Previously, D₂ dopamine receptors (D₂ DARs) have been shown to undergo G-protein-coupled receptor kinase phosphorylation in an agonist-specific fashion. We have now investigated the ability of the second messenger-activated protein kinases, protein kinase A (PKA) and protein kinase C (PKC), to mediate phosphorylation and desensitization of the D₂ DAR. HEK293T cells were transiently transfected with the D₂ DAR and then treated with pharmacological activators and inhibitors of PKA or PKC. Treatment with agents that increase cAMP, and activate PKA, had no effect on the phosphorylation state of the D₂ DAR, suggesting that PKA does not phosphorylate the D₂ DAR in HEK293T cells. In contrast, cellular treatment with phorbol 12-myristate 13-acetate (PMA), a PKC activator, resulted in a ~3-fold increase in D₂ DAR phosphorylation. The phosphorylation was specific for PKC as the PMA effect was mimicked by phorbol 12,13-dibutyrate, but not by 4α-phorbol 12,13-didecanoate, active and inactive, phorbol diesters, respectively. The PMA-mediated D₂ DAR phosphorylation was completely blocked by co-treatment with the PKC inhibitor, bisindolylmaleimide II, and augmented by co-transfection with PKCβI. In contrast, PKC inhibition had no effect on agonist-promoted phosphorylation, suggesting that PKC is not involved in this response. PKC phosphorylation of the D₂ DAR was found to promote receptor desensitization as reflected by a decrease in agonist potency for inhibiting cAMP accumulation. Most interestingly, PKC phosphorylation also promoted internalization of the D₂ DAR through a β-arrestin- and dynamin-dependent pathway, a response not usually associated with PKC phosphorylation of G-protein-coupled receptors. Site-directed mutagenesis experiments resulted in the identification of two domains of PKC phosphorylation sites within the third intracellular loop of the receptor. Both of these domains are involved in regulating sequestration of the D₂ DAR, whereas only one domain is involved in receptor desensitization. These results indicate that PKC can mediate phosphorylation of the D₂ DAR, resulting in both functional desensitization and receptor internalization.

G-protein-coupled receptors (GPCRs) represent a large family of seven transmembrane-spanning proteins that transduce cellular responses to numerous extracellular signals, including hormones, neurotransmitters, odorants, and light (1, 2). Most cells maintain homeostatic control of their responsiveness to signals through regulating the expression and functional activity of their cell surface GPCRs. One widely studied form of GPCR regulation is that of agonist-induced desensitization. In this process, activation of the GPCR by an agonist also triggers a sequence of events that results in the dampening of the receptor-mediated signal. The mechanisms associated with this “homologous” form of desensitization have been most thoroughly investigated for the β₂-adrenergic receptor-coupled adenyl cyclase system (3, 4), resulting in the following paradigm. Agonist occupancy of the receptor promotes its phosphorylation by a member of the G-protein-coupled receptor kinase (GRK) family leading to the binding of an arrestin-like protein, ultimately resulting in uncoupling of the receptor from its cognate G-protein and decreased functional signaling. The binding of an arrestin-like molecule also promotes internalization of the receptor through clathrin-coated pits into an endosomal compartment where it may be de-phosphorylated and recycled to the cell surface or degraded via a lysosomal pathway. Although in many instances this desensitization paradigm has been shown to be operative for other G-protein-coupled receptors, recent studies have suggested that there may be significant exceptions and variations to this general scheme.

Much less is known about “heterologous” forms of regulation in which activation of one GPCR, or other receptor system(s), may lead to the desensitization of multiple unrelated receptors in the same cell. Evidence to date suggests that heterologous regulation of receptor-mediated signaling pathways frequently involves phosphorylation of various signaling components by second messenger-regulated protein kinases that are activated by the initial signal (4–7). Typically, phosphorylation of GPCRs by second messenger activated kinases such as protein kinase A (PKA) or C (PKC) has been suggested to dampen the ability of the receptor to couple to G-proteins and elicit a response, although precise mechanistic details are lacking. It is highly likely that heterologous forms of desensitization may play a critical role in fine-tuning GPCR responses in cells such as neurons that may concurrently receive numerous hormonal or neurotransmitter signals.

Dopamine receptors (DARs) are members of the GPCR superfamily and consist of five structurally distinct subtypes (8). These can be divided into two subgroups on the basis of their structure and pharmacological and transductional properties. The first subgroup, termed “D₁-like” comprises the D₁ and D₅ DARs that stimulate adenyl cyclase and raise intracellular levels of cAMP. The second DAR subgroup includes the D₂, D₃, D₄ dopamine receptor.

