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Dexamethasone-Induced Ras Protein 1 Negatively Regulates Protein Kinase C δ: Implications for Adenylyl Cyclase 2 Signaling

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ABSTRACT

We identified dexamethasone-induced Ras protein 1 (Dexras1) as a negative regulator of protein kinase C (PKC) δ, and the consequences of this regulation have been examined for adenylyl cyclase (EC 4.6.1.1) type 2 (AC2) signaling. Dexras1 expression in human embryonic kidney 293 cells completely abolished dopamine D_2 receptor-mediated potentiation of AC2 activity, which is consistent with previous reports of its ability to block receptor-mediated $G\beta\gamma$ signaling pathways. In addition, Dexras1 significantly reduced phorbol 12-myristate 13-acetate (PMA)-stimulated AC2 activity but did not alter $G\alpha_s$-mediated cAMP accumulation. Dexras1 seemed to inhibit PMA stimulation of AC2 by interfering with PKCδ autophosphorylation. This effect was selective for the δ isoform because Dexras1 did not alter autophosphorylation of PKCα or PKCε. Dexras1 disruption of PKCδ autophosphorylation resulted in a significant blockade of PKC kinase activity as measured by [γ-^{32}P]ATP incorporation using a PKC-specific substrate. Moreover, Dexras1 and PKCδ coimmunoprecipitated from whole-cell lysates. Dexras1 did not alter the membrane translocation of PKCδ; however, the ability of Dexras1 to interfere with PKCδ autophosphorylation was isoprenylation-dependent as determined using the farnesyltransferase inhibitor methyl {*N*-[2-phenyl-4-*N* [2(*R*)-amino-3-mecaptopropylamino] benzoyl]}-methionate (FTI-277) and a CAAX box-deficient Dexras1 (C277S) mutant. PMA-stimulated AC2 activity was also not affected by Dexras1 C277S. Taken as a whole, these data suggest that Dexras1 functionally interacts with PKCδ at the cellular membrane through an isoprenylation-dependent mechanism to negatively regulate PKCδ activity. Moreover our study suggests that Dexras1 acts to modulate the activation of AC2 in an indirect fashion by inhibiting both $G\beta\gamma$- and PKC-stimulated AC2 activity. The current study provides a novel role for Dexras1 in signal transduction.

Dexras1/activator of G protein signaling 1/Ras dexamethasone-induced 1 was originally identified as a dexamethasone-inducible member of the Ras superfamily of monomeric G proteins (Kemppainen and Behrend, 1998). Dexras1 possesses the consensus guanine nucleotide-binding motif identified in Ras proteins and a membrane-targeting CAAX box at its carboxyl terminus (Cismowski et al., 2000). It has been proposed that Dexras1 may function as a guanine nucleotide exchange factor (GEF) for $G\alpha_{i/o}$ proteins and, consequently, compete with G protein-coupled receptors to disrupt receptor-G protein signaling (Graham et al., 2002, 2004; Takesono et al., 2002). Although Dexras1 has been shown to possess GEF activity for $G\alpha_{i/o}$ subunits in vitro (Cismowski et al., 2000), direct data supporting its role as a GEF in vivo are still lacking. In mammalian cells, Dexras1 blocks agonist-stimulated $G_{i/o}$-coupled receptor activation of extracellular signal-regulated kinase (Graham et al., 2002; Nguyen and Watts, 2005) and $G\beta\gamma$-regulated inwardly rectifying potassium channels (Takesono et al., 2002). Dexras1 also inhibits $G\beta\gamma$-dependent heterologous sensitization of adenylyl cyclase type 1 (AC1) without interfering with the short-term inhibition of AC1 activity by $G\alpha_{i/o}$ subunits (Nguyen and Watts, 2005). In human cancer cell lines, Dexras1 suppresses clonogenic growth through a pertussis toxin-insensitive pathway, although the exact mechanism for this effect is unknown (Vaidyanathan et al., 2004). The contrasting observation that Dexras1 interferes with receptor-mediated $G\beta\gamma$ signaling pathways, without altering receptor-stimulated $G\alpha_{i/o}$ signaling, and that Dexras1 can also regulate aberrant cellular

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ABBREVIATIONS: Dexras1, dexamethasone-induced Ras protein 1; GEF, guanine nucleotide exchange factor; AC, adenylyl cyclase; HEK, human embryonic kidney; PDK1, phosphoinositide-dependent kinase 1; PMA, phorbol 12-myristate 13-acetate; FTI-277, methyl {*N*-[2-phenyl-4-*N* [2(*R*)-amino-3-mecaptopropylamino] benzoyl]}-methionate; PKC, protein kinase C; MAP, mitogen-activated protein; IBMX, 3-isobutyl-1-methylxanthine; NP-40, Nonidet P-40; PBS, phosphate-buffered saline; ANOVA, analysis of variance; cPKC, conventional protein kinase C; nPKC, novel protein kinase C.

growth in a pertussis toxin-insensitive manner suggests the possibility of a more complex role for Dexras1 in signal transduction than was hypothesized previously.

Adenylyl cyclase 2 belongs to the G$\beta\gamma$-stimulated subfamily of adenylyl cyclase isoforms (Hanoune and Defer, 2001). Adenylyl cyclase 2 signaling can be promoted by activators of protein kinase C (PKC) such as diacylglycerol or phorbol esters (Jacobowitz and Iyengar, 1994; Bol et al., 1997). Protein kinase C is a family of serine/threonine kinases composed of at least 12 members that are categorized into three groups based on their regulatory properties: conventional PKCs (cPKCs; α, β, and γ), novel PKCs (nPKCs; ϵ, η, δ, and θ), and atypical PKCs (λ and ζ) (Newton, 2001). The protein kinase C isoform(s) responsible for phosphorylating AC2 has not been identified, although convention suggests that it is a member of the cPKC or nPKC subfamily because atypical PKCs are insensitive to diacylglycerol stimulation. The ability of Dexras1 to inhibit $G_{i/o}$-coupled receptor-stimulated G$\beta\gamma$ signaling pathways suggests that Dexras1 may interfere with the conditional activation of AC2 by G$\beta\gamma$ dimers (Graham et al., 2002; Takesono et al., 2002; Nguyen and Watts, 2005).

In this report, we reveal that Dexras1 abolishes dopamine D_{2L} receptor-mediated potentiation of AC2 activity, presumably by interfering with agonist-stimulated G$\beta\gamma$ signaling. More significantly, we demonstrate that Dexras1 inhibits phorbol ester stimulation of AC2, thereby implicating a role for Dexras1 in PKC-dependent signaling pathways. To provide insight into the mechanisms for this blockade, we used a series of biochemical, pharmacological, and genetic approaches to demonstrate that Dexras1 inhibits PKCδ activity. Taken together, our data reveal that Dexras1 negatively modulates AC2 signaling in an indirect fashion by inhibiting both G$\beta\gamma$- and PKC-stimulated AC2 activity.

