0026-895X/97/020181-06$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, **52:**181–186 (1997).

ACCELERATED COMMUNICATION

Activation of Type II Adenylate Cyclase by D_2 and D_4 but Not D_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

Received March 3, 1997; Accepted April 17, 1997

SUMMARY

The D_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, $G_{i/o}$, 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 α subunits, and G protein $\beta\gamma$ subunits. The ability of $\beta\gamma$ subunits to activate this enzyme in the presence of activated α_s has been particularly well characterized. Although stimulation by $\beta\gamma$ subunits has been described as conditional on the presence of activated α_s, $\beta\gamma$ subunits also potentiate ACII activity after activation of protein kinase C. We created stable cell lines expressing ACII and the D_{2L} receptor, the D_3 receptor, or the $D_{4.4}$ receptor. Activation of D_{2L} or $D_{4.4}$ receptors, but not D_3 receptors, potentiated β-adrenergic receptor/G_s-stimulated activity of ACII, as measured by the intracellular accumulation of cAMP. Similarly, stimulation of D_{2L} or $D_{4.4}$ receptors potentiated phorbol ester-stimulated ACII activity in the absence of activated α_s, whereas stimulation of D_3 receptors did not. The effect of D_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_{2L} and $D_{4.4}$ 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_3 receptor-mediated effects suggests that stimulation of D_3 receptors does not result in an appreciable release of $\beta\gamma$ subunits.

The D_2-like receptor family is composed of D_2, D_3,and D_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_2 and D_3 receptors inhibit dopamine synthesis in a dopamine-producing cell line, whereas D_4 receptors do not (1, 2), and D_2 and D_4 receptors mediate robust inhibition of cAMP accumulation in a variety of cell lines, whereas inhibition of cAMP accumulation by the D_3 receptor is modest or absent (2–5).[1] On the other hand, D_2, D_3, and D_4 receptors all activate K^+ channels in *Xenopus laevis* oocytes and stimulate mitogenesis in Chinese hamster ovary cells in a pertussis toxin-sensitive manner (3, 6). Thus, all D_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 $G\alpha_i$ (10), it is stimulated by α_s (11), phorbol esters (12), and G protein $\beta\gamma$ subunits (13) in reconstituted systems. Additionally, ACII is synergistically activated by α_s and PMA (8, 12) as well as by α_s and $\beta\gamma$ subunits (11, 13, 14). In intact cells, ACII is activated by $\beta\gamma$ subunits in combination with activated α_s,

This work was supported by the Johnson and Johnson Focused Giving Program, the Veterans Affairs Merit Review and Research Career Scientist Programs, a Young Investigator Award from the National Alliance for Research on Schizophrenia and Depression, and T32 DA07262.

[1] V. J. Watts and K. A. Neve, unpublished observations.

ABBREVIATIONS: ACII, type II adenylate cyclase; CBS, calf bovine serum; D_{2L}, long (444-amino acid) form of D_2 receptors; $D_{4.4}$, a variant of the D_4 dopamine receptor with four copies of a direct imperfect repeat in the third cytoplasmic loop; DMEM, Dulbecco's modified Eagle's media; FBS, fetal bovine serum; HEK, human embryonic kidney; PMA, phorbol-12-myristate-13-acetate; ANOVA, analysis of variance.

whereas $\beta\gamma$ stimulation alone has no detectable effect on ACII activity (7, 9, 15–17). In those studies $\beta\gamma$ subunits were supplied by stimulating $G_{i/o}$-coupled receptors (e.g., the D_2 dopamine receptor), and activated α_s was provided by stimulating G_s-coupled receptors or by co-transfection with a constitutively active mutant of α_s, α_s-Q227L. The synergy between activated α_s and either PMA or G protein $\beta\gamma$ subunits has led to ACII being described as a coincidence detector that integrates multiple signals (8, 18). Further, liberation of $\beta\gamma$ subunits *via* activation of $G_{i/o}$-coupled receptors enhances ACII stimulation by G_q-coupled receptors or phorbol esters (19).