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The abbreviations used are: GPCRs, G-protein-coupled receptors; PKA, protein kinase A; PKC, protein kinase C; DMEM, Dulbecco’s modification of Eagle’s medium; EBSS, Earle’s balanced salt solution; PDBu, phorbol 12,13-dibutyrate; 4αPDD, 4α-phorbol 12,13-didecanoate; BIMII, bisindolylmaleimide II; WT, wild type; D₂ DAR, D₂ dopamine receptor.

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and \(D_2\) receptors and is termed “\(D_2\)-like.” The \(D_2\)-like DARs are coupled to the inhibition of adenyl cyclase as well as the modulation of potassium and calcium ion channels. As with other GPCRs, DARs are subject to a wide variety of regulatory mechanisms, which can either positively or negatively modulate their expression and functional activity (9).

The mechanisms underlying various forms of DAR regulation have only begun to be elucidated. The greatest information has been derived from studies using the \(D_2\) DAR, which appears to be phosphorylated and internalized in a GRK-dependent fashion upon agonist activation, although some notable differences from the scheme described above exist (10–15). In contrast, studies of the \(D_2\) DAR have revealed that regulation of this receptor is extremely complex with agonist activation variability resulting in functional desensitization, sensitization, receptor up- or down-regulation, or no effect at all (reviewed in Ref. 9). Given that the \(D_2\) DAR is the primary target for all known antipsychotic drugs (agonists) and drugs used to treat Parkinson’s disease (agonists), more information concerning the regulatory mechanisms for this receptor may lead to improved therapies for treating \(D_2\) DAR-related diseases (16, 17). Recent information has, in fact, suggested that the \(D_2\) DAR is a substrate for GRKs in cellular expression systems and that GRK-mediated phosphorylation promotes receptor internalization (18, 19). In contrast, little information is available concerning phosphorylation and regulation of the \(D_2\) DAR by second messenger-activated protein kinases, although earlier studies have suggested potential roles for PKA and PKC (20–24). In our present study, we have now investigated phosphorylation of the \(D_2\) DAR by second messenger-activated protein kinases, and we demonstrate that PKC phosphorylates the \(D_2\) DAR on multiple sites within two domains of the third intracellular loop. This PKC-mediated phosphorylation of the \(D_2\) DAR was demonstrated to promote both functional desensitization and internalization of the receptor protein via a \(\beta\)-arrestin- and dynamin-dependent pathway.

**EXPERIMENTAL PROCEDURES**

**Materials—**HEK293-Cl were a gift of Dr. Vanitha Ramakrishnan. [\(^3\H\)]Sulpiride (69–77 Ci/mmol), [\(^{18}\)F]methyldopasipiperone (80 Ci/mmol), and [\(^{35}\)P]orthophosphate (carrier-free) were purchased from PerkinElmer Life Sciences. CAMP assay kits were from Diagnostic Products Corp. (Los Angeles, CA). Dulbecco’s modification of Eagle’s medium (DMEM) was from Cellgro® Mediatech, Inc. (Herndon, VA). [\(^3\H\)]Methylsulpiride was purchased from Invitrogen. Calcium phosphate transfection kits were from Clontech. MiniComplete™ protease inhibitor mixture was purchased from Roche Applied Science. Site-directed mutagenesis kits were obtained from Stratagene (La Jolla, CA). Anti-FLAG M2 affinity gel and all other reagents were purchased from Sigma.

**Plasmids and Mutagenesis—**To create an amino-terminal FLAG-tagged construct of the rat \(D_2L\) DAR (psF-\(D_2L\)), a synthetic oligonucleotide encoding a signal sequence and an antigenic epitope of the “FLAG” has been derived from studies using the \(D_1\) DAR, which have only begun to be elucidated. The greatest information late their expression and functional activity (9).