Materials and Methods

Materials. [^{3}H]cAMP was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Fetalclone1 serum and bovine calf serum were purchased from Hyclone (Logan, UT). Rabbit Dexras1 antibody was purchased from Calbiochem (San Diego, CA). Isoform-specific phospho-PKC antibodies (PKCα, Thr638; PKCδ, Ser643; PKCϵ, Thr710) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-PKCδ and anti-PKCϵ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG antibody, isoproterenol, phorbol 12-myristate 13-acetate (PMA), quinpirole, isobutylmethylxanthine, and most other reagents were purchased from Sigma-Aldrich (St. Louis, MO). The cDNA for Dexras1 was obtained from Guthrie cDNA Resource Center (http://www.cdna.org).

Cell Culture. HEK293T cells stably expressing the rat dopamine D_{2L} receptor were generated as described previously (Neve et al., 2001). HEK-AC2 cells were generated by transfection using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and selection using G418 (900 μg/ml). G418-resistant colonies were screened and selected based on the response to PMA stimulation. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetalclone1 serum, 5% bovine calf serum, 0.05 U/ml penicillin, and 50 mg/ml streptomycin. Cells were grown in a humidified incubator in the presence of 5% CO_2 at 37°C.

Transfections. At 90 to 95% confluence, transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. For experiments examining AC2 activity in the presence of only wild-type Dexras1 (Fig. 1), cotransfections were performed using the dual expression vector pBudCE4 (Invitrogen) containing AC2 alone or in combination with Dexras1. All other cotransfections were performed using separate plasmids encoding individual cDNAs and transfections were equalized by mass using pcDNA3.

cAMP Accumulation Assay. Cells were seeded in 24-well cluster plates at a concentration of approximately 150,000 cells/well and transfections were performed as described above. At 48 h after transfection, cells were washed with Earle's balanced salt solution assay buffer (Earle's balanced salt solution containing 2% bovine calf serum, 0.025% ascorbic acid, and 15 mM Na^+-HEPES) for 5 min at room temperature. The medium was decanted, and the cells were placed on ice. All stimulations were performed in the presence of 500 μM IBMX at 37°C for 15 min. The stimulation medium was decanted and the reaction was terminated by the addition of 100 to 200 μl of 3% ice-cold trichloroacetic acid. The plates were stored at 4°C overnight before quantification of cAMP using a competitive binding assay adapted from Watts and Neve (1996).

Immunodetection. Cells were seeded in six-well cluster plates at a concentration of approximately 750,000 cells/well and transfections were performed as described above. At 48 h after transfection (72 h if cells were serum-starved overnight), the plates were placed on ice and lysed with ice-cold lysis buffer [1 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 0.3 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 1 μg/ml leupeptin, and 1% Nonidet P-40 (NP-40)] for 10 min. The cells were then scraped from the plates, centrifuged at 13,000*g* for 10 min, and the supernatant was retained for analysis. For subcellular fractionation experiments, cells were lysed with ice-cold lysis buffer (without NP-40) and centrifuged at 100,000*g* for 30 min at 4°C to generate the pellet (membrane) and supernatant (cytosol) fractions. Membrane pellets were then solubilized in ice-cold lysis buffer containing 1% NP-40. Total protein content was determined using a BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL). Protein samples were equalized by dilution and resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene diflouride membranes (Bio-Rad, Hercules, CA). Nonspecific antibody binding was blocked by incubating membranes overnight in 5% nonfat dried milk at 4°C. Membranes were washed with Tris-buffered saline and incubated with the indicated primary antibody for 3 h. The membranes were washed, and immunodetection was accomplished using an enhanced chemifluorescence Western blotting kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) according to the manufacturer's protocol. After final antibody incubation, membranes were again washed, exposed to enhanced chemifluorescence substrate, and then scanned using the Storm Imaging System (Molecular Dynamics, Sunnyvale, CA). Immunoblots were quantified using ImageQuant software according to manufacturer's instructions. Where indicated, the amount of PKCδ autophosphorylation was normalized as the ratio of phospho-PKCδ to total PKCδ expression (phospho-PKCδ/total PKCδ).

Coimmunoprecipitation. Cells in 150-mm tissue culture plates were washed with PBS and then incubated with dithiobis[succinimidylpropionate] (25 mM) at room temperature for 30 min. The reaction was terminated by the addition of 20 mM Tris-HCl, pH 7.5, for 15 min. The cells were then collected in PBS by centrifuging at 200*g* for 10 min and washed twice with PBS. Cells were then incubated with lysis buffer (PBS, 5 mM EDTA, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, and 25 μg/ml aprotinin) at 4°C for 30 min on a rotating platform. The lysate was cleared by centrifuging at 13,000*g* for 30 min at 4°C. The supernatant was incubated with protein G Sepharose beads in the absence of antibody for 1 h at 4°C to remove nonspecifically bound proteins. The supernatant was cleared by centrifugation and transferred to a new tube containing Protein G Sepharose beads and 5 μg of primary antibody at 4°C overnight. The beads were pelleted and washed five times with PBS for 2 min and then resuspended in 2× Laemmli sample buffer for immunoblot analysis as described above.

In Vitro PKC Kinase Assay. PKC activity was measured with the SignaTECT PKC assay kit according to the manufacturer's protocol (Promega, Madison, WI) with minor modifications to directly examine PKC activity. In brief, PKC was enriched from cell lysates with DEAE ion exchange chromatography. The eluate was used to measure [γ-^{32}P]ATP incorporation into the PKC-specific substrate, neurogranin, under control and PKC-stimulated conditions. The PKC stimulation buffer contained (final concentration) 0.25 mM EGTA, 0.1 mg/ml bovine serum albumin, 0.3 mg/ml phosphatidylserine, 0.03 mg/ml diacylglycerol, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 nM PMA, 100 μM biotinylated neurogranin peptide substrate, 100 μM ATP, and 0.5 μCi [γ-^{32}P]ATP (3000 Ci/mM). The control buffer was essentially identical with the stimulation buffer but did not contain phosphatidylserine, diacylglycerol, or PMA. All reactions were performed in the absence of Ca^{2+} to selectively activate nPKC isoforms. The reaction was incubated in a 30°C water bath for 5 min and terminated by the addition of 7.5 M guanidine hydrochloride. An equal amount of reaction mix was spotted onto a streptavidin-coated membrane (*S*-adenosylmethionine synthetase-2 biotin capture membrane; Promega), washed three times each with 2 M NaCl, 2 M NaCl, with 1% H_3PO_4, and once with distilled H_2O. The membrane was dried and counted in an LS6500 scintillation counter (Beckman Coulter, Fullerton, CA). Total protein content from the DEAE eluate was determined using a BCA protein assay kit.

Data and Statistical Analysis. Statistical analyses were performed using Prism and InStat software (GraphPad Software Inc., San Diego, CA). A p value of <0.05 defined significance.

