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Dexras1 Negatively Regulates Protein Kinase C δ:

Implications for Adenylyl Cyclase 2 Signaling

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Running Title: Dexras1 Inhibits PKCδ

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Abbreviations: AC (1-9), adenylyl cyclase type 1-9; AGS1, activator of G protein signaling 1; Dexras1, dexamethasone-induced Ras protein 1; ERK 1/2, extracellular-signal regulated kinase; GEF, guanine nucleotide exchange factor; GIRK, Gβγ-regulated inwardly rectifying potassium channel; GPCR, G protein coupled receptor; HEK, human embryonic kidney; PDK1, phosphoinositide-dependent kinase 1; PMA, phorbol 12-myristate 13-acetate

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Abstract

We have identified 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 HEK293 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 Gβγ signaling pathways. Additionally, Dexras1 significantly reduced PMA-stimulated AC2 activity but did not alter $G\alpha_s$-mediated cyclic AMP accumulation. Dexras1 appeared to inhibit PMA stimulation of AC2 by interfering with PKCδ autophosphorylation. This effect was selective for the δ isoform, as 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 [^{32}P]γ-ATP incorporation using a PKC-specific substrate. Moreover, Dexras1 and PKCδ co-immunoprecipitated 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, FTI-277, and a CAAX box-deficient Dexras1 (C277S) mutant. PMA-stimulated AC2 activity was also not affected by Dexras1C277S. Collectively, 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 Gβγ- and PKC-stimulated AC2 activity. The current study provides a novel role for Dexras1 in signal transduction.

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Dexras1/AGS1/RASD1 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, as well as a membrane-targeting CAAX box at its carboxy 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 (GPCR) to disrupt receptor-G protein signaling (Graham et al., 2002; Graham et al., 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* is still lacking. In mammalian cells, Dexras1 blocks agonist-stimulated $G_{i/o}$-coupled receptor activation of extracellular signal-regulated kinase (ERK 1/2) (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 acute 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 growth in a pertussis toxin-insensitive manner suggests the possibility of a more complex role for Dexras1 in signal transduction than previously hypothesized.

Adenylyl cyclase 2 belongs to the $G\beta\gamma$-stimulated subfamily of adenylyl cyclase isoforms (for review, see Hanoune and Defer, 2001). Adenylyl cyclase 2 signaling can be promoted by

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

In this report we reveal that Dexras1 abolishes dopamine D_{2L} receptor-mediated potentiation of AC2 activity, presumably by interfering with agonist-stimulated Gβγ 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 have employed a series of biochemical, pharmacological, and genetic approaches to demonstrate that Dexras1 inhibits PKCδ activity. Collectively, our data reveal that Dexras1 negatively modulates AC2 signaling in an indirect fashion by inhibiting both Gβγ- and PKC-stimulated AC2 activity.

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MATERIALS & METHODS

Materials – [^{3}H]Cyclic AMP was purchased from PerkinElmer Life Science Products (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 was generated as described previously (Neve et al., 2001). HEK-AC2 cells were generated by transfection using Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) 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 90-95 % confluency, transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. For experiments examining AC2 activity in the presence of only wild-type Dexras1 (Figure 1), co-transfections were performed using the dual expression vector, pBudCE4 (Invitrogen), containing AC2 alone or in combination with Dexras1. All other co-transfections

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were performed using separate plasmids encoding individual cDNAs and transfections were equalized by mass using pcDNA3.

Cyclic AMP 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 hours post-transfection, cells were washed with Earle's balanced salt solution (EBSS) assay buffer (EBSS 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 media was decanted and the reaction was terminated by the addition of 100-200 μl of 3 % ice-cold trichloroacetic acid. The plates were stored at 4°C overnight before quantification of cyclic AMP using a competitive binding assay adapted from Watts and Neve (Watts and Neve, 1996).

Immunodetection – Cells were seeded in 6-well cluster plates at a concentration of approximately 750 000 cells/well and transfections were performed as described above. At 48 hours post-transfection (72 hr 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 DTT, 0.3 mM PMSF, 20 ug/ml aprotinin, 1 μg/ml leupeptin, plus 1 % NP-40) for 10 min. The cells were then scraped from the plates, centrifuged at 13 000 x *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 x *g* for 30 minutes 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

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equalized by dilution and resolved by SDS-polyacrylamide gel electrophoresis and electro-transferred to Bio-Rad polyvinylidene difluoride (PVDF) membranes (Hercules, CA). Nonspecific antibody binding was blocked by incubating membranes overnight in 5 % non fat dried milk at 4°C. Membranes were washed with Tris-buffered saline and incubated with the indicated primary antibody for 3 hr. The membranes were washed and immunodetection was accomplished using the Amersham Life Sciences enhanced chemifluorescence (ECF) western blotting kit (Buckinghamshire, UK) according to the manufacturer's protocol. Following final antibody incubation, membranes were again washed, exposed to ECF substrate, and then scanned using the Molecular Dynamics Storm Imaging System (Sunnyvale, CA). Immunoblots were quantified using ImageQuant software according to manufacturer instructions. Where indicated the amount of PKCδ autophosphorylation was normalized as the ratio of phospho-PKCδ to total PKCδ expression (phospho-PKCδ/total PKCδ).