**Whole-cell Phosphorylation Assays—**Metabolic labeling of cells and subsequent immunoprecipitation of the \(D_2\) DAR was carried out as described previously (10). Briefly, HEK293T cells were transfected with psF-\(D_2L\) using the calcium-phosphate method. One day after transfection, cells were seeded at 1–5 \(\times\) 10⁴ per well of a poly(D)-lysine-coated 2-well plate for phosphorylation assay and ~2 × 10⁵ cells on a 100-mm dish for radioligand binding assay to quantify the level of receptor expression. The next day, the cells were washed with Earle’s balanced salt solution (EBSS) and incubated for 1 h in phosphate-free DMEM with 10% fetal calf serum. Media were removed and replaced with 1 ml of fresh media supplemented with 200 \(\mu\)g/ml [\(^{35}\)P]orthophosphate. After 45 min at 37 °C, the cells were washed with 1 ml of phosphate-free DMEM 500 mM NaCl, once with solubilization buffer (50 mM HEPES, 1 mM EDTA, 10% glycerol, 1% Triton X-100, pH 7.4, at 4 °C) + 150 mM NaCl supplemented with Complete protease inhibitor mixture and phosphatase inhibitors (40 mM sodium pyrophosphate, 50 mM NaF). The samples were cleared by centrifugation in a microcentrifuge at 13,000 × g for 10 min. The protein concentration was determined using the bicinchoninic acid protein assay (Pierce). The level of \(D_2\) DAR expression for each transfection was quantified via radioligand binding assays using the cells from the same transfection. After receptor/protein quantification, equal amounts of receptor protein were then transferred to fresh tubes with 40 \(\mu\)l of washed M2-agarose and incubated overnight with mixing at 4 °C. The samples were then washed once with solubilization buffer and once with 500 mM NaCl, once with 150 mM NaCl, and once with Tris-EDTA, pH 7.4, at 4 °C. Samples were then incubated 2× SDS-PAGE loading buffer for 1 h at 37 °C before being resolved by 4–20% Tris-glycine SDS-PAGE. The gels were dried and subjected to autoradiography. After developing, the band intensity was quantitated by LabWorks™ software (UVP Inc., Upland, CA).

**Intact Cell [\(3\H\)]Sulpiride Binding—**HEK293T cells expressing rat \(D_2\) DAR were seeded on 1 day after transfection at a density of 2 × 10⁵ cells/well in poly(D)-lysine-coated 24-well plates. The next day, cells were incubated in the absence (control) and presence of 1 \(\mu\)M PMA in DMEM for 2 h. Stimulation was terminated by quickly cooling the plates and washing the cells three times with ice-cold EBSS. Cells were then incubated with 0.5 ml of [\(3\H\)]sulpiride in EBSS (final concentration, 6.4 nM) at 4 °C for 3 h 30 min. For saturation binding assays, cells were incubated with 0.5–50 nM [\(3\H\)]sulpiride and 10 μM to 100 μM dopamine. The association constant was determined in the presence of 5 μM (+)-butaclamol. Cells were washed three times with ice-cold EBSS, and 0.5 ml of 1% Triton X-100 was added. Samples were mixed with 5 ml of liquid scintillation mixture and counted with a Beckman LS6500 scintillation counter.

**Membrane [\(3\H\)]Methylsulpiride Binding—**HEK293T cells were harvested and washed in EBSS after 24 h of incubation in the absence of cAMP or forskolin. The cells were resuspended in lysis buffer (5 mM Tris, pH 7.4, at 4 °C; 5 mM MgCl₂) and were disrupted using a Dounce homogenizer followed by centrifugation at 34,000 × g for 10 min. The resulting membrane pellet was resuspended in binding buffer (50 mM Tris, pH 7.4). The membrane suspension was then added to assay tubes containing [\(3\H\)]methylsulpiride in a final concentration of 1.0 μM (+)-butaclamol was added at the final concentration of 3 μM to determine nonspecific binding. The assay tubes were incubated at room temperature for 1.5 h, and the reaction was terminated by rapid filtration through GF/C filters pretreated with 0.3% polyethyleneimine. Radioactivity bound to the filters was quantitated by liquid scintillation spectroscopy.

**Determination of cAMP Production—**HEK293T cells were seeded into poly(D)-lysine-coated 24-well plates 1 day before the assay at a density of 2 × 10⁵ cells per well. To assess the effect of PMA, the cultures were first incubated for 10 min in the absence (control) and presence of 1 \(\mu\)M PMA in DMEM. Subsequently, the cells were washed once with pre-warmed EBSS. For the control group, the cells were further incubated with various concentrations of dopamine in a total volume of 500 μl at 37 °C for 10 min in the presence of 3 μM forskolin, 30 μM Ro-20-1724 (phosphodiesterase inhibitor), 0.2 mM sodium metabsulitole (to prevent oxidation of dopamine), and 10 μM propranolol (to block endogenous \(\beta\)-adrenergic receptors) in 20 mM HEPES-buffered DMEM. For the PMA-treated group, the cells were incubated with the same composition of buffer as in the control group except for containing 1 \(\mu\)M PMA. The supernatant was aspirated, and perchloric acid (3%, 200 \(\mu\)l/well) was added. After incubating on ice for 30 min, 80 \(\mu\)l of 15%
interestingly, treatment of the cells with bisindolylmaleimide (BIMII; lane 2), an inhibitor of PKC, decreases the phosphorylation obtained in A was quantified by scanning the autoradiographs followed by analysis with LabWorks™ software (UVP, Inc., Upland, CA). Data are presented as a percentage above basal phosphorylation and expressed as the mean ± S.E. from more than three independent experiments. *, p < 0.05; **, p < 0.01, compared with the basal, unpaired Student’s t test. C, autoradiogram in which the cells were pretreated with the indicated drugs: lane 1, vehicle (basal); lane 2, 1 μM forskolin; lane 3, 1 μM PDBu; lane 4, 0.1 μM 4α-PDD. The autoradiogram shown is representative of two independent experiments.