The divergent signaling pathways of the D_2-like dopamine receptors and the unique regulatory properties of ACII provide the basis for the current study. We examined and compared the ability of D_2, D_3, and D_4 dopamine receptors to potentiate (presumably *via* $\beta\gamma$ subunits) isoproterenol- and PMA-induced activation of ACII. To this end, we created cells stably expressing ACII and the D_{2L} dopamine receptor (ACII/D2L), ACII and the D_3 dopamine receptor (ACII/D3), or ACII and the $D_{4.4}$ dopamine receptor (ACII/D4). We now report that D_2 agonists potentiated isoproterenol-stimulated cAMP accumulation in HEK293 cells expressing the D_{2L} or the $D_{4.4}$ dopamine receptor together with ACII, whereas the D_3 receptor did not. Consistent with the hypothesis that $\beta\gamma$ subunits enhance the responsiveness of ACII to a variety of stimuli, we also found that activation of D_{2L} and $D_{4.4}$ receptors potentiated protein kinase C-activated ACII activity.

Experimental Procedures

Materials. [^{3}H]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 D_{2L} (Dr. Olivier Civelli, University of California at Irvine) and human $D_{4.4}$ 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-D_{2L}, HEK-D_3, and HEK-$D_{4.4}$ cells was carried out by electroporation (0.17 kV, 950 μF, 0.4-cm cuvette gap). HEK 293 cells (8×10^6) 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-D_{2L} cDNA (15 μg), pcDNA1-D_3 cDNA (15 μg), or pcDNA1-$D_{4.4}$ cDNA (15 μg) with pBabe Puro (2 μg), to confer resistance to puromycin (20). Transfectants were isolated and screened by radioligand binding as described previously (21). Creation of ACII/D2L, ACII/D3, and ACII/D4 cells, was carried out by transfection of HEK-ACII cells with pcDNA1-D_{2L}, pcDNA1-D_3, or pcDNA1-$D_{4.4}$ as described above.

Cell culture. HEK 293 cells expressing D_2-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% CO_2.

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 $1000 \times g$ 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 D_2 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). [^{3}H]cAMP (1 nM final concentration) was added to each tube, followed by cAMP-binding protein ($\sim$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 BetaPlate 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 D_{2L} receptor. HEK-D_{2L} 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 G_s 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 D_2 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-D_{2L} cells, activation of D2L receptors inhibits cAMP accumulation (22).

Potentiation of α_s-stimulated ACII by D_{2L}, D_3, and $D_{4.4}$ receptors. To study the effects of $\beta\gamma$ subunits on α_s-stimulated ACII activity we created cells stably expressing ACII and the $G_{i/o}$-coupled dopamine receptors, D_{2L} (ACII/D2L cells), D_3 (ACII/D3), or $D_{4.4}$ (ACII/D4 cells). In ACII/D2L cells, D_2 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

TABLE 1

Blockade of dopamine agonist-mediated potentiation of isoproterenol-stimulated cyclic AMP accumulation

cAMP accumulation was stimulated with 100 nM isoproterenol in the absence (control) and presence of dopamine (1 μM) or quinpirole (1 μM) for 15 min. As indicated, some experiments were carried out in the presence of spiperone (1 μM), staurosporine (1 μM), or following treatment with pertussis toxin (25 ng/ml, 18 hr). Data shown are the mean ± standard error for three or more independent experiments, each conducted with duplicate determinations. *$p < 0.01$ compared to control cells (Dunnett's postrepeated measures ANOVA). **$p < 0.01$ compared to vehicle pretreatment (paired Student's t test).