Results

Dexras1 Inhibits G$\beta\gamma$- and PMA-Stimulated AC2 Activity. AC2 is conditionally activated by G$\beta\gamma$ subunits upon the short-term activation of the $G_{i/o}$-coupled dopamine D_{2L} receptor (Watts and Neve, 1997). Because Dexras1 has been shown to block receptor-mediated G$\beta\gamma$-dependent signaling pathways, we investigated the effects of Dexras1 on dopamine D_{2L} receptor-mediated potentiation of AC2 activity. The activity of AC2 was examined by stimulating cells with isoproterenol to activate endogenous $G\alpha_s$-coupled β-adrenergic receptors. Isoproterenol-stimulated cAMP accumulation was significantly increased above basal levels in AC2 transfected cells (Fig. 1A). The $G\alpha_s$-mediated activation of AC2 was retained in Dexras1-transfected cells, because cAMP accumulation values were comparable with those observed in the absence of Dexras1 (Fig. 1A). In agreement with its short-term regulatory properties, costimulation of cells with isoproterenol and the D_2 receptor agonist quinpirole resulted in greater than 50% potentiation of AC2 activity (Fig. 1A). This effect was completely abolished in cells cotransfected with Dexras1, which is consistent with the ability of Dexras1 to block receptor-mediated activation of G$\beta\gamma$ signaling effectors (Fig. 1A).

We examined further the role for Dexras1 in regulating AC2 activity by stimulating cells with the AC2 activator PMA. Cells transfected with only AC2 exhibited robust cAMP accumulation in response to stimulation with PMA compared with basal cAMP levels (Fig. 1B). Similar to the results obtained from $G\alpha_s$ stimulation, cAMP accumulation was potentiated greater than 50% after costimulation with PMA and quinpirole compared with the response from PMA stimulation alone (Fig. 1B). It is surprising that Dexras1 cotransfection significantly reduced PMA-stimulated cAMP accumulation by approximately 80% (Fig. 1B). The ability of D_{2L} receptor activation to potentiate AC2-mediated cAMP accumulation was abolished in Dexras1-cotransfected cells (Fig. 1B). These results suggest that Dexras1 inhibits PKC-mediated activation of AC2 and is the first evidence that Dexras1 may act to obstruct PKC-dependent signaling pathways.

PMA-Stimulated AC2 Activity Is PKCδ-Dependent. As a first step in identifying the mechanisms by which Dexras1 may be inhibiting PMA stimulation of AC2, we used pharmacological inhibitors of PKC to provide insight into the PKC isoform(s) that may be activating AC2. We used two PKC inhibitors with selectivity for phorbol ester-sensitive PKC isoforms: bisindolylmaleimide is a broad-range PKC inhibitor that targets many members of cPKC and nPKC isoforms, whereas rottlerin has greater specificity for PKCδ (Gschwendt et al., 1994). HEK-AC2 cells were examined for PMA-stimulated cAMP accumulation in the absence and presence of bisindolylmaleimide or rottlerin. PMA stimulation of AC2 resulted in a greater than 6-fold increase in cAMP accumulation above basal (Fig. 2A). The PKC inhibitors bisindolylmaleimide and rottlerin significantly reduced the PMA-stimulated cAMP response to approximately 2-fold above basal (Fig. 2A). In contrast, neither PKC inhibitor had any effect on PKC-independent activation of AC2 by $G\alpha_s$ (Fig. 2A). Isoproterenol-stimulated cAMP accumulation remained

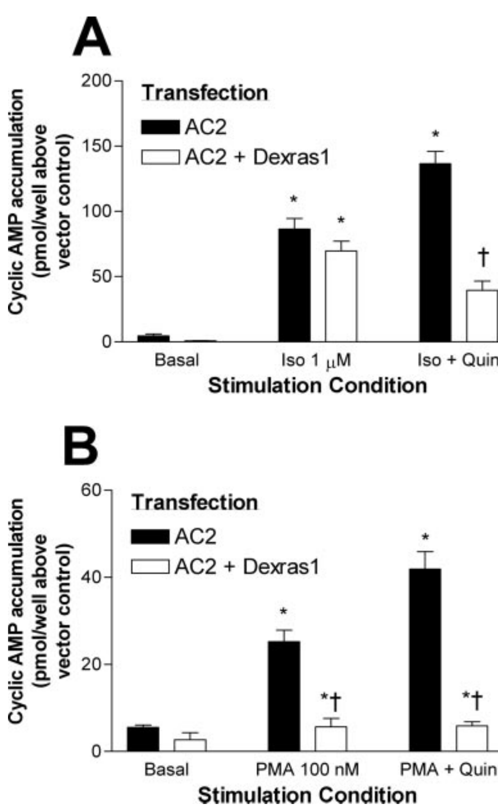

Fig. 1. Dexras1, $G\alpha_s$, G$\beta\gamma$, and PMA modulation of AC2. HEK-D_{2L} cells were cotransfected with AC2 in the absence or presence of Dexras1 using the dual expression vector, pBudCE4, as described under *Materials and Methods*. At 48 h after transfection, cells were stimulated with isoproterenol (A) or PMA (B) in the absence and presence of 1 μM quinpirole as indicated for 15 min at 37°C in the presence of IBMX. cAMP accumulation was measured as described under *Materials and Methods*. The data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. cAMP accumulation is presented as picomoles per well above vector-transfected cells. *, $p < 0.05$ versus matched basal cAMP accumulation. †, $p < 0.05$ versus AC2 transfected cells for each stimulation condition (repeated-measures ANOVA with Bonferroni post-test).

at greater than 6-fold above basal in the presence of both inhibitors (Fig. 2A). These data suggest that PKCδ activity is necessary for phorbol ester stimulation of AC2.

In a second approach to investigate the role of PKCδ in phorbol ester activation of AC2, we performed the converse experiment to determine whether the expression of recombinant PKCδ was sufficient to enhance PMA-stimulated AC2 activity. Cells were transiently transfected with AC2 in the absence and presence of PKCδ and PMA-stimulated AC2 activity was examined. Cells transfected with only AC2 exhibited a significant increase in cAMP accumulation after stimulation with PMA (Fig. 2B). Likewise, when cells were cotransfected with AC2 and PKCδ, a robust increase in PMA-stimulated cAMP accumulation was also observed; however, the magnitude of cAMP accumulation in response to PMA stimulation in the presence of recombinant PKCδ was more than double that from cells transfected with AC2 alone (Fig. 2B). These data confirm that stimulation of PKCδ is sufficient to activate AC2. Taken together, these data suggest that PKCδ plays a prominent role in phorbol ester stimulation of AC2.