FIG. 1. PKC-mediated phosphorylation of D₂ DARs expressed in HEK293T cells. ³²P-labeled HEK293T cells were transiently transfected with the FLAG-tagged D₂ DAR and incubated with the indicated drugs for 20 min. Cells were solubilized, and the samples were subjected to immunoprecipitation as described under “Experimental Procedures.” Receptors were quantified, and equal amounts of receptor protein were loaded into each gel lane and resolved by 4–20% SDS-PAGE. The extent of receptor phosphorylation was visualized by autoradiography and quantitated as described below. A, representative autoradiogram is shown. Lane 1, vehicle (basal); lane 2, 1 μM forskolin; lane 3, 10 μM BIMII; lane 4, 4 μM PDD. The autoradiograph shown is representative of two independent experiments.

PKC-mediated Phosphorylation of D₂ DARs in HEK293T Cells—As an initial approach to investigating the role of second messenger-activated protein kinases in D₂ DAR phosphorylation, we treated transiently transfected HEK293T cells with various activators and inhibitors of PKA and PKC (Fig. 1). The major phosphorylated protein in FLAG-tagged D₂ DAR-expressing cells runs as a broad band of 60–85 kDa and is not present in immunoprecipitates of untransfected cells (data not shown). As seen in Fig. 1A, the D₂ DAR is phosphorylated under basal conditions, and treatment of the cells with forskolin, a phorbol ester that directly activates PKC, increases the phosphate content of the D₂ DAR by ~3-fold (Fig. 1, A and B). Most interestingly, treatment of the cells with bisindolylmaleimide (BIMII), an inhibitor of PKC, decreases the phosphorylation state of the D₂ DAR by about 50% (Fig. 1, A and B). In contrast, cellular treatment with agents that lead to PKA activation, including forskolin, which raises intracellular cAMP levels, and 8-4(4-chlorophenylthio)adenosine 3′,5′ cyclic AMP (cAMP), a membrane-permeable cAMP analog, have no effect on D₂ DAR phosphorylation (Fig. 1, A and B). Similarly, treatment with H89, a PKA inhibitor, does not affect the phosphorylation state of the D₂ DAR (Fig. 1, A and B). These results suggest that PKC activation promotes D₂ DAR phosphorylation and that the basal receptor phosphorylation might be partially explained by constitutive PKC phosphorylation. The phosphorylation was specific for PKC, as the PMA effect was mimicked by PDBu but not by the biologically inactive phorbol ester 4α-PDD (Fig. 1C).

Agonist activation of the D₂ DAR has also been shown to promote its phosphorylation, an effect that is known to involve G-protein-coupled receptor kinases (18, 19). Because in some cells D₂ DARs can stimulate phospholipase C-β to elevate intracellular calcium and diacylglycerol levels through activation of G-protein βγ subunits (28, 29), it is possible that agonist-induced D₂ DAR phosphorylation at least partly involves PKC.

To investigate whether PKC-mediated phosphorylation is involved in the agonist-induced D₂ DAR phosphorylation, we incubated the cells with the PKC inhibitor BIMII before and during dopamine treatment. As shown in Fig. 2, dopamine-induced D₂ DAR phosphorylation was not affected by BIMII treatment, but PMA-stimulated D₂ DAR phosphorylation was totally abolished by the PKC inhibitor. Agonist activation using dopamine resulted in a doubling of the phosphorylation state of the D₂ DAR in both the absence or presence of BIMII (Fig. 2). Overall, this suggests that PKC is not involved in homologous phosphorylation of the D₂ DAR but may occur heterologously through the activation of Gq-linked or other receptors that lead to PKC activation.