Cell type	Drug	Condition/pretreatment			
		Vehicle	+ Spiperone (1 μM)	+ Staurosporine (1 μM)	+ Pertussis toxin (25 ng/ml)
		pmol/well			
ACII/D2L	Control	9.92 ± 1.8	6.7 ± 1.3	7.9 ± 0.8	9.6 ± 2.0
	Dopamine	22.0 ± 3.3*	5.7 ± 1.3	19.6 ± 1.7*	5.8 ± 1.3
	Quinpirole	24.4 ± 4.9*	8.0 ± 1.9	18.4 ± 2.1*	8.5 ± 1.7
ACII/D4	Control	15.2 ± 2.3	6.7 ± 0.6	31.6 ± 3.3**	9.8 ± 0.8
	Dopamine	37.1 ± 5.1*	8.4 ± 2.6	62.3 ± 10.5*	12.9 ± 1.4
	Quinpirole	36.0 ± 5.8*	8.2 ± 1.9	68.4 ± 13.2*	10.6 ± 2.1

of α_s (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, spiperone, 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. 2). 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 spiperone or by pretreatment with pertussis toxin (Table 1). Staurosporine 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 μM failed to result in significant potentiation of isoproterenol-stimulated ACII activity in ACII/D3 cells (data not shown).

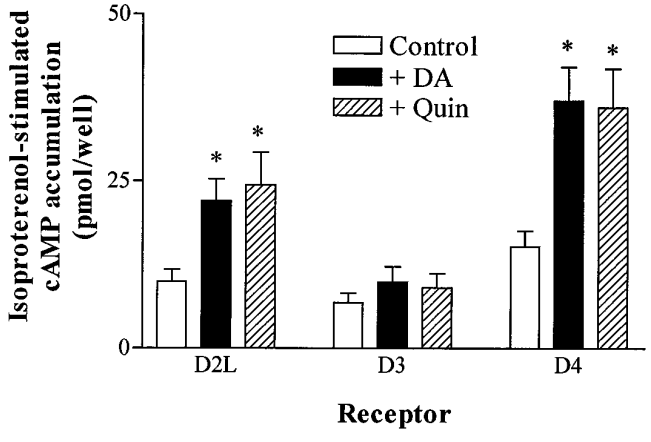

Fig. 1. Potentiation of isoproterenol-stimulated cAMP accumulation in ACII/D2L, ACII/D3, and ACII/D4 cells. cAMP accumulation was stimulated with 100 nM isoproterenol in the absence and presence of dopamine (*DA*, 1 μM) or quinpirole (*Quin*, 1 μM) for 15 min. Data shown are the mean ± standard error for three or more independent experiments, each conducted with duplicate determinations. *, $p < 0.01$ compared with control cells (Dunnett's postrepeated measures ANOVA).

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 α_s (*via* G_s-coupled receptors or co-transfection with a constitutively active α_s, α_s-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 α_s. The role of $\beta\gamma$ subunits is supported by the observation that co-expression of α_t, 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 α_s (11, 13) and also by $\beta\gamma$ subunits alone, albeit to a lesser extent (13). The α subunits of $G_{i/o}$ do not directly modulate ACII, because transfection of constitutively active mutants of these α subunits has little effect on basal or receptor-stimulated activity of ACII in HEK 293 cells (9).

D_2 dopaminergic and α_2-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 α_2-adrenergic receptor stimulates ACII in the absence of constitutively active α_s-Q227L. It has been suggested that the ability of the α_2-adrenergic receptor to couple to G_s 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 α_2-adrenergic receptors was not (7). In the present study, we have confirmed the finding that potentiation of G_s-stimulated cAMP accumulation by D_{2L} receptors is blocked by pretreatment with pertussis toxin and have extended this observation to the $D_{4.4}$ receptor.

TABLE 2

Blockade of dopamine agonist-mediated potentiation of PMA-stimulated cyclic AMP accumulation

cAMP accumulation was stimulated with 100 nM PMA in the absence (control) and presence of dopamine (1 μM) or quinpirole (1 μM) for 15 min. As indicated, some experiments were carried out in the presence of spiperone (1 μM), staurosporine (1 μM), or after treatment with pertussis toxin (25 ng/ml, 18 hr). Data shown are the mean ± standard error for three or more independent experiments, each conducted with duplicate determinations. *$p < 0.01$ compared to control cells (Dunnett's postrepeated measures ANOVA). **$p < 0.05$ compared to vehicle pretreatment (Student's t test).

