ACCELERATED COMMUNICATION

Activation of Type II Adenylate Cyclase by D₂ and D₄ but Not D₃ Dopamine Receptors

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SUMMARY

The D₂-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 βγ 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 βγ subunits. The ability of βγ subunits to activate this enzyme in the presence of activated αs has been particularly well characterized. Although stimulation by βγ subunits has been described as conditional on the presence of activated αs, βγ subunits also potentiate ACII activity after activation of protein kinase C. We created stable cell lines expressing ACII and the D₂L receptor, the D₃ receptor, or the D₄.₄ receptor. Activation of D₂L or D₄.₄ receptors, but not D₃ receptors, potentiated β-adrenergic receptor/Gₐ-stimulated activity of ACII, as measured by the intracellular accumulation of cAMP. Similarly, stimulation of D₂L or D₄.₄ receptors potentiated phorbol ester-stimulated ACII activity in the absence of activated αs, whereas stimulation of D₃ receptors did not. The effect of D₂-like receptor stimulation was blocked by pretreatment with pertussis toxin and by inhibition of protein kinase C. We propose that activation of both D₂L and D₄.₄ dopamine receptors potentiated phorbol-12-myristate-13-acetate-stimulated ACII activity through the release of βγ subunits from pertussis toxin-sensitive G proteins. In contrast, the lack of D₃ receptor-mediated effects suggests that stimulation of D₃ receptors does not result in an appreciable release of βγ subunits.

The D₂-like receptor family is composed of D₂, D₃, and D₄ 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, D₂ and D₃ receptors inhibit dopamine synthesis in a dopamine-producing cell line, whereas D₄ receptors do not (1, 2), and D₂ and D₄ receptors mediate robust inhibition of cAMP accumulation in a variety of cell lines, whereas inhibition of cAMP accumulation by the D₃ receptor is modest or absent (2–5).¹ On the other hand, D₂, D₃, and D₄ 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 D₂-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 Gαi (10), it is stimulated by αs (11), phorbol esters (12), and G protein βγ subunits (13) in reconstituted systems. Additionally, ACII is synergistically activated by αs and PMA (8, 12) as well as by αs and βγ subunits (11, 13, 14). In intact cells, ACII is activated by βγ subunits in combination with activated αs.

¹ V. J. Watts and K. A. Neve, unpublished observations.

ABBREVIATIONS: ACII, type II adenylate cyclase; CBS, calf bovine serum; D₂L, long (444-amino acid) form of D₂ receptors; D₄.₄, a variant of the D₄ 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 βγ stimulation alone has no detectable effect on ACII activity (7, 9, 15–17). In those studies βγ subunits were supplied by stimulating Gαq-coupled receptors (e.g., the D2 dopamine receptor), and activated αs was provided by stimulating Gq-coupled receptors or by co-transfection with a constitutively active mutant of αs, αs-Q227L. 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 Gαq-coupled receptors enhances ACII stimulation by Gq-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 D4 agonists potentiated isoproterenol-stimulated cAMP accumulation in HEK293 cells expressing the D4r or the D4.4 dopamine receptor together with ACII, whereas the D2 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 D4L 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-bromo-3,4-dihydroxyphenylacetic acid)-ethyl)-2-aminoethanesulfonic acid in a total volume of 400 μl was supplied by stimulating Gs-coupled receptors (e.g., the D2 receptor) and the D4.4 dopamine receptor (ACII/D4). We now report that D4 agonists potentiated isoproterenol-stimulated cAMP accumulation in HEK293 cells expressing the D4r or the D4.4 dopamine receptor together with ACII, whereas the D2 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 D4L and D4.4 receptors potentiated protein kinase C-activated ACII activity.

**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 Gα0-coupled dopamine receptors, D2L (ACII/D2L cells), D3 (ACII/D3), or D4.4 (ACII/D4 cells). In ACII/D2L cells, D2 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

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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).

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of $\alpha_2$ (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 $D_4$ 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 $D_4$ receptor could stimulate ACII activity. As we observed with the $D_{2L}$ receptor, activation of the $D_4$ 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). Stauroporine did not prevent the $D_2_L$-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 $D_4$ 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 $\alpha_2$ (via $G_i/o$-coupled receptors or co-transfection with a constitutively active $\alpha_2$, $\alpha_2$-Q227L), stimulates ACII (7, 9, 15, 16). The activation of $G_i/o$-coupled receptor releases $\beta y$ subunits, which in turn potentiate the activation of ACII by $\alpha_2$. The role of $\beta y$ subunits is supported by the observation that co-expression of $\alpha_2$, which presumably sequesters released $\beta y$ 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 $\beta y$ subunits in combination with activated $\alpha_2$ (11, 13) and also by $\beta y$ 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 $\alpha_2$-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 $\alpha_2$-adrenergic receptor stimulates ACII in the absence of constitutively active $\alpha_2$-Q227L. It has been suggested that the ability of the $\alpha_2$-adrenergic receptor to couple to $G_i/o$ 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 $\alpha_2$-adrenergic receptors was not (7). In the present study, we have confirmed the finding that potentiation of $G_i/o$-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 1**

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<td>Vehicle</td>
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<td>+ 1 $\mu$M</td>
<td>6.7 ± 1.3</td>
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<td>+ 25 ng/ml</td>
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<td>15.2 ± 2.3</td>
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<td>+ Spiperone</td>
<td>+ 1 $\mu$M</td>
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<td>12.9 ± 1.4</td>
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**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 (control) 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 D2L, D3, and D4.4 receptors. Results from reconstitution studies have demonstrated that protein kinase C phosphorylates and activates ACII independently of βγ subunits (12). Furthermore, short term PMA treatment of intact cells does not activate βγ as assessed by reconstituted adenylate cyclase activity in the membranes of COS cells (25). To examine the hypothesis that βγ subunits released by the activation of Gβγ-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 D2 agonists potentiated the actions of PMA in ACII/D2L cells (Fig. 3 and Table 2). The effects of D2 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 EC50 value of 132 nM (Fig. 2). Similarly, activation of D4.4 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 D4.4 receptor activation on PMA-stimulated cAMP accumulation was also blocked by the D4 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 D2-like receptors and further suggest that spiperone is an inverse agonist at these receptors.

In contrast to the ability of D2 and D4 receptors to potentiate cAMP accumulation, D3 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 D3 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 D4 receptor activation potentiates cAMP accumulation stimulated by either αβ (i.e., isoproterenol) or protein kinase C (PMA) in a pertussis toxin-sensitive manner strongly suggests that activated αβ 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 D2-like dopamine receptor family. The results of these studies are similar to those examining D2L, D3, and D4-mediated inhibition of cAMP accumulation in which D2 and D4 receptors display robust inhibition and D3 receptors show little or no effect (2–4). Thus, it seems that the inefficient coupling of D3 receptors to Gαo proteins provides a

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**TABLE 2**

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<th>Condition/pretreatment</th>
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<td></td>
<td>+ Stauroporine (1 μM)</td>
<td>6.0 ± 1.1***</td>
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<td>11.1 ± 5.2***</td>
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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 EC50 for dopamine + PMA (●) was 132 nM and for dopamine + isoproterenol (○) was 32 nM.
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, neutri- 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 D₄ 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 G_{i,ω}. The protein kinase C pathway has also been implicated in D₃ receptor-potentiated arachidonic acid release in Chinese hamster ovary cells and in inhibition of cell proliferation in GH₃ZR₇ cells, because inhibitors of protein kinase C block both of these D₃ 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 dopamine receptors potentiated isoproterenol- and PMA-stimu-


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