Co-immunoprecipitation – 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 addition of 20 mM Tris-HCl (pH 7.5) for 15 minutes. The cells were then collected in PBS by centrifuging at 200 x *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 PMSF, 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 x *g* for 30 min at 4°C. The supernatant was incubated with Protein G sepharose beads in the absence of antibody for 1 hr 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 primary antibody at 4°C overnight.

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The beads were pelleted and washed 5 times with PBS for 2 minutes, and then resuspended in 2x laemmli sample buffer for immunoblot analysis as described above.

In vitro PKC Kinase Assay – PKC activity was measured with the Promega SignaTECT PKC assay kit according to the manufacturer's protocol with minor modifications to directly examine PKCδ activity. Briefly, PKC was enriched from cell lysates with DEAE ion exchange chromatography. The eluate was used to measure [^{32}P]γ-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 BSA, 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 [^{32}P]γ-ATP (3000Ci/mM). The control buffer was essentially identical to 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 (Promega SAM^2 biotin capture membrane), washed three times each with 2 M NaCl, 2 M NaCl with 1 % H_3PO_4, and once with dH_2O. The membrane was dried and counted in a Beckman LS6500 scintillation counter. Total protein content from the DEAE eluate was determined using a BCA protein assay kit.

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

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RESULTS

Dexras1 Inhibits Gβγ- and PMA-stimulated AC2 Activity

AC2 is conditionally activated by Gβγ subunits upon acute activation of the $G_{i/o}$-coupled dopamine D_{2L} receptor (Watts and Neve, 1997). Since Dexras1 has been shown to block receptor-mediated Gβγ-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 cyclic AMP accumulation was significantly increased above basal levels in AC2 transfected cells (Figure 1a). The $G\alpha_s$-mediated activation of AC2 was retained in Dexras1 transfected cells, as cyclic AMP accumulation values were comparable to those observed in the absence of Dexras1 (Figure 1a). In agreement with its acute regulatory properties, co-stimulation of cells with isoproterenol and the D_2 receptor agonist, quinpirole, resulted in greater than 50 % potentiation of AC2 activity (Figure 1a). This effect was completely abolished in cells co-transfected with Dexras1, which is consistent with the ability of Dexras1 to block receptor-mediated activation of Gβγ signaling effectors (Figure 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 cylic AMP accumulation in response to stimulation with PMA when compared to basal cyclic AMP levels (Figure 1b). Similar to the results obtained from $G\alpha_s$ stimulation, cyclic AMP accumulation was potentiated greater than 50 % following co-stimulation with PMA and quinpirole in comparison to the response from PMA stimulation alone (Figure 1b). Surprisingly, Dexras1 co-transfection significantly reduced PMA-stimulated cyclic AMP accumulation approximately 80 % (Figure 1b). The ability of D_{2L} receptor activation to potentiate AC2-mediated cyclic AMP

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accumulation was abolished in Dexras1 co-transfected cells (Figure 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 employed 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 cyclic AMP accumulation in the absence and presence of bisindolylmaleimide or rottlerin. PMA stimulation of AC2 resulted in greater than 6-fold increase in cyclic AMP accumulation above basal (Figure 2a). The PKC inhibitors bisindolylmaleimide and rottlerin significantly reduced the PMA-stimulated cyclic AMP response to approximately 2-fold above basal (Figure 2a). In contrast, neither PKC inhibitor had any effect on PKC-independent activation of AC2 by $G\alpha_s$ (Figure 2a). Isoproterenol-stimulated cyclic AMP accumulation remained at greater than 6-fold above basal in the presence of both inhibitors (Figure 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 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 cyclic AMP accumulation

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following stimulation with PMA (Figure 2b). Likewise, when cells were co-transfected with AC2 and PKCδ, a robust increase in PMA-stimulated cyclic AMP accumulation was also observed; however, the magnitude of cyclic AMP accumulation in response to PMA stimulation in the presence of recombinant PKCδ was more than double that from cells transfected with AC2 alone (Figure 2b). These data confirm that stimulation of PKCδ is sufficient to activate AC2. Collectively, these data suggest that PKCδ plays a prominent role in phorbol ester stimulation of AC2.