Cell type	Drug	Condition/pretreatment			
		Vehicle	+ Spiperone (1 μM)	+ Staurosporine (1 μM)	+ Pertussis toxin (25 ng/ml)
		pmol/well			
ACII/D2L	Control	36.1 ± 4.8	26.6 ± 8.2	6.0 ± 1.1**	11.1 ± 5.2**
	Dopamine	160 ± 25*	15.3 ± 4.5	3.9 ± 0.8	6.8 ± 2.4
	Quinpirole	94.2 ± 13*	19.7 ± 8.5	3.6 ± 0.6	7.3 ± 1.9
ACII/D4	Control	71.3 ± 11	36.6 ± 7.1	4.9 ± 1.4**	24.0 ± 6.7**
	Dopamine	157 ± 17*	32.7 ± 2.1	5.1 ± 1.5	30.6 ± 5.1
	Quinpirole	139 ± 19*	47.5 ± 8.26	5.9 ± 1.6	22.5 ± 5.9

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_s (12). Furthermore, short term PMA treatment of intact cells does not activate α_s, as assessed by reconstituted adenylate cyclase activity in the membranes of S49 cyc$^-$ cells (25). To examine the hypothesis that $\beta\gamma$ subunits released by the activation of $G_{i/o}$-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_4 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_2-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_{2L} receptor activation potentiates cAMP accumulation stimulated by either α_s (i.e., isoproterenol) or protein kinase C (PMA) in a pertussis toxin-sensitive manner strongly suggests that activated α_s is not an absolute requirement for stimulation of ACII by $\beta\gamma$ subunits, but that the binding of $\beta\gamma$ 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 $\beta\gamma$ subunits requires co-activation of the protein kinase C pathway. PMA activation of ACII was enhanced by $\beta\gamma$ 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_2-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 $G_{i/o}$ proteins provides a

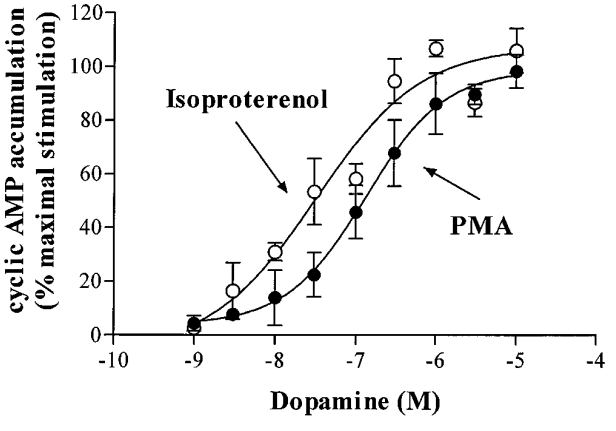

Fig. 2. Dopamine-stimulated cAMP accumulation in presence of isoproterenol or PMA in ACII/D2L cells. Data are expressed as the percentage of maximal stimulation and represent the average ± standard error of five (+ PMA) or six (+ isoproterenol) independent experiments conducted in duplicate. The resulting EC_{50} for dopamine + PMA (●) was 132 nM and for dopamine + isoproterenol (○) was 32 nM.

