscript{3}, and D\textsubscript{4} receptors all activate K\textsuperscript{+} channels in Xenopus laevis oocytes and stimulate mitogenesis in Chinese hamster ovary cells in a pertussis toxin-sensitive manner (3, 6). Thus, all D\textsubscript{2-like} receptors couple to pertussis toxin-sensitive pathways, but the efficiency and specificity of coupling are not identical for all subtypes.

ACII is widely expressed in the central nervous system, and its activity is regulated by a variety of biochemical signals (7–9). Although ACII is not inhibited by G\textsubscript{s} (10), it is stimulated by \( \alpha_{s} \) (11), phorbol esters (12), and G protein \( \beta\gamma \) subunits (13) in reconstituted systems. Additionally, ACII is synergistically activated by \( \alpha_{s} \) and PMA (8, 12) as well as by \( \alpha_{s} \) and \( \beta\gamma \) subunits (11, 13, 14). In intact cells, ACII is activated by \( \beta\gamma \) subunits in combination with activated \( \alpha_{s} \),

\textsuperscript{1} V. J. Watts and K. A. Neve, unpublished observations.
whereas βγ stimulation alone has no detectable effect on ACII activity (7, 9, 15–17). In those studies βγ subunits were supplied by stimulating Gαs-coupled receptors (e.g., the D2 dopamine receptor), and activated αs was provided by stimulating Gs-coupled receptors or by co-transfection with a constitutively active mutant of αs, αQ227L. The synergy between activated αs and either PMA or G protein βγ subunits has led to ACII being described as a coincidence detector that integrates multiple signals (8, 18). Further, liberation of βγ subunits via activation of Gαs-coupled receptors enhances ACII stimulation by Gs-coupled receptors or phorbol esters (19).

The divergent signaling pathways of the D2-like dopamine receptors and the unique regulatory properties of ACII provide the basis for the current study. We examined and compared the ability of D2L, D3, and D4 dopamine receptors to potentiate (presumably via βγ subunits) isoproterenol- and PMA-induced activation of ACII. To this end, we created cells stably expressing ACII and the D2L dopamine receptor (ACII/D2L), ACII and the D3 dopamine receptor (ACII/D3), or ACII and the D4.4 dopamine receptor (ACII/D4). We now report that D3 agonists potentiated isoproterenol-stimulated cAMP accumulation in HEK293 cells expressing the D2L or the D4.4 dopamine receptor together with ACII, whereas the D2L receptor did not. Consistent with the hypothesis that βγ subunits enhance the responsiveness of ACII to a variety of stimuli, we also found that activation of D3L and D4.4 receptors potentiated protein kinase C-activated ACII activity.

**Experimental Procedures**

**Materials.** [3H]cAMP was purchased from Dupont NEN. Spiperone, quinpirole, and forskolin were purchased from Research Biochemicals International. HEK 293 cells expressing ACII (HEK-ACII) were obtained from Dr. Daniel Storm and Mark Nielsen (University of Washington, Seattle). Rat D2L (Dr. Olivier Civelli, University of California at Irvine) and human D4.4 cDNAs (Dr. Hubert Van Tol, University of Toronto, and Dr. David Grandy, Oregon Health Sciences University, Portland, OR) were generous gifts. Dopamine (3-hydroxytyramine) and most other reagents were purchased from Sigma Chemical (St. Louis, MO).

**Production of cell lines.** Creation of HEK-D2L, HEK-D3, and HEK-D4.4 cells was carried out by electroporation (0.17 kV, 950 μF, 0.4 cm cuvette gap). HEK 293 cells (8 × 10⁶) were resuspended in DMEM supplemented with 10% FBS and 5 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid in a total volume of 400 μl, including pcDNA1-D2L cDNA (15 μg), pcDNA1-D3 cDNA (15 μg), or pcDNA1-D4.4 cDNA (15 μg) with pBabe Puro (2 μg), to confer resistance to puromycin (20). Transfectants were isolated and screened by resistance to puromycin (20). Transfectants were isolated and screened by resistance to puromycin (20). Transfectants were isolated and screened by resistance to puromycin (20). Transfectants were isolated and screened by resistance to puromycin (20). Transfectants were isolated and screened by resistance to puromycin (20). Transfectants were isolated and screened by resistance to puromycin (20).