Because HEK293 cells do not abundantly express any known Gq-linked receptors, we co-transfected the M1 muscarinic receptor, which couples to Gq and leads to phospholipase C and PKC activation. Fig. 3, A and B, shows that in M1 receptor-
PKC-mediated D2 Receptor Phosphorylation

Effect of PKC-mediated Phosphorylation on D2 DAR Sequestration and Desensitization—As a first approach to investigating the effects of PKC-mediated phosphorylation on D2 DAR function, we examined the expression of the receptor, both total cellular expression as well as just cell surface expression (Fig. 4). In order to assess the cell surface expression of the D2 DAR, we used the radioligand, [3H]sulpiride, and employed an intact cell binding assay. [3H]Sulpiride is a hydrophilic antagonist ligand, which is membrane-impermeable, and restricted to binding only those receptors at the surface of intact cells (18, 19, 33). Fig. 4A shows that cellular treatment with PMA results in a 15–20% loss of D2 DAR expression on the cell surface. In contrast, there is no effect of PMA treatment on total D2 DAR expression as assessed using the hydrophobic antagonist [3H]methylspiperone and membrane binding assays (Fig. 4B). Table I shows average radioligand binding data from multiple experiments and further shows that only [3H]sulpiride binding to cell surface D2 DARs is reduced by PMA treatment. Total cellular receptor binding detected with [3H]methylspiperone is unaffected. We also investigated the effect of co-expressing PKCβ on the PMA-induced receptor internalization, and the results are shown in Table I. Overexpression of PKCβ significantly increased this response with about 50% of the receptors being sequestered upon treatment with PMA. Taken together, these results demonstrate that PKC activation results in se-
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Cell surface D2 DAR expression was assessed by measuring [3H]sulpiride binding on intact HEK293T cells transiently transfected with the D2 DAR alone or with the D2 DAR plus PKCβ. Cells were incubated with vehicle (control) or 1 μM PMA for 2 h. After washing with ice-cold EBSS, the cells were subjected to intact cell [3H]sulpiride binding assays as described under "Experimental Procedures." Total cellular D2 DAR expression was measured by a saturation radioligand binding of [3H]methylypiperone to HEK293T cell crude membranes. Data are represented as mean ± S.E. of three to four experiments, indicated in parentheses. ND, not determined.

| [3H]Sulpiride binding | [3H]Methylypiperone binding |
|-----------------------|----------------------------|
| fmoles/10⁶ cells       | fmoles/mg protein          |
| Control               | 75.0 ± 2.1 (3)              | 3.04 ± 0.38 (4)            |
| PMA                   | 61.5 ± 3.0 (3)              | 3.10 ± 0.48 (4)            |
| PKCβ control          | 65.0 ± 10.5 (3)             | ND                         |
| PKCβ PMA              | 31.7 ± 2.6 (3)              | ND                         |

*p < 0.01, compared with the control, paired Student’s t test.

Effects of dominant negative mutants of dynamin and β-arrestin on PMA-stimulated D2 DAR sequestration

Cell surface D2 DAR expression was assessed by measuring [3H]sulpiride-binding sites on intact HEK293T cells transiently transfected with D2L DAR with pcDNA (empty vector) or expression constructs for dynaminK44A or β-arrestinK341–419. Cells were incubated with vehicle (control) or 1 μM PMA for 2 h. After washing with ice-cold EBSS, the cells were subjected to intact cell [3H]sulpiride binding assays. Data are represented as mean ± S.E. of 4–7 independent experiments, indicated in parentheses.

| D2L with            | [3H]Sulpiride binding |
|---------------------|-----------------------|
|                     | fmoles/10⁶ cells       |
| pcDNA (7)           | 58.0 ± 2.7             |
| DynaminK44A (4)     | 69.2 ± 9.3             |
| β-Arrestin(318–419) (5) | 64.4 ± 6.0           |

*p < 0.01, compared with the control, unpaired Student’s t test.

Fig. 5. Dopamine inhibition of forskolin-stimulated cAMP accumulation. Cells were incubated in the presence or absence of 1 μM PMA for 10 min. Subsequently, the cells were washed once with pre-warmed EBSS. For the control group, the cells were further incubated with various concentrations of dopamine for 10 min in the presence of 3 μM forskolin, 30 μM Ro-20-1724 (phosphodiesterase inhibitor), 0.2 mM sodium metabisulfite (to prevent oxidation of dopamine), and 10 μM propranolol (to block endogenous β-adrenergic receptors) in 20 mM HEPES-buffered DMEM. For the PMA-treated group, the cells were incubated with the same composition of buffer as in the control group except containing 1 μM PMA. Whole-cell cAMP assays were performed as described under "Experimental Procedures." Data shown represent the means ± S.E. values from three experiments and are expressed as a percentage of forskolin-stimulated cAMP accumulation in the absence of dopamine. The average of estimated EC₅₀ parameters was 11.3 ± 0.5 nM for control and 33.8 ± 3.3 nM for PMA-treated (p < 0.001, Student’s t test, unpaired).