Dexras1 Interferes with PKCδ Autophosphorylation

The results from our pharmacological experiments prompted us to investigate the effects of Dexras1 on PKCδ autophosphorylation, as 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 (Figure 3a). However, when cells were co-transfected with PKCδ and Dexras1, PKCδ autophosphorylation was reduced (Figure 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 were also explored. Immunoblot analysis revealed that the levels of endogenous PKCδ in the presence of transfected Dexras1 were 98 ± 11 % (n=4) when compared to vector transfected control cells (Figure 3a, lanes 1 & 2). Likewise, when cells were co-transfected 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 (Figure 3a, lanes 3 & 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

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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% when compared to cells transfected with PKCδ alone (Figure 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 (Figure 3). Co-transfection of Dexras1 did not alter the autophosphorylation of PKCα (Figure 3c) or PKCε (Figure 3d). Accordingly, total protein expression of each PKC isoform was not altered when co-transfected with Dexras1 (data not shown). Dexras1 was robustly co-expressed with PKCα and PKCε, indicating that the lack of an effect on the autophosphorylation of these two PKC isoforms was not due to impaired Dexras1 expression (Figure 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 if the ability of Dexras1 to disrupt autophosphorylation at this residue translated into an ability to inhibit its

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enzymatic activity. Cells were transiently transfected with PKCδ in the absence and presence of 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 greater than 300 % increase in PKC specific enzymatic activity above that of endogenous nPKC isoforms (Figure 4). Co-transfection of Dexras1 reduced PKC kinase activity by approximately 50 % (Figure 4). Immunoblot analysis of eluates following DEAE chromatography revealed that total PKCδ levels were comparable, whereas there was reduced levels of autophosphorylated PKCδ in eluates from Dexras1-transfected cells (Figure 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 co-immunoprecipitate with PKCδ. Cells were co-transfected 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 following PKCδ immunoprecipitation (Figure 5). The band was more reactive from cells transfected with Dexras1 cDNA compared to that of vector transfected control cells, although Dexras1 immunoreactivity was also observed in the absence of transfected Dexras1 (Figure 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.

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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 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 (Figure 6, lanes 1 & 3). Stimulating cells with PMA for 30 minutes resulted in the translocation of PKCδ to the membrane fractions (Figure 6, lanes 2 & 4). Although Dexras1 expression decreased the autophosphorylation of PKCδ, the PMA-induced membrane translocation of PKCδ was retained in cells co-transfected with Dexras1 (Figure 6, lanes 3 & 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

Since Dexras1 did not appear 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 employed 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,

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transfection of PKCδ exhibited robust autophosphorylation at serine 643 that was significantly reduced by Dexras1 (Figure 7). In contrast, treatment of cells with the peptidomimetic, FTI-277, abolished the ability of Dexras1 to negatively regulate PKCδ autophosphorylation (Figure 7b). PKCδ autophosphorylation was comparable in the absence and presence of Dexras1 when cells were treated with FTI-277 (Figure 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 utilized a CAAX box-deficient Dexras1 mutant (C277S) to determine if Dexras1 must be targeted to the cellular membrane to disrupt PKCδ autophosphorylation. Initial experiments confirmed the Dexras1C277S mutant failed to localize to membrane fractions (Figure 8). In contrast, wild-type Dexras1 was abundantly expressed in both the cytosolic and membrane fractions (Figure 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 Dexras1C277S on PKCδ autophosphorylation. Transient transfection of PKCδ revealed robust autophosphorylation of this novel PKC isoform that was significantly decreased by co-transfecting wild-type Dexras1 (Figure 9). In contrast, co-transfection of cells with the Dexras1C277S mutant failed to alter PKCδ autophophorylation (Figure 9). Phospho-PKCδ immunoreactivity in the presence of Dexras1C277S was comparable to 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 (Figure 9c).

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Dexras1-mediated Inhibition of AC2 Signaling is Isoprenylation-dependent

We continued our studies with Dexras1C277S and investigated its effects on PMA-stimulated AC2 activity. Cells were co-transfected with AC2 and either wild-type Dexras1 or Dexras1C277S and subsequently examined for PMA-stimulated cyclic AMP accumulation. The results of these studies parallel the effects of Dexras1 and Dexras1C277S on PKCδ autophosphorylation. Transfection of Dexras1 resulted in a significant decrease in PMA stimulation of AC2 (Figure 10), which is consistent with our earlier findings. In contrast, Dexras1C277S failed to inhibit PMA-stimulated AC2 activity; PMA-stimulated cyclic AMP accumulation was comparable in the absence and presence of Dexras1C277S (Figure 10). These data demonstrate that an intact CAAX box is also required for Dexras1-mediated inhibition of PMA-stimulated AC2 activity. Collectively, our study suggests that Dexras1 functionally interacts with PKCδ at the cellular membrane to interfere with PKC-dependent regulation of AC2 signaling.