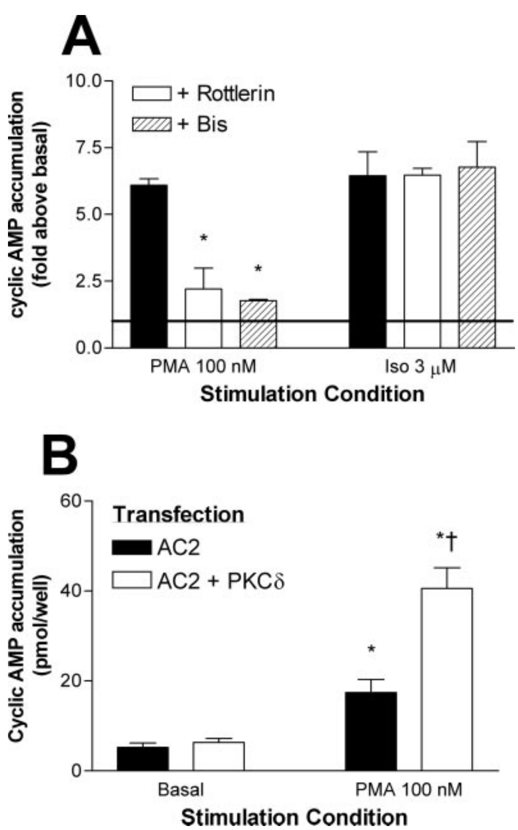

Fig. 2. PKCδ and PMA-dependent activation of AC2. A, HEK-AC2 cells were stimulated with 100 nM PMA or 3 μM isoproterenol in the absence and presence of 25 μM rottlerin or 1 μM bisindolylmaleimide for 15 min at 37°C in the presence of IBMX. cAMP accumulation was measured as described under *Materials and Methods*. The data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. cAMP accumulation is presented as the -fold increase above basal. Basal cAMP accumulation values were the following: 6.3 ± 0.7; + rottlerin, 4.8 ± 0.8; + bis, 5.9 ± 0.3 pmol/well. ∗, $p < 0.05$ versus PMA-stimulated cAMP accumulation alone (repeated-measures ANOVA with Bonferroni posttest). B, HEK-D_{2L} cells were transiently transfected with AC2 in the absence and presence of PKCδ. At 48 h after transfection cells were stimulated with 100 nM PMA for 15 min at 37°C in the presence of IBMX. cAMP accumulation was subsequently measured as described under *Materials and Methods*. The data are presented as mean ± S.E.M. of three independent experiments performed in duplicate. ∗, $p < 0.05$ versus matched basal cAMP accumulation. †, $p < 0.05$ versus AC2 transfected cells for each stimulation condition (repeated-measures ANOVA with Bonferroni post-test).

Dexras1 Interferes with PKCδ Autophosphorylation. The results from our pharmacological experiments prompted us to investigate the effects of Dexras1 on PKCδ autophosphorylation, because autophosphorylation has been identified as a crucial component in regulating PKC catalytic activity (Newton, 2001). Transfection of PKCδ into cells resulted in robust immunoreactivity of phospho-serine 643 (Fig. 3A). However, when cells were cotransfected with PKCδ and Dexras1, PKCδ autophosphorylation was reduced (Fig. 3A). Because a decrease in PKCδ expression levels would decrease PKCδ autophosphorylation levels, the effect of Dexras1 transfection on the expression levels of total PKCδ was also explored. Immunoblot analysis revealed that the levels of endogenous PKCδ in the presence of transfected Dexras1 were 98 ± 11% ($n = 4$) compared with vector-transfected control cells (Fig. 3A, lanes 1 and 2). Likewise, when cells were cotransfected with PKCδ and Dexras1, the expression of total PKCδ (endogenous and recombinant) was 100 ± 4% of that from control cells that were transfected with PKCδ alone (Fig. 3A, lanes 3 and 4). These data provide evidence that Dexras1 interferes with PKCδ autophosphorylation and not PKCδ expression. The effect of Dexras1 on PKCδ autophosphorylation was further analyzed by normalizing the amount of PKCδ autophosphorylation as a ratio of total PKCδ expression (phospho-PKCδ/total PKCδ) for each transfection condition. This analysis was designed to control directly for any effects that total PKCδ expression may have on PKCδ autophosphorylation. The analyses of the normalized data revealed that transfection of Dexras1 reduced PKCδ autophosphorylation by 27 ± 2% compared with cells transfected with PKCδ alone (Fig. 3B). The results of this analysis support our initial immunoblot studies and provide stronger evidence that the effects of Dexras1 on PKCδ autophosphorylation are independent of Dexras1-induced changes in PKCδ expression.

The specificity of Dexras1 to interfere with PKCδ autophosphorylation was examined by evaluating its effects on other PKC isoforms. Cells were transiently transfected with either the conventional PKCα or the novel PKCϵ in the absence and presence of Dexras1. Autophosphorylation of PKCα and PKCϵ was very robust in cells transfected with the respective cDNA (Fig. 3). Cotransfection of Dexras1 did not alter the autophosphorylation of PKCα (Fig. 3C) or PKCϵ (Fig. 3D). Therefore, total protein expression of each PKC isoform was not altered when cotransfected with Dexras1 (data not shown). Dexras1 was robustly coexpressed with PKCα and PKCϵ, indicating that the lack of an effect on the autophosphorylation of these two PKC isoforms was not a result of impaired Dexras1 expression (Fig. 3). These data reveal that Dexras1 acts to interfere with the autophosphorylation of the novel PKC family member PKCδ in an isoform-specific manner.

Dexras1 Inhibits PKC Enzymatic Activity. PKCδ autophosphorylation at serine 643 has been identified to be a key event in regulating its kinase activity (Li et al., 1997). Therefore, we investigated whether the ability of Dexras1 to disrupt autophosphorylation at this residue translated into an ability to inhibit its enzymatic activity. Cells were transiently transfected with PKCδ in the absence and presence of