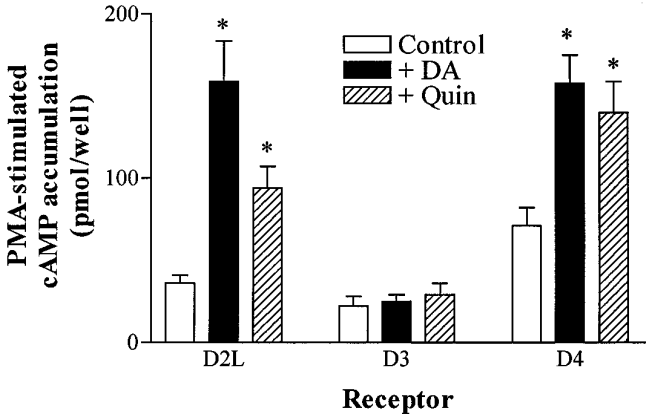

Fig. 3. Potentiation of PMA-stimulated cAMP accumulation in ACII/D2L, ACII/D3, and ACII/D4 cells. cAMP accumulation was stimulated with 100 nM PMA in the absence and presence of dopamine (*DA*, 1 μM) or quinpirole (*Quin*, 1 μM) for 15 min. Data shown are the mean $\pm$ standard error for three or more independent experiments, each conducted with duplicate determinations. *, $p < 0.01$ compared with control cells (Dunnett's postrepeated measures ANOVA).

concentration of $\beta\gamma$ subunits that is not sufficient to potentiate α_s- or PMA-stimulated ACII activity. Although D_3 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 D_3 receptor activation is reduced compared with the response that is mediated by D_2 and D_4 receptors (1, 2, 6, 26, 27). Specifically, the muscarinic receptor-gated atrial potassium channel, Girk1, is activated by D_2, D_3, and D_4 dopamine receptors, but the maximal current is 3-fold larger for D_2 and D_4 receptors than for D_3 receptors (6). Because Girk1 is also activated by $\beta\gamma$ subunits (28), the results of the present study support the hypothesis that smaller current induced by D_3 receptor activation could be due to diminished release of $\beta\gamma$ subunits. The reasons for the functional differences among D_2, D_3, and D_4 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 D_{2L} receptors potentiate the actions of PMA on ACII is important considering the evidence that has linked D_2 dopamine receptors and the protein kinase C pathway. For example, D_2 dopamine receptors have been shown to stimulate phosphoinositide hydrolysis in Ltk^- fibroblasts expressing D_2 dopamine receptors (29). Thus, in Ltk^- fibroblasts expressing ACII and D_2 receptors, it is possible that D_2 agonists could stimulate cAMP accumulation due to increased protein kinase C activity in combination with the release of $\beta\gamma$ subunits from $G_{i/o}$. The protein kinase C pathway has also been implicated in D_2 receptor-potentiated arachadonic acid release in Chinese hamster ovary cells and in inhibition of cell proliferation in GH_4ZR_7 cells, because inhibitors of protein kinase C block both of these D_2 receptor effects (30, 31). Taken together, these observations and the current study suggest that the interactions between the protein kinase C pathway and D_2 dopamine receptors are important in modulating neurotransmission in a variety of cell types.

In summary, we have demonstrated that activation of D_{2L} and $D_{4.4}$ receptors potentiated isoproterenol- and PMA-stimulated cAMP synthesis by ACII, whereas D_3 receptors did not. Furthermore, our data confirm that ACII can be synergistically activated by multiple signals, including PMA and $\beta\gamma$ subunits, α_s and $\beta\gamma$ subunits, or PMA and α_s. However, the activation of ACII by $\beta\gamma$ subunits is conditional, requiring co-activation by either protein kinase C or α_s, suggesting that the binding of $\beta\gamma$ to ACII results in an enhancement of responsiveness of the enzyme to other activators. Potentiation of PMA-stimulated ACII activity by $\beta\gamma$ subunits represents another example of coincident signal detection and may influence the interactions between D_{2L} and $D_{4.4}$ dopamine receptors and the protein kinase C pathway.

Acknowledgments

We acknowledge Drs. Aaron Janowsky and Amy Eshleman for careful reading of the manuscript, and Dr. Brenda Wiens and Mr. Minh Vu for assistance with the D_3 and $D_{4.4}$ receptor cDNAs. We would also like to thank Drs. Hubert Van Tol and David Grandy for providing us with the cDNA for the human $D_{4.4}$ receptor and Dr. Daniel Storm and Mark Nielsen for providing us with HEK-ACII cells.