**Cell culture.** HEK 293 cells expressing D2L-like receptors were maintained in DMEM supplemented with 5% FBS and 5% CBS, penicillin/streptomycin, and puromycin (2 μg/ml). ACII/D2L, ACII/D3, and ACII/D4 cells were maintained in DMEM supplemented with 5% FBS and 5% CBS, penicillin/streptomycin, puromycin (2 μg/ml), and hygromycin (460 units/ml). HEK-ACII cells were maintained in DMEM supplemented with 5% FBS and 5% CBS, penicillin/streptomycin, and hygromycin (460 units/ml). Cells were grown in a humidified incubator at 37° in the presence of 10% CO2.

**cAMP accumulation assays.** Cells were plated at densities between 100,000 and 150,000 cells/well in 48-well tissue culture clusters. Confluent cells were preincubated with 200 μl of assay buffer (Earle’s balanced salt solution, containing 0.02% ascorbic acid and 2% CBS) for 10 min, then placed on ice. All drugs were added at 4°, then each cluster was transferred to a 37° water bath. After 15 min, the medium was decanted, and the cells were placed on ice and lysed with 3% trichloroacetic acid. The 48-well plates were then stored at 4° for at least 1 hr and centrifuged at 1000 × g for 15 min before quantification of cAMP. For pertussis toxin experiments, the toxin was added to the growth medium (25 ng/ml) 18 hr before the cAMP accumulation assay. This treatment has been determined to eliminate detectable coupling of D3 dopamine receptors to inhibition of cAMP accumulation (22).

**Quantification of cAMP.** cAMP was quantified using a competitive binding assay adapted with minor modifications from Nordstedt and Fredholm (23). Duplicate samples of the cell lysate (10–20 μl) were added to reaction tubes containing cAMP assay buffer (100 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA). [3H]cAMP (1 nM final concentration) was added to each tube, followed by cAMP-binding protein (~100 μg of crude extract from bovine adrenal cortex in 200 μl of cAMP buffer). The reaction tubes were incubated on ice for 3 hr. The tubes were then harvested by filtration (Whatman GF/C filters) using a 96-well Tomtec cell harvester. Filters were allowed to dry, and BetaPlate scintillation fluid (50 μl) was added to each sample. Radioactivity on the filters was determined using an Wallac Beta-Plate scintillation counter. The concentration of cAMP in each sample was estimated in duplicate assays from a standard curve ranging from 0.1 to 100 pmol cAMP/assay.

**Data analysis.** Dose-response curves for cAMP were analyzed by nonlinear regression using the program Prism 2.0 (GraphPad Software, San Diego, CA). Statistical comparisons were made using ANOVA followed by Dunnett’s post hoc t test comparing control with drug groups, except where indicated in the figure legends.

**Results and Discussion**

We examined cAMP accumulation in HEK 293 cells expressing only the D2L receptor. HEK-D2L cells were treated with isoproterenol (100 nM) or PMA (100 nM) in the absence or presence of dopamine (1 μM) or quinpirole (1 μM), and cAMP accumulation was determined. There was no significant stimulation of cAMP accumulation above basal levels by isoproterenol (100 nM) or PMA (100 nM), reflecting the low level of adenylate cyclase activity that has made the HEK 293 cell line valuable for the characterization of recombinant adenylate cyclases (24) (data not shown). We also examined cAMP accumulation in HEK 293 cells expressing ACII (HEK-ACII). We found that isoproterenol acting via endogenously expressed β-adrenergic receptors stimulated cAMP accumulation 3-fold above basal levels in HEK-ACII cells (data not shown). PMA, which is thought to bypass Gs and activate ACII by protein kinase C-dependent phosphorylation of the enzyme (12), stimulated cAMP accumulation 40-fold above basal levels in HEK-ACII cells (data not shown). The D2 agonists, quinpirole or dopamine, had no effect on isoproterenol- or PMA-stimulated cAMP accumulation in HEK-ACII cells (data not shown). However, when cAMP accumulation is stimulated by forskolin in HEK-D2L cells, activation of D2L receptors inhibits cAMP accumulation (22).