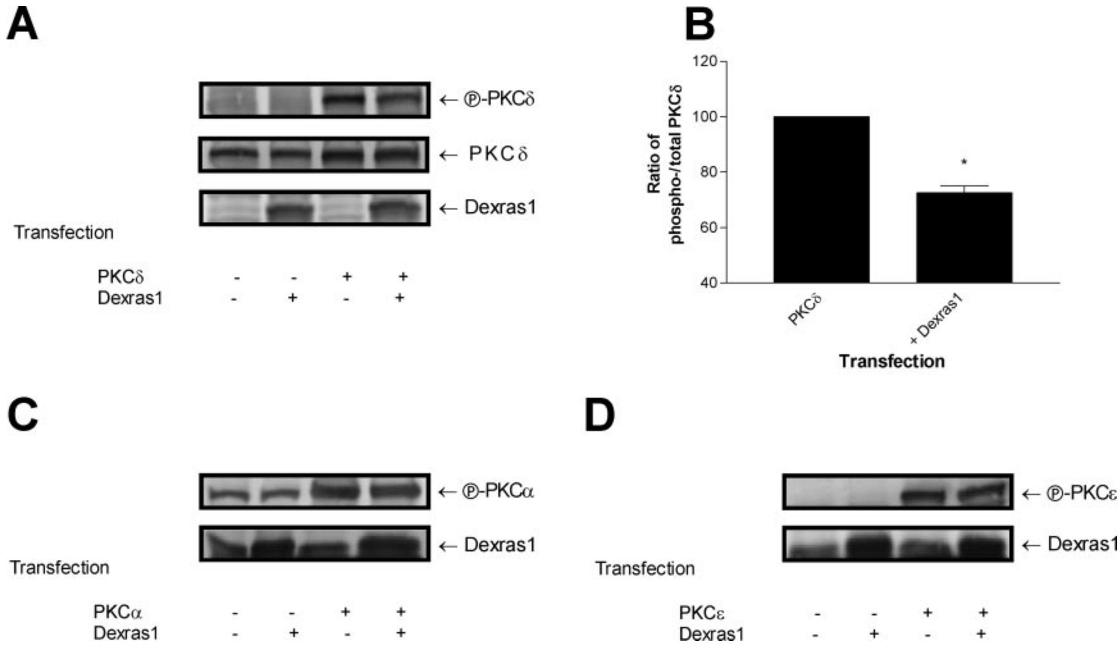

Fig. 3. Dexras1 expression and PKC autophosphorylation. The effect of Dexras1 on PKC autophosphorylation was examined in HEK-D_{2L} cells transiently transfected with PKCδ, PKCα, or PKCϵ in the absence and presence of Dexras1. At 48 h after transfection, cells were serum-starved overnight. Cells were then lysed with ice-cold lysis buffer containing 1% NP-40, and the detergent-soluble cell extracts were examined for autophosphorylation by immunoblot analysis using isoform-specific phospho-PKC antibodies as indicated. The blots were then stripped and reprobed with anti-PKCδ and/or anti-Dexras1 antibody as indicated. Immunoblots shown are representative of at least three independent experiments. B, the phospho-PKCδ and PKCδ blots were examined for pixel intensity for the area under the curve generated for each individual band. The data are presented as phospho-/total PKCδ and have been normalized to values obtained from PKCδ transfection alone. The data are presented as mean ± S.E.M. of four independent experiments. *, $p < 0.05$ compared with PKCδ transfection alone (one-sample *t* test).

Dexras1. DEAE Sepharose ion exchange chromatography was used to enrich PKCδ before performing an in vitro kinase assay designed to examine nPKC enzymatic activity using neurogranin as a PKC-specific substrate. Transfection of PKCδ resulted in a greater than 300% increase in PKC-specific enzymatic activity greater than that of endogenous nPKC isoforms (Fig. 4). Cotransfection of Dexras1 reduced PKC kinase activity by approximately 50% (Fig. 4). Immunoblot analysis of eluates after DEAE chromatography revealed that total PKCδ levels were comparable, whereas there were reduced levels of autophosphorylated PKCδ in eluates from Dexras1-transfected cells (Fig. 4, inset). These data provide evidence that Dexras1 inhibits PKC kinase activity.

Dexras1 Interacts with PKCδ in Intact Cells. We next explored the possibility that Dexras1 may be physically interacting with PKCδ to disrupt its autophosphorylation and enzymatic activity. We therefore examined the ability of Dexras1 to coimmunoprecipitate with PKCδ. Cells were cotransfected with PKCδ and either Dexras1 or vector control. Cell lysates were then subjected to immunoprecipitation with anti-PKCδ antibody, and the immunoprecipitates were examined for the presence of Dexras1 by immunoblot analysis. The blot revealed an approximately 31-kDa band immunoreactive to anti-Dexras1 antibody after PKCδ immunoprecipitation (Fig. 5). The band was more reactive from cells transfected with Dexras1 cDNA compared with that of vector-transfected control cells, although Dexras1 immunoreactivity was also observed in the absence of transfected Dexras1 (Fig. 5). These data suggest that PKCδ can physically associate with endogenous and recombinant Dexras1 in intact cellular systems. Moreover, the combined data implicate this interaction as a mechanism to negatively regulate PKCδ signaling.

Dexras1 Does Not Inhibit PKCδ Membrane Translocation. Although Dexras1 possesses a membrane-targeting CAAX motif, there have been conflicting reports as to its localization within the cell (Cismowski et al., 2000; Fang et al., 2000). Furthermore, studies have suggested that not all

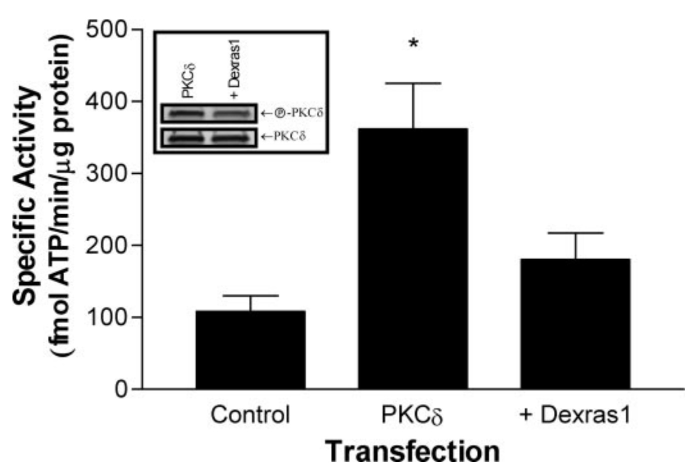

Fig. 4. Dexras1 and PKC kinase activity. HEK-D_{2L} cells were transiently transfected with PKCδ in the absence and presence of Dexras1. At 48 h after transfection, cells were prepared for an in vitro PKC kinase assay as described under *Materials and Methods*. PKC-specific activity was obtained by subtracting nonspecific activity from total catalytic activity. The data are expressed as mean ± S.E.M. of five independent experiments performed in duplicate. *, $p < 0.05$ versus untransfected control cells (repeated-measures ANOVA with Bonferroni post-test). Inset, immunoblot analysis of phospho-PKCδ and total PKCδ from the eluates after DEAE chromatography. Immunoblots shown are representative of two independent experiments.

of Dexras1 effects are isoprenylation-dependent (Graham et al., 2001). One possible mechanism for the ability of Dexras1 to interact and disrupt PKCδ signaling may be that it is acting in the cytosol to interfere with membrane translocation of this kinase. We investigated this possibility by transfecting cells with PKCδ in the absence and presence of Dexras1 to examine the ability of PMA to promote membrane translocation of phospho-PKCδ. Under resting conditions, phosphorylated PKCδ was found predominantly in the cytoplasmic fractions (Fig. 6, lanes 1 and 3). Stimulating cells with PMA for 30 min resulted in the translocation of PKCδ to the membrane fractions (Fig. 6, lanes 2 and 4). Although Dexras1 expression decreased the autophosphorylation of PKCδ, the PMA-induced membrane translocation of PKCδ was retained in cells cotransfected with Dexras1 (Fig. 6, lanes 3 and 4). These data suggest that Dexras1 does not interfere with the membrane translocation of PKCδ to inhibit PMA-stimulated AC2 activity.

Dexras1 Regulation of PKCδ Autophosphorylation Is Isoprenylation-Dependent. Because Dexras1 did not seem to interfere with cytosolic PKCδ to inhibit its membrane translocation, we continued our efforts by investigating the requirement for Dexras1 to be membrane-localized to regulate PKCδ. Our initial experiments used a pharmacological approach to examine the isoprenylation-dependent regulation of PKCδ by Dexras1. Previous studies have determined that H-Ras is a target for farnesylation at its CAAX box; therefore, we used the farnesyl transferase inhibitor FTI-277 based on the high sequence homology between the CAAX box of Dexras1 (CVIS) and that of H-Ras (CVLS). Consistent with our previous data, transfection of PKCδ exhibited robust autophosphorylation at serine 643 that was significantly reduced by Dexras1 (Fig. 7). In contrast, treatment of cells with the peptidomimetic FTI-277 abolished the ability of Dexras1 to negatively regulate PKCδ autophosphorylation (Fig. 7B). PKCδ autophosphorylation was comparable in the absence and presence of Dexras1 when cells were treated with FTI-277 (Fig. 7). The expression of PKCδ was not altered by Dexras1 or FTI-277 (data not shown). These data suggest that Dexras1 is targeted to the cellular membrane through an isoprenylation-dependent mechanism to negatively regulate PKCδ.