Identification of PKC Phosphorylation Sites within the D2 DAR—We were next interested in testing for potential effects of PMA treatment on the functional coupling of the D2 DAR. We thus examined D2 DAR-mediated inhibition of forskolin-stimulated cAMP accumulation in the HEK293T cells. Fig. 5 shows that under basal conditions, dopamine is able to inhibit the forskolin-stimulated cAMP response by about 70%. Treatment of the cells with PMA did not affect the forskolin response per se (16.4 ± 1.6 pmol/well for control; 14.3 ± 2.4 pmol/well for PMA-treated) nor did it affect the maximum inhibition by dopamine (Fig. 5). In contrast, after PMA treatment, the EC₅₀ for dopamine inhibition of cAMP accumulation was shifted by about 3-fold to lower potency (Fig. 5). This appears to be because of a reduction in the potency of dopamine for eliciting this response rather than a decreased affinity for the receptor as competition radioligand binding assays did not reveal an alteration in the receptor binding affinity of dopamine (data not shown). These findings suggest that another consequence of PKC phosphorylation of the D2 DAR is to attenuate functional G-protein coupling.

Identification of PKC Phosphorylation Sites within the D2 DAR—We were next interested in attempting to identify the putative PKC phosphorylation sites in the D2 DAR protein. PKC recognition and phosphorylation of proteins is known to require basic amino acid residues near the serine or threonine phosphoacceptor group(s) (39). We thus scanned the D2 DAR sequence for all possible PKC recognition motifs, and the results are shown in Fig. 6. It should be noted that we discounted those serine and threonine residues within the 28-amino acid insertion sequence defining the D2L DAR (see Fig. 6), were mutated, there was no change in phosphorylation between the D2S and D2L isoforms (data not shown). When the residues indicated in Fig. 6, either individually or in combination, comparable expression levels of all mutants were confirmed by radioligand binding assays, and identical results were obtained for immunoprecipitation and immunoblots (data not shown). When the residues indicated in black, and with an asterisk (Fig. 6), were mutated, there was no change in PMA-stimulated receptor phosphorylation (data not shown), indicating that they do not serve as substrates for PKC. In contrast, when serines 228 and 229 (Fig. 6) were mutated to alanines, both basal and PMA-stimulated D2 DAR phosphorylation was significantly diminished (Fig. 7, A and B). Individual mutation of either serine 228 or 229 resulted in a small reduc...
tion of receptor phosphorylation, whereas simultaneous mutation of both residues (mutant A) decreased the phosphorylation by about 50% compared with the wild-type receptor (Fig. 7, A and B). These results suggest that serines 228 and 229 in the D<sub>2</sub> DAR are involved in the PKC-mediated phosphorylation but that additional residues are involved as well.

In order to identify the remaining PKC phosphorylation sites, we examined a cluster of residues, threonines 352 and 354 and serine 355, in a more distal segment of the third cytoplasmic loop of the receptor (Fig. 6). Fig. 7, C and D, shows that single mutations of each of these residues reduces the PKC-mediated phosphorylation of the receptor, although the greatest effect was observed with the single S355A mutant. These results suggest that there are two domains of PKC phosphorylation within the D<sub>2</sub> DAR. Domain I consists of serines 228 and 229 and is located in the proximal region of the third cytoplasmic loop (Fig. 6). Domain II consists of serine 355 and threonines 352 and 354 and is located in a more distal segment of the loop (Fig. 6).

To confirm that the domain I and II residues can fully account for the PKC-mediated phosphorylation, we analyzed various combinations of mutations, and the results are shown in Fig. 7, E and F. Each one of the mutant constructs shown in Fig. 7, E and F, has both of the domain I serines (228 and 229) mutated along with selected residues of domain II. Mutant B additionally includes serine 355; mutant D includes threonine 354 and serine 355; mutant E includes threonine 352 and serine 355; and mutant F represents mutation of all five of the

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**Fig. 6.** Diagram of the rat D<sub>2</sub>L dopamine DAR sequence. Gray residues represent those absent in the D<sub>2S</sub> isoform. Black residues represent putative PKC phosphorylation sites. Black residues with asterisks represent potential PKC phosphorylation sites and were mutated to alanine or valine residues, yet these mutations did not result in an alteration of PMA-induced phosphorylation. The numbered black residues correspond to the functionally identified PKC phosphorylation sites as determined by mutational analysis and as discussed in the text. The combination mutants, where more than one residue was mutated, are delineated at the bottom of the figure and are referred to as mutants A–F.
phosphoacceptor amino acids within domains I and II (cf. Fig. 6). Clearly, the vast majority of the PMA-induced phosphorylation is reduced in mutant B, which involves serines 228, 229, and 355; however, when only threonines 352 and 354 are additionally mutated is there a complete loss of the PMA effect (Fig. 7, E and F). These results confirm the notion that PKC phosphorylation sites within the D₂ DAR are involved in regulating its sequestration from the cell surface. Previously, little information has been available concerning the role of regulatory phosphorylation of the D₂ DAR or specific kinases involved. GRKs 2 and 3