**Potentiation of αs-stimulated ACII by D2L, D3, and D4.4 Receptors.** To study the effects of βγ subunits on αs-stimulated ACII activity we created cells stably expressing ACII and the Gαs-coupled dopamine receptors, D2L (ACII/D2L cells), D3 (ACII/D3), or D4.4 (ACII/D4 cells). In ACII/D2L cells, D2L agonists alone did not alter cAMP accumulation (data not shown). In contrast, when dopamine (1 μM) or quinpirole (1 μM) was added in combination with an activator...
of $\alpha_\gamma$ (100 nm isoproterenol), there was marked potentiation of isoproterenol-stimulated cAMP accumulation (Fig. 1 and Table 1). This potentiation seemed to be mediated via $D_{2L}$ receptors acting through a $G_{i/o}$ protein because it was blocked by the $D_2$ antagonist, spiradoline, and by pretreatment of the cells with pertussis toxin (25 ng/ml for 18 hr; Table 1). The effect of dopamine on isoproterenol-stimulated cAMP accumulation was dose-dependent, with an EC$_{50}$ value of 32 nM (Fig. 1). Like the $D_{2L}$ receptor, the $D_{4.4}$ receptor inhibits the activity of endogenous adenylate cyclases in a variety of cell lines (e.g., see Ref. 5); recent work, however, has suggested that the $D_2$ and $D_4$ receptors may act through different pertussis toxin-sensitive $G$ proteins (1). In light of this, we examined whether the $D_{4.4}$ receptor could stimulate ACII activity. As we observed with the $D_{2L}$ receptor, activation of the $D_{4.4}$ receptor potentiated isoproterenol activation of ACII in ACII/D4 cells (Fig. 1), and the effects were blocked by spiradoline or by pretreatment with pertussis toxin (Table 1). Stauroporine did not prevent the $D_2$-like receptor potentiation of isoproterenol-stimulated activity, indicating that the potentiation is not mediated by dopamine receptor activation of protein kinase C (Table 1). In contrast to the effects of $D_{2L}$ and $D_{4.4}$ receptors, activation of $D_3$ receptors was without effect on cAMP accumulation (Fig. 1). The lack of a $D_3$-mediated effect does not seem to be due to low receptor density. Although the ACII/D2L and ACII/D4 cells had receptor densities of 600 and 1500 fmol/mg of membrane protein, respectively, we tested nine ACII/D3 clones ranging in receptor density from 200-1300 fmol/mg of protein, and none produced significant potentiation of isoproterenol-stimulated cAMP accumulation in the presence of dopamine agonists (data not shown). The data presented in Figs. 1 and 3 are from clone ACII/D3-17, which expressed the $D_3$ receptor at a density of approximately 1100 fmol/mg of membrane protein. Additionally, increasing the concentration of dopamine agonists to 10 $\mu$M failed to result in significant potentiation of isoproterenol-stimulated ACII activity in ACII/D3 cells (data not shown).

The results of the studies with ACII/D2L and ACII/D4 cells are consistent with studies demonstrating that stimulation of $G_{i/o}$-coupled receptors, in combination with activation of $\alpha_\gamma$ (via $G_{s}$-coupled receptors or co-transfection with a constitutively active $\alpha_\gamma$, $\alpha_\gamma$-Q227L), stimulates ACII (7, 9, 15, 16). The activation of $G_{i/o}$-coupled receptors releases $\beta\gamma$ subunits, which in turn potentiate the activation of ACII by $\alpha_\gamma$. The role of $\beta\gamma$ subunits is supported by the observation that co-expression of $\alpha_\gamma$, which presumably sequesters released $\beta\gamma$ subunits, blocks activation of ACII by $G_{i/o}$-coupled receptors (7, 9, 17, 19). Moreover, reconstitution studies indicate that ACII is directly stimulated by $\beta\gamma$ subunits in combination with activated $\alpha_\gamma$ (11, 13) and also by $\beta\gamma$ subunits alone, albeit to a lesser extent (13). The $\alpha$ subunits of $G_{i/o}$ do not directly modulate ACII, because transfection of constitutively active mutants of these $\alpha$ subunits has little effect on basal or receptor-stimulated activity of ACII in HEK 293 cells (9).