References

1. O'Hara, C. M., A. Uhland-Smith, K. L. O'Malley, and R. D. Todd. Inhibition of dopamine synthesis by dopamine D_2 and D_3 but not D_4 receptors. *J. Pharmacol. Exp. Ther.* **277:**186–192 (1996).
2. Tang, L., R. D. Todd, A. Heller, and K. L. O'Malley. Pharmacological and functional characterization of D_2, D_3 and D_4 dopamine receptors in fibroblast and dopaminergic cell lines. *J. Pharmacol. Exp. Ther.* **268:**495–502 (1994).
3. Chio, C. L., M. E. Lajiness, and R. M. Huff. Activation of heterologously expressed D_3 dopamine receptors: comparison with D_2 dopamine receptors. *Mol. Pharmacol.* **45:**51–60 (1994).
4. MacKenzie, R. G., D. VanLeeuwen, T. A. Pugsley, Y.-H. Shih, S. Demattos, L. Tang, R. D. Todd, and K. L. O'Malley. Characterization of the human dopamine D_3 receptor expressed in transfected cell lines. *Eur. J. Pharmacol. Mol. Pharmacol.* **266:**79–85 (1994).
5. Asghari, V., S. Sanyal, S. Buchwaldt, A. Paterson, V. Jovanovic, and H. H. M. Van Tol. Modulation of intracellular cyclic AMP levels by different human dopamine D_4 receptor variants. *J. Neurochem.* **65:**1157–1165 (1995).
6. Werner, P., N. Hussy, G. Buell, K. A. Jones, and R. A. North. D_2, D_3, and D_4 dopamine receptors couple to G protein-regulated potassium channels in *Xenopus* oocytes. *Mol. Pharmacol.* **49:**656–661 (1996).
7. Federman, A. D., B. R. Conklin, K. A. Schrader, R. R. Reed, and H. R. Bourne. Hormonal stimulation of adenylyl cyclase through G_i-protein $\beta\gamma$ subunits. *Nature (Lond.)* **356:**159–161 (1992).
8. Lustig, K. D., B. R. Conklin, P. Herzmark, R. Taussig, and H. R. Bourne. Type II adenylylcyclase integrates coincident signals from G_s, G_i, and G_q. *J. Biol. Chem.* **268:**13900–13905 (1993).
9. Tsu, R. C., R. A. Allen, and Y. H. Wong. Stimulation of type II adenylyl cyclase by chemoattractant formyl peptide and C5a receptors. *Mol. Pharmacol.* **47:**835–841 (1995).
10. Taussig, R., W.-J. Tang, J. R. Hepler, and A. G. Gilman. Distinct patterns of bidirectional regulation of mammalian adenylyl cyclases. *J. Biol. Chem.* **269:**6093–6100 (1994).
11. Tang, W.-J., and A. G. Gilman. Type-specific regulation of adenylyl cyclase by G protein $\beta\gamma$ subunits. *Science (Washington D. C.)* **254:**1500–1502 (1991).
12. Jacobowitz, O., and R. Iyengar. Phorbol ester-induced stimulation and phosphorylation of adenylyl cyclase 2. *Proc. Natl. Acad. Sci. USA* **91:** 10630–10634 (1994).
13. Taussig, R., L. M. Quarmby, and A. G. Gilman. Regulation of purified type I and type II adenylylcyclases by G protein $\beta\gamma$ subunits. *J. Biol. Chem.* **268:**9–12 (1993).
14. Premont, R. T., J. Chen. H.-W. Ma, M. Ponnapalli, and R. Iyengar. Two members of a widely expressed subfamily of hormone-stimulated adenylyl cyclases. *Proc. Natl. Acad. Sci. USA* **89:**9809–9813 (1992).
15. Inglese, J., L. M. Luttrell, J. A. Iñiguez-Lluhi, K. Touhara, W. J. Koch, and R. J. Lefkowitz. Functionally active targeting domain of the β-adrenergic receptor kinase: An inhibitor of $G_{\beta\gamma}$-mediated stimulation of type II adenylyl cyclase. *Proc. Natl. Acad. Sci. USA* **91:**3637–3641 (1994).
16. Thomas, J. M., and B. B. Hoffman. Isoform-specific sensitization of adenylyl cyclase activity by prior activation of inhibitory receptors: role of $\beta\gamma$ subunits in transducing enhanced activity of the type VI isoform. *Mol. Pharmacol.* **49:**907–914 (1996).
17. Chan, J. S. C., T. T. Chiu, and Y. H. Wong. Activation of type II adenylyl