**Fig. 8. Role of specific PKC phosphorylation sites in PMA regulation of the D₂ Receptor.**—Because PKC can phosphorylate and regulate many cellular signaling molecules involved in D₂ receptor signaling such as Go₁ (40, 41), GRK2 (42), or adenyl cyclase 2 (43), we wanted to correlate the specific PKC phosphorylation sites on the receptor with the PMA-induced regulatory effects. We initially examined various phosphorylation-defective mutant receptors on PMA-induced receptor sequestration (Fig. 8). There was no difference in the basal cell surface receptor expression levels between wild-type and all the mutant receptors (data not shown). Mutation of serines 228 and 229 to alanines (MutA) diminished PMA-induced D₂ DAR sequestration compared with that of wild-type receptor. In contrast, mutation of serine 355 appeared to have no effect on PMA-induced receptor sequestration as evidenced by the single mutant receptor (S355A) or when mutated in combination with serines 228/229 (MutB). Most interestingly, a mutant with the three domain II substitutions (MutC), which includes S355A, also showed diminished receptor sequestration. This result would imply a role for threonine residues 352 and 354 in the sequestration response. When all five domain I and II residues are mutated (MutF), the PMA-induced sequestration is essentially abolished (Fig. 8). Taken together, these results suggest that both of the PKC phosphorylation sites on the D₂ DAR are involved in regulating its sequestration from the cell surface.

We next evaluated PKC phosphorylation site-defective D₂ DARs for their ability to undergo PMA-induced desensitization (Fig. 9). When all potential PKC phosphorylation sites from domains I and II are simultaneously mutated (MutF), the PMA-induced EC₅₀ shift for dopamine is abolished. The loss of this regulatory response appears to be due to mutation of a single residue, serine 355, as the single S355A mutant shows the same complete loss of response as MutF (Fig. 9). These results indicate that, in contrast to the PMA-induced sequestration response, only serine 355 in domain II is involved in the PMA-induced desensitization.

**DISCUSSION**

In our present study, we have identified and characterized two domains of PKC phosphorylation sites within the D₂ DAR and correlated their phosphorylation with receptor desensitization and internalization. Previously, little information has been available concerning the role of regulatory phosphorylation of the D₂ DAR or specific kinases involved. GRKs 2 and 3
PKC-mediated $D_2$ Receptor Phosphorylation

have been shown to phosphorylate the $D_2$ DAR leading to enhanced agonist-stimulated receptor internalization (18, 19). GRKs 5 and 6 have been suggested to regulate $D_2$ DAR sequestration or desensitization, although direct phosphorylation of the receptor by these kinases has not yet been demonstrated (18, 44). Our investigation is now the first to demonstrate PKC-mediated phosphorylation of the $D_2$ DAR and the first to delineate specific phosphorylation sites within the $D_2$ DAR that regulate functional uncoupling and intracellular trafficking. These results may explain previous reports of phorbol ester effects on $D_2$ DAR functioning (20–24, 45) and also provide a mechanism for how the $D_2$ DAR may be regulated by heterologous desensitization through activation of $G_q$-coupled GPCRs. In fact, our present results show that agonist activation of the M1 muscarinic receptor can lead to $D_2$ DAR phosphorylation. Furthermore, recent studies using endogenous tissues have suggested that the $G_q$-coupled cholecystokinin CCK$_2$ (46) and the neurotensin NT1 (47) receptors can negatively modulate $D_2$ DAR function, possibly through a PKC-mediated mechanism.

It was interesting to find that two domains of PKC phosphorylation sites exist within the $D_2$ DAR, both of which control receptor sequestration, whereas only one (domain II) is associated with receptor desensitization. Furthermore, within domain II only serine 355 seemed to be associated with functional uncoupling of the receptor. How phosphorylation of this residue leads to decreased G-protein coupling is unclear at this time. However, this residue is in close proximity to the distal end of the 3rd cytoplasmic loop near the sixth transmembrane domain. We (48) and others (49) have shown that both the proximal and distal regions of the 3rd cytoplasmic loop of the $D_2$ DAR, near the transmembrane segments, are involved in G-protein coupling. Thus, one hypothesis is that phosphorylation of serine 355 disrupts G-protein coupling of this distal segment of the loop either through an electrostatic or conformational mechanism leading to impaired G-protein coupling and decreased potency for inhibition of adenylyl cyclase activity. This would be similar to the proposed mechanism for PKA-mediated phosphorylation and desensitization of the $\beta_2$-adrenergic receptor (50).