$D_2$ dopaminergic and $\alpha_\gamma$-adrenergic receptors are among the $G_{i/o}$-coupled receptors that activate ACII, but they differ in their mode of activation (7). Unlike the $D_2$ receptor, the $\alpha_\gamma$-adrenergic receptor stimulates ACII in the absence of constitutively active $\alpha_\gamma$-Q227L. It has been suggested that the ability of the $\alpha_\gamma$-adrenergic receptor to couple to $G_\alpha$ is responsible for this difference between the two receptors (7). Consistent with this suggestion, the activation of ACII by $D_2$ receptors was abolished by pretreatment with pertussis toxin, whereas the activation by $\alpha_\gamma$-adrenergic receptors was not (7).

### Table 1

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**Footnotes:**
- *p < 0.01 compared to control cells (Dunnett’s postrepeated measures ANOVA).
- †p < 0.01 compared to vehicle pretreatment (paired Student’s t test).

**Fig. 1.** Potentiation of isoproterenol-stimulated cAMP accumulation in ACII/D2L, ACII/D3, and ACII/D4 cells. cAMP accumulation was stimulated with 100 nM isoproterenol in the absence and presence of dopamine (DA, 1 $\mu$M) or quinpirole (Quin, 1 $\mu$M) for 15 min. Data shown are the mean ± standard error for three or more independent experiments, each conducted with duplicate determinations. *$p < 0.01$ compared with control cells (Dunnett’s postrepeated measures ANOVA).
Potentiation of PMA-stimulated ACII by D_{2L}, D_{3}, and D_{4.4} receptors. Results from reconstitution studies have demonstrated that protein kinase C phosphorylates and activates ACII independently of α_{2a} (12). Furthermore, short term PMA treatment of intact cells does not activate α_{2a} as assessed by reconstituted adenylate cyclase activity in the membranes of S49 cyc^−^ cells (25). To examine the hypothesis that βγ subunits released by the activation of G_{αγ}-coupled receptors can potentiate the actions of protein kinase C on ACII, we assessed the ability of dopamine agonists to enhance PMA-stimulated cAMP accumulation in ACII/D2L, ACII/D3, and ACII/D4 cells. When stimulation by PMA (100 nm) was conducted in the presence of dopamine (1 μm) or quinpirole (1 μm), cAMP accumulation was 2–4-fold greater, compared with PMA alone, indicating that D_{2} agonists potentiated the actions of PMA in ACII/D2L cells (Fig. 3 and Table 2). The effects of D_{2} agonists on PMA-stimulated cAMP accumulation were blocked by co-incubation with spiperone (1 μm) and by overnight treatment with pertussis toxin (Table 2). Analysis of dose response curves revealed that the potentiation by dopamine was dose-dependent, with an EC_{50} value of 132 nm (Fig. 2). Similarly, activation of D_{4.4} receptors potentiated PMA-stimulated cAMP accumulation in ACII/D4 cells, and this effect was blocked by spiperone and by pretreatment with pertussis toxin (Fig. 3 and Table 2). The effect of D_{4.4} receptor activation on PMA-stimulated cAMP accumulation was also blocked by the D_{3} antagonist clozapine (data not shown). We also observed that pertussis toxin treatment significantly reduced PMA-stimulated cAMP accumulation in ACII/D2L and ACII/D4 cells. There was also a trend for spiperone to decrease PMA-stimulated activity in both cell lines (Table 2), and to decrease isoproterenol-stimulated activity in ACII/D4 cells (Table 1). These results may reflect constitutive activity of the D_{2L}-like receptors and further suggest that spiperone is an inverse agonist at these receptors.

In contrast to the ability of D_{2} and D_{4} receptors to potentiate cAMP accumulation, D_{3} receptor activation did not augment PMA-stimulated cAMP accumulation in ACII/D3 cells (Fig. 3). One difference between the current study and that of Tsu and Wong (19) is that in the latter study the cells were pretreated with PMA before addition of dopamine agonists. In an effort to demonstrate modulation of ACII by D_{3} receptors, we also completed studies in which ACII/D3 cells were pretreated with PMA for 10 min before ACII stimulation in the presence of dopamine agonists. These studies demonstrated that pretreatment with PMA significantly enhanced basal and stimulated (isoproterenol and PMA) ACII activity, but there was no potentiation of cAMP accumulation by dopamine agonists in ACII/D3 cells (data not shown).