cyclase by the cloned μ-opioid receptor: coupling to multiple G proteins. *J. Neurochem.* **65:**2682–2689 (1995).
18. Sunahara, R. K., C. W. Dessauer, and A. G. Gilman. Complexity and diversity of mammalian adenylyl cyclases. *Annu. Rev. Pharmacol. Toxicol.* **36:**461–480 (1996).
19. Tsu, R. C., and Y. H. Wong. G_i-mediated stimulation of type II adenylyl cyclase is augmented by G_q-coupled receptor activation and phorbol ester treatment. *J. Neurosci.* **16:**1317–1323 (1996).
20. Morgenstern, J. P., and H. Land. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18:** 3587–3596 (1990).
21. Cox, B. A., M. P. Rosser, M. R. Kozlowski, K. M. Duwe, R. L. Neve, and K. A. Neve. Regulation and functional characterization of a rat recombinant dopamine D_3 receptor. *Synapse* **21:**1–9 (1995).
22. Watts, V. J., and K. A. Neve. Sensitization of endogenous and recombinant adenylate cyclase by activation of D_2 dopamine receptors. *Mol. Pharmacol.* **50:**966–976 (1996).
23. Nordstedt, C., and B. B. Fredholm. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal. Biochem.* **189:**231–234 (1990).
24. Choi, E.-J., W. T. Wong, T. R. Hinds, and D. R. Storm. Calcium and muscarinic agonist stimulation of type I adenylylcyclase in whole cells. *J. Biol. Chem.* **267:**12440–12442 (1992).
25. Zhou, X.-M., P. Curran, J. Baumgold, and P. H. Fishman. Modulation of adenylylcyclase by protein kinase C in human neurotumor SK-N-MC cells: evidence that the α isozyme mediates both potentiation and desensitization. *J. Neurochem.* **63:**1361–1370 (1994).
26. Pilon, C., D. Lévesque, V. Dimitriadou, N. Griffon, M.-P. Martres, J.-C. Schwartz, and P. Sokoloff. Functional coupling of the human dopamine D_3 receptor in a transfected NG 108–15 neuroblastoma-glioma hybrid cell line. *Eur. J. Pharmacol. Mol. Pharmacol.* **268:**129–139 (1994).
27. Swarzenski, B. C., K. L. O'Malley, and R. D. Todd. PTX-sensitive regulation of neurite outgrowth by the dopamine D_3 receptor. *Neuroreport* **7:**573–576 (1996).
28. Wickman, K. D., J. A. Iñiguez-Lluhi, P. A. Davenport, R. Taussig, G. B. Krapivinsky, M. E. Linder, A. G. Gilman, and D. E. Clapham. Recombinant G-protein $\beta\gamma$ subunits activate the muscarinic-gated atrial potassium channel. *Nature (Lond.)* **368:**255–257 (1994).
29. 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 phosphatidylinositol 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).
30. Kanterman, R. Y., L. C. Mahan, E. M. Briley, F. J. Monsma, D. R. Sibley, J. Axelrod, and C. C. Felder. Transfected D_2 dopamine receptors mediate the potentiation of arachidonic acid release in Chinese hamster ovary cells. *Mol. Pharmacol.* **39:**364–369 (1991).
31. Senogles, S. E. The D_2 dopamine receptor mediates inhibition of growth in GH_4ZR_7 cells: involvement of protein kinase-Cϵ. *Endocrinology* **134:**783–789 (1994).

Send reprint requests to: Val J. Watts, Medical Research Service (151LL), VA Medical Center, 3710 SW US Veterans Hospital Road, Portland, OR 97201. E-mail: wattsv@ohsu.edu