To provide support for our pharmacological evidence, we used a CAAX box-deficient Dexras1 mutant (C277S) to determine whether Dexras1 must be targeted to the cellular membrane to disrupt PKCδ autophosphorylation. Initial experiments confirmed the Dexras1 C277S mutant failed to localize to membrane fractions (Fig. 8). In contrast, wild-type

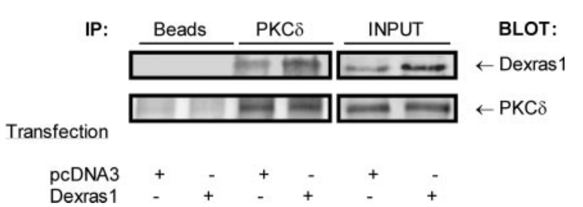

Fig. 5. Dexras1 and PKCδ interactions in intact cells. HEK-D_{2L} cells were cotransfected with PKCδ and either Dexras1 or pcDNA3 control. At 48 h after transfection, cells were treated with 25 mM dithiobis(succinimidyl)-propionate at room temperature for 30 min. Cell lysates were then prepared for an immunoprecipitation assay as described under *Materials and Methods*. The lysate samples were incubated with beads alone or in combination with anti-PKCδ antibody to immunoprecipitate PKCδ. Samples were then examined for Dexras1 immunoreactivity by immunoblot analysis as described under *Materials and Methods*. The blot was then stripped and reprobed with anti-PKCδ antibody. The input lane represents 5 μl of cell lysate samples used for immunoprecipitation. Blot shown is representative of two independent coimmunoprecipitation experiments.

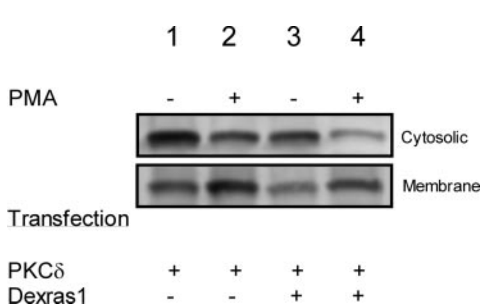

Fig. 6. Dexras1 and PMA-dependent translocation of PKCδ. HEK-D_{2L} cells were transiently transfected with PKCδ in the absence and presence of Dexras1. At 48 h after transfection, cells were serum-starved overnight. Thereafter, cells were stimulated with 100 nM PMA for 30 min at 37°C. The reaction was terminated by removing the media and lysing the cells with ice-cold lysis buffer. Cells were then scraped from the plates and centrifuged at 100,000*g* for 30 min at 4°C to separate the membrane and cytosolic fractions. PKCδ translocation was examined by immunoblot analysis using a phosphospecific PKCδ antibody as described under *Materials and Methods*. Shown is a representative immunoblot from three independent experiments.

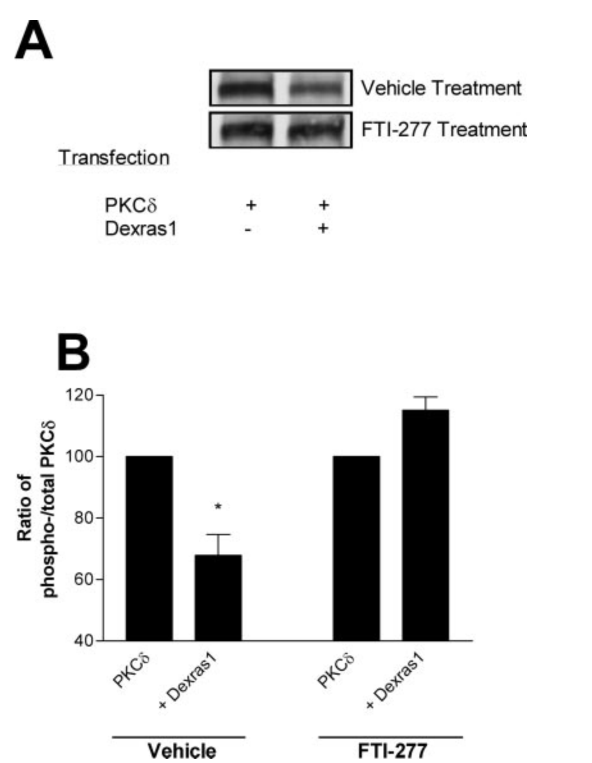

Fig. 7. Dexras1 isoprenylation and PKCδ autophosphorylation. The effect of FTI-277 was examined on Dexras1-mediated disruption of PKCδ autophosphorylation. A, HEK-D_{2L} cells were transiently transfected with PKCδ in the absence and presence of Dexras1. At 5 h after transfection, 10 μM FTI-277 was added to the culture media. At 48 h after transfection, cells were serum-starved overnight, lysed with ice-cold lysis buffer containing 1% NP-40, and the detergent-soluble cell extracts were examined for autophosphorylation by immunoblot analysis using a phosphospecific PKCδ antibody. Immunoblots shown are representative of three independent experiments. B, the phospho-PKCδ and PKCδ blots were examined for pixel intensity for the area under the curve generated for each individual band. The data are presented as phospho-/total PKCδ and have been normalized to values obtained from matched PKCδ transfection alone. The data are presented as mean ± S.E.M. of three independent experiments. *, $p < 0.05$ compared with PKCδ transfection alone (one-sample *t* test).

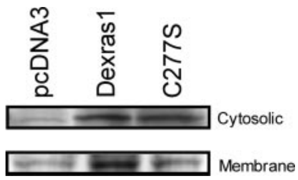

Fig. 8. Cellular localization of wild-type and a CAAX box-deficient Dexras1 mutant (C277S). HEK-D_{2L} cells were transiently transfected with pcDNA3, Dexras1, or Dexras1 C277S. At 48 h after transfection, cells were scraped from the plates and centrifuged at 100,000*g* for 30 min at 4°C to separate the membrane and cytosolic fractions. Dexras1 expression was examined by immunoblot analysis using an anti-Dexras1 antibody as described under *Materials and Methods*. Shown is a representative blot from three independent experiments.