The finding that D_{3} receptor activation potentiates cAMP accumulation stimulated by either α_{2a} (i.e., isoproterenol) or protein kinase C (PMA) in a pertussis toxin-sensitive manner strongly suggests that activated α_{2a} is not an absolute requirement for stimulation of ACII by βγ subunits, but that the binding of βγ subunits to ACII enhances the effects of other activators. In studies with ACII/D2L and ACII/D4 cells, the protein kinase C inhibitor, staurosporine, abolished both PMA-stimulated ACII activity and the potentiation of that pathway by dopamine agonists (Table 2), further indicating that the activation of ACII by βγ subunits requires co-activation of the protein kinase C pathway. PMA activation of ACII was enhanced by βγ subunits in both ACII/D2L and ACII/D4 cells, but not in ACII/D3 cells.

The current study adds another divergent signaling pathway to the D_{2L}-like dopamine receptor family. The results of these studies are similar to those examining D_{2}, D_{3}, and D_{4}-mediated inhibition of cAMP accumulation in which D_{2} and D_{4} receptors display robust inhibition and D_{3} receptors show little or no effect (2–4). Thus, it seems that the inefficient coupling of D_{3} receptors to G_{αγ} proteins provides a
concentration of βγ subunits that is not sufficient to potentiate αs or PMA-stimulated ACII activity. Although D3 receptors couple to several pertussis toxin-sensitive signaling events including K+ channel conductance, mitogenesis, neurite outgrowth, dopamine synthesis, and dopamine release, in many instances the functional response to D3 receptor activation is reduced compared with the response that is mediated by D2 and D1 receptors (1, 2, 6, 26, 27). Specifically, the muscarinic receptor-gated atrial potassium channel, Girk1, is activated by D2, D3, and D4 dopamine receptors, but the maximal current is 3-fold larger for D2 and D4 receptors than for D3 receptors (6). Because Girk1 is also activated by βγ subunits (28), the results of the present study support the hypothesis that smaller current induced by D3 receptor activation could be due to diminished release of βγ subunits. The reasons for the functional differences among D2, D3, and D4 receptors are largely unknown, but most likely reflect differences among the receptor subtypes in the efficiency of activation of various G proteins. The observation that D2L receptors potentiate the actions of PMA on ACII is important considering the evidence that has linked D2 dopamine receptors and the protein kinase C pathway. For example, D2 dopamine receptors have been shown to stimulate phosphoinositide hydrolysis in Ltk− fibroblasts expressing D2 dopamine receptors (29). Thus, in Ltk− fibroblasts expressing ACII and D2 receptors, it is possible that D2 agonists could stimulate cAMP accumulation due to increased protein kinase C activity in combination with the release of βγ subunits from Giα. The protein kinase C pathway has also been implicated in D2 receptor-potentiated arachidonic acid release in Chinese hamster ovary cells and in inhibition of cell proliferation in GH3Z4R7 cells, because inhibitors of protein kinase C block both of these D2 receptor effects (30, 31). Taken together, these observations and the current study suggest that the interactions between the protein kinase C pathway and D2 dopamine receptors are important in modulating neurotransmission in a variety of cell types.

In summary, we have demonstrated that activation of D2L and D4 receptors potentiated isoproterenol- and PMA-stimulated cAMP synthesis by ACII, whereas D3 receptors did not. Furthermore, our data confirm that ACII can be synergistically activated by multiple signals, including PMA and βγ subunits, αs and βγ subunits, or PMA and αs. However, the activation of ACII by βγ subunits is conditional, requiring co-activation by either protein kinase C or αs, suggesting that the binding of βγ to ACII results in an enhancement of responsiveness of the enzyme to other activators. Potentiation of PMA-stimulated ACII activity by βγ subunits represents another example of coincident signal detection and may influence the interactions between D2 and D4 dopamine receptors and the protein kinase C pathway.

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References