Similarly, the mechanism by which PKC phosphorylation of residues in domains I and II leads to receptor sequestration/internalization remains to be determined. Our results indicate that the PKC-promoted receptor internalization occurs through a $\beta$-arrestin- and dynamin-dependent pathway suggesting the involvement of clathrin-coated pits. Previously, the $D_2$ DAR has been shown to undergo agonist-induced internalization via a dynamin-dependent mechanism (19, 51, 52; however, see 53). One possibility is that phosphorylation of the 3rd cytoplasmic loop by PKC promotes $\beta$-arrestin association in a fashion similar to, but independent from, that proposed for GRK phosphorylation (19). In this case, multisite phosphorylation of the 3rd cytoplasmic loop by PKC would enable its direct association with $\beta$-arrestin leading to receptor internalization. This would represent a novel mechanism for PKC-mediated regulation of GPCRs, which is more usually associated with functional desensitization rather than receptor internalization.

Alternatively, another consideration is that the $D_2$ DAR is proposed to exhibit constitutive activity (54, 55) that may be associated with constitutive GRK phosphorylation and internalization as suggested for constitutively active GPCRs (56, 57). Indeed, the $D_2$ DAR has been shown previously to exhibit constitutive agonist-independent internalization and recycling (53). Thus, another possibility is that PKC phosphorylation facilitates or enhances the constitutive GRK-mediated pathway leading to increased basal internalization. As GRKs prefer to phosphorylate serine or threonine residues in close proximity to negatively charged residues, one possibility is that prior phosphorylation by PKC enhances the affinity of GRKs for neighboring serines or threonines, the phosphorylation of which leads to $\beta$-arrestin association. Experiments designed to differentiate these various possibilities are currently in progress.

It was of interest that we did not observe any evidence for $D_2$ receptor phosphorylation by PKA. This was somewhat surprising given that there are multiple consensus recognition sequences for PKA within the $D_2$ DAR protein, and an earlier report (22) using brain membranes suggested that PKA could negatively modulate $D_2$ DAR function. One possible explanation for this observation would be if HEK293 cells did not express high levels of PKA; however, these cells have been used previously to study PKA-mediated phosphorylation of $\beta_2$-adrenergic receptors (50). Another possibility is that HEK293 cells
lack a necessary adaptor or auxiliary protein that might specifically link PKA to the D₂ DAR. This might be evaluated by using other cell lines to examine D₂ DAR phosphorylation in response to PKA activation. Alternatively, the D₂ DAR may not be a good substrate for PKA and might not be endogenously regulated by this protein kinase.

Our present results also suggest that there is specificity associated with PKC phosphorylation of the D₂ DAR. Previously, HEK293 cells were reported as expressing the μ, ζ, and β₁ isoforms of PKC (31). Consequently, we overexpressed each of these isoforms, as well as PKCβ, and found that only the β₁ isoform leads to increased D₂ DAR phosphorylation. Co-expression of this isoform also leads to increased receptor internalization in response to PMA. Additionally, a PKCβ-specific inhibitor was found to block PMA-induced D₂ DAR phosphorylation. Although it is possible that other, untested, isoforms of PKC might also be capable of phosphorylating the D₂ DAR, it is clear that the β₁ isoform can specifically mediate this response. PKCβ is expressed ubiquitously in multiple tissues and is found at high levels in the brain (58). It will be interesting in future experiments to determine the exact cellular localization of the D₂ DAR and β₁ isoform of PKC. In cells expressing high levels of PKCβ, the D₂ DAR may be particularly susceptible to this form of heterologous regulation.

In summary, we have found that PKC can mediate phosphorylation of the D₂ DAR producing novel functional effects. This regulatory event appears to be specific for the β₁ isoform of PKC, although additional isoforms remain to be tested. Two phosphorylation domains, each containing a cluster of serine or threonine residues, were identified within the 3rd cytoplasmic domain of the receptor. Both of these domains regulate internalization of the receptor, whereas only one is involved in receptor desensitization. These results provide a mechanism by which the D₂ DAR can be regulated in a heterologous fashion through receptors that activate PKC signaling pathways.

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