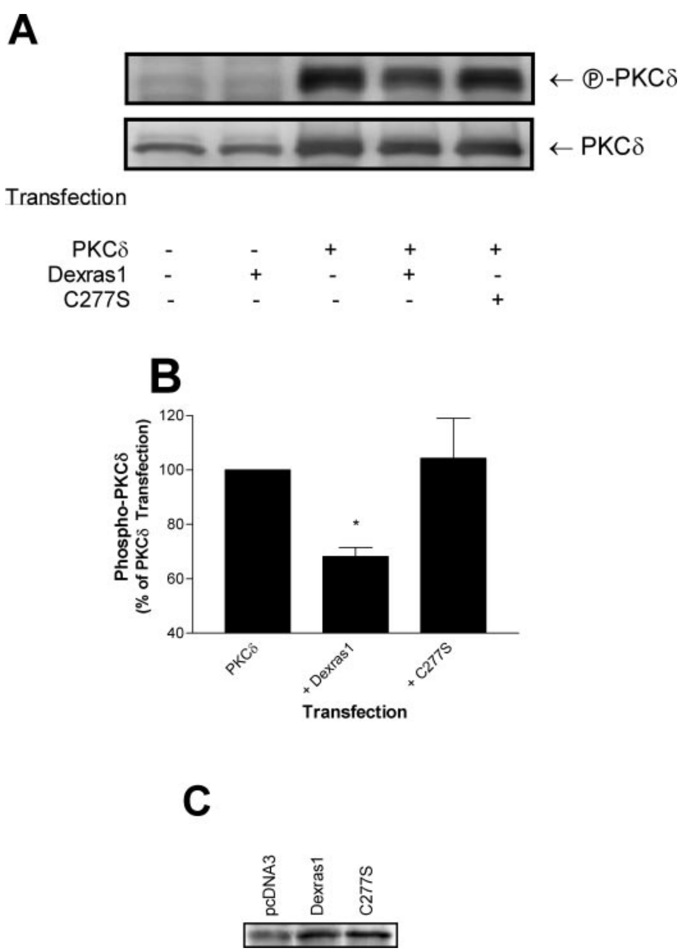

Fig. 9. Dexras1, Dexras1 C277S, and PKCδ autophosphorylation. A, HEK-D_{2L} cells were cotransfected with PKCδ, and either wild-type Dexras1 or Dexras1 C277S as indicated. At 48 h after transfection, cells were serum-starved overnight. Cells were then lysed with ice-cold lysis buffer containing 1% NP-40, and the detergent-soluble cell extracts were examined for PKCδ autophosphorylation by immunoblot analysis as described under *Materials and Methods*. Immunoblot shown is representative of four independent experiments. B, the phospho-PKCδ blots were examined for pixel intensity for the area under the curve generated for each individual band. The data have been normalized to values obtained for phospho-PKCδ immunoreactivity from PKCδ transfection alone. The data are presented as mean ± S.E.M. of four independent experiments. *, $p < 0.05$ compared with PKCδ-transfected conditions (one-sample *t* test). C, HEK-D_{2L} cells were transiently transfected with pcDNA3, Dexras1, or Dexras1 C277S and examined for Dexras1 expression by immunoblot analysis as described under *Materials and Methods* using an anti-Dexras1 antibody. Shown is a representative blot from three independent experiments.

Dexras1 was abundantly expressed in both the cytosolic and membrane fractions (Fig. 8). These data demonstrate that in the absence of an intact CAAX box, Dexras1 fails to localize to the cellular membrane. Our subsequent experiments examined the effect of Dexras1 C277S on PKCδ autophosphorylation. Transient transfection of PKCδ revealed robust autophosphorylation of this novel PKC isoform that was significantly decreased by cotransfecting wild-type Dexras1 (Fig. 9). In contrast, cotransfection of cells with the Dexras1 C277S mutant failed to alter PKCδ autophosphorylation (Fig. 9). Phospho-PKCδ immunoreactivity in the presence of Dexras1 C277S was comparable with that observed when cells were transfected with PKCδ alone. Immunoblot analysis confirmed the expression of wild-type and mutant Dexras1 proteins in whole-cell lysates (Fig. 9C).

Dexras1-Mediated Inhibition of AC2 Signaling Is Isoprenylation-Dependent. We continued our studies with Dexras1 C277S and investigated its effects on PMA-stimulated AC2 activity. Cells were cotransfected with AC2 and either wild-type Dexras1 or Dexras1 C277S and subsequently examined for PMA-stimulated cAMP accumulation. The results of these studies parallel the effects of Dexras1 and Dexras1 C277S on PKCδ autophosphorylation. Transfection of Dexras1 resulted in a significant decrease in PMA stimulation of AC2 (Fig. 10), which is consistent with our earlier findings. In contrast, Dexras1 C277S failed to inhibit PMA-stimulated AC2 activity; PMA-stimulated cAMP accumulation was comparable in the absence and presence of Dexras1 C277S (Fig. 10). These data demonstrate that an intact CAAX box is also required for Dexras1-mediated inhibition of PMA-stimulated AC2 activity. Taken as a whole, our study suggests that Dexras1 functionally interacts with PKCδ at the cellular membrane to interfere with PKC-dependent regulation of AC2 signaling.

Discussion

In this report, we provide evidence that Dexras1 may have a dual role in modulating the activation of AC2 signaling by concurrently blocking PKC and Gβγ activity—two proteins that function as activators of AC2. Dexras1 seemed to preferentially target Gβγ- and PKC-dependent activation of AC2, because $G\alpha_s$-mediated cAMP accumulation was not significantly altered. The ability of Dexras1 to block $G_{i/o}$-coupled

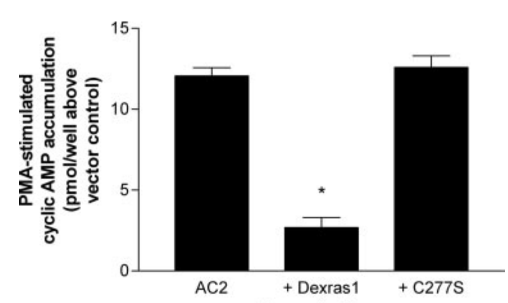

Fig. 10. Dexras1, Dexras1 C277S, and AC2 activity. HEK-D_{2L} cells were cotransfected with PKCδ and either wild-type Dexras1 or Dexras1 C277S as indicated. At 48 h after transfection, cells were stimulated with 300 nM PMA for 15 min at 37°C in the presence of IBMX. cAMP accumulation was subsequently measured as described under *Materials and Methods*. The data are presented as mean ± S.E.M. of three independent experiments performed in duplicate (repeated-measures ANOVA with Bonferroni post-test).

receptor-mediated potentiation of AC2 activity is consistent with previous reports that Dexras1 may function to negatively regulate $G\beta\gamma$-dependent signaling pathways (Cismowski et al., 2000; Graham et al., 2002; Takesono et al., 2002; Nguyen and Watts, 2005). In contrast, the ability of Dexras1 to interfere with phorbol ester regulation of AC2 activity presents a novel role for Dexras1 in signal transduction.

We provide evidence that Dexras1 acts to negatively regulate PKCδ signaling in intact cells. Dexras1 significantly reduced PKCδ autophosphorylation at serine 643 and the functional consequence was a loss of PKCδ catalytic activity. This is in agreement with a previous study that identified serine 643 of PKCδ as an important autophosphorylation site for its enzymatic activity (Li et al., 1997). The role for Dexras1 in regulating PKC function seems to be selective for the δ isoform, as Dexras1 did not interfere with the autoregulation of PKCα or PKCϵ. Moreover, Dexras1 regulation of PKCδ signaling was dependent on isoprenylation-mediated membrane localization, as autophosphorylation of PKCδ was neither altered by a CAAX box-deficient Dexras1 mutant nor when cells were treated with the farnesyltransferase inhibitor FTI-277. These results are consistent with observations that a constitutively active, but CAAX box-deficient Dexras1 mutant (A178V/C277term) failed to inhibit cAMP-stimulated human growth hormone secretion in AtT-20 corticotroph cells in comparison to the 86% reduction of secretion induced by Dexras1A178V alone (Graham et al., 2001). The data presented in this report support a model in which Dexras1 negatively regulates PKCδ through an isoprenylation-dependent mechanism: 1) Dexras1 seems to be post-translationally modified by farnesylation of its CAAX box and localizes to the cellular membrane. 2) At the membrane, Dexras1 functionally interacts with PKCδ and interferes with its autoregulatory mechanisms. 3) The disruption of autophosphorylation results in a decrease in PKC kinase activity. The precise mechanism by which Dexras1 disrupts PKCδ autophosphorylation (step 2 above) is unclear at this time. Whether Dexras1 blocks PKCδ-mediated autophosphorylation at serine 643 or if Dexras1 acts to promote the activity of a phosphatase has yet to be determined. It should be noted that Dexras1 regulation of PKCδ autophosphorylation does not seem to be the sole factor involved in its ability to inhibit phorbol ester-stimulated AC2 activity. The moderate effect of Dexras1 on PKCδ autoregulation is more likely to be a contributing factor toward its larger effects on AC2 activity. As Dexras1 can also regulate $G\beta\gamma$ signaling (Graham et al., 2002; Takesono et al., 2002; Nguyen and Watts, 2005), it may be that Dexras1 interferes with multiple inputs to AC2 that function in an additive or synergistic manner for maximal AC2 activity. Dexras1 may also be involved in other aspects of adenylyl cyclase signaling that have yet to be characterized.

Dexras1 has been proposed to function as a GEF for $G\alpha_{i/o}$ proteins (Cismowski et al., 2000), although there is evidence to suggest that Dexras1 may also regulate pertussis toxin-insensitive pathway (Vaidyanathan et al., 2004). In combination with the results of this study, one interpretation for these data may be that Dexras1 can also regulate $G_{i/o}$ protein signaling through an indirect pathway that involves PKC. For example, the ability of Dexras1 to inhibit receptor-stimulated activation of extracellular signal-regulated kinase (Graham et al., 2002) and heterologous sensitization of AC1 (Nguyen and Watts, 2005) may be partly attributed to the block of PKC activity. Many $G_{i/o}$-coupled receptors have been reported to transactivate MAP kinases through a complex signaling pathway that involves $G\beta\gamma$ regulation of PKC (Wetzker and Bohmer, 2003). Likewise, pertussis toxin-sensitive sensitization of AC isoforms has also been proposed to occur via an intricate $G\beta\gamma$- and PKC-dependent pathway (Thomas and Hoffman, 1996; Varga et al., 2003; Nguyen and Watts, 2005). Therefore, inhibition of PKCδ activity may contribute toward the selective blockade of agonist-stimulated $G\beta\gamma$-dependent signaling pathways by Dexras1. Thus, the model for Dexras1 in G protein-coupled receptor signal transduction might be amended to include an indirect regulation of pertussis toxin-sensitive $G_{i/o}$ protein signaling through a PKCδ-dependent pathway.

Molecular modeling studies have revealed that serine 643 of PKCδ is situated at the apex of a “turn motif” that is conserved in ABC kinases (Newton, 2003). Autophosphorylation of PKC isoforms at this serine/threonine residue in the turn motif locks the enzyme in a catalytically competent conformation (Bornancin and Parker, 1996; Edwards et al., 1999). Our discovery that PKCδ coimmunoprecipitates with endogenous and recombinant Dexras1 from whole-cell lysates suggests that Dexras1 may be interacting with PKCδ at or near its turn motif to interfere with autophosphorylation of serine 643 and inhibit proper catalytic function. It is currently unclear, however, why Dexras1 specifically targets the δ isoform. One possible explanation may be that Dexras1 functions as a physiological regulator of PKCδ activity. In contrast to most PKC isoforms that require “priming” by the upstream phosphoinositide-dependent kinase 1 (PDK1) for catalytic function, PKCδ has been shown to possess modest kinase activity in the absence of phosphorylation by PDK1 (Stempka et al., 1997; Gschwendt, 1999). This effect has been attributed to a glutamic acid residue situated five positions upstream of the PDK1 phosphorylation site (threonine 505) that may provide the negative charge required for structural integrity and catalytic function (Stempka et al., 1999). Because PKCδ seems to be processed as a semiactive enzyme, Dexras1 may serve to suppress its basal kinase activity until the proper signal is relayed.

PKCδ is involved in many cellular processes such as growth, differentiation, and apoptosis (Kikkawa et al., 2002). PKCδ has been implicated to have a prominent role in oncogenesis. For example, regulation of PKCδ activity in rat primary tumors using a PKCδ inhibitory peptide was shown to decrease the metastatic potential of primary mammary tumor as determined by the development of secondary lung metastases (Kiley et al., 1999). In the MDA-MB-231 and MCF-7 human breast cancer cell lines, PKCδ has been shown to act as a prosurvival and proproliferative factor (McCracken et al., 2003; De Servi et al., 2005). Furthermore, PKCδ has been identified to be the predominant isoform expressed in MCF-7 cells, and antiestrogen resistance of these cells is associated with the up-regulation of PKCδ expression (Shanmugam et al., 1999; Nabha et al., 2005). Together, these studies suggest factors that act to impair PKCδ signaling may have a role in regulating oncogenesis. Dexras1 expression has been shown to inhibit clonogenic growth of MCF-7 and A549 cells (Vaidyanathan et al., 2004), which suggests that Dexras1 may have a regulatory role in oncogenesis by inhibiting PKCδ activity. Although protein kinase C has historically been believed to be pro-oncogenic by activating MAP kinase pathways (Hofmann, 2004), PKC isoforms might also regulate cellular proliferation by promoting adenylyl cyclase signaling, because studies support a positive role for cAMP in cell growth and proliferation (Stork and Schmitt, 2002). Thus, the antiproliferative effects of Dexras1 might be associated with its ability to inhibit PKCδ activity to negatively regulate two distinct oncogenic pathways: cAMP signaling, as we demonstrate in this report, and MAP kinase activation (Graham et al., 2002; Nguyen and Watts, 2005).

In summary, the current study identifies a novel role for Dexras1 in cellular communication. We provide evidence that Dexras1 acts to negatively modulate AC2 signaling by interfering with PKCδ activity through an isoprenylation-dependent mechanism. These results are consistent with reports of a pertussis toxin-independent mechanism for Dexras1 in intact cells (Vaidyanathan et al., 2004). The role for Dexras1 in regulating PKCδ activity may provide novel therapeutic targets for drug therapy, because many physiological and pathophysiological processes are associated with altered PKCδ signaling.

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