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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 appeared to preferentially target Gβγ- and PKC-dependent activation of AC2, as $G\alpha_s$-mediated cyclic AMP accumulation was not significantly altered. The ability of Dexras1 to block $G_{i/o}$-coupled receptor-mediated potentiation of AC2 activity is consistent with previous reports that Dexras1 may function to negatively regulate Gβγ-dependent signaling pathways (Cismowski et al., 2000; Graham et al., 2002; Nguyen and Watts, 2005; Takesono et al., 2002). 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 appears 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 hGH 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

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which Dexras1 negatively regulates PKCδ through an isoprenylation-dependent mechanism: (1) Dexras1 appears 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 appear 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 towards its larger effects on AC2 activity. As Dexras1 can also regulate Gβγ signaling (Graham et al., 2002; Nguyen and Watts, 2005; Takesono et al., 2002), 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 ERK 1/2 (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

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signaling pathway that involves Gβγ regulation of PKC (Wetzker and Bohmer, 2003). Similarly, pertussis toxin-sensitive sensitization of AC isoforms has also been proposed to occur via an intricate Gβγ- and PKC-dependent pathway (Nguyen and Watts, 2005; Thomas and Hoffman, 1996; Varga et al., 2003). Therefore, inhibition of PKCδ activity may contribute towards the selective blockade of agonist-stimulated Gβγ-dependent signaling pathways by Dexras1. Thus, the model for Dexras1 in GPCR 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 co-immunoprecipitates 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 (Gschwendt, 1999; Stempka et al., 1997). 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). As PKCδ appears to be

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processed as a semi-active 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 pro-survival and pro-proliferative factor (De Servi et al., 2005; McCracken et al., 2003). Furthermore, PKCδ has been identified to be the predominant isoform expressed in MCF-7 cells, and anti-estrogen resistance of these cells is associated with the upregulation of PKCδ expression (Nabha et al., 2005; Shanmugam et al., 1999). Collectively, 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 classically been thought to be pro-oncogenic by activating MAP kinase pathways (Hofmann, 2004), PKC isoforms might also regulate cellular proliferation by promoting adenylyl cyclase signaling, as studies support a positive role for cyclic AMP in cell growth and proliferation (Stork and Schmitt, 2002). Thus, the anti-proliferative effects of Dexras1 might be associated with its ability to inhibit PKCδ activity to negatively regulate two distinct oncogenic pathways: cyclic AMP 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, as many physiological and pathophysiological processes are associated with altered PKCδ signaling.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Dexras1, $G\alpha_s$, $G\beta\gamma$, and PMA modulation of AC2. HEK-D_{2L} cells were co-transfected with AC2 in the absence or presence of Dexras1 using the dual expression vector, pBudCE4, as described in Materials and Methods. At 48 hr post-transfection cells were stimulated with (A) isoproterenol or (B) PMA in the absence and presence of 1 μM quinpirole as indicated, for 15 min at 37°C in the presence of IBMX. Cyclic AMP accumulation was measured as described in Materials and Methods. The data are presented as mean ± S.E.M. of 3 independent experiments performed in duplicate. Cyclic AMP accumulation is presented as pmol/well above vector-transfected cells. * $p < 0.05$ versus matched basal cyclic AMP accumulation. † $p < 0.05$ versus AC2 transfected cells for each stimulation condition (Repeated measures ANOVA with Bonferroni posttest).

Figure 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. Cyclic AMP accumulation was measured as described in Materials and Methods. The data are presented as mean ± S.E.M. of 3 independent experiments performed in duplicate. Cyclic AMP accumulation is presented as fold above basal. Basal cyclic AMP accumulation values were: 6.3 ± 0.7; + Rottlerin, 4.8 ± 0.8; + Bis, 5.9 ± 0.3 pmol/well. * $p < 0.05$ versus PMA-stimulated cyclic AMP 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 hr post-transfection cells were stimulated with 100 nM PMA for 15 min at 37°C in the presence of

IBMX. Cyclic AMP accumulation was subsequently measured as described in Materials and Methods. The data are presented as mean ± S.E.M. of 3 independent experiments performed in duplicate. * $p < 0.05$ versus matched basal cyclic AMP accumulation. † $p < 0.05$ versus AC2 transfected cells for each stimulation condition (Repeated measures ANOVA with Bonferroni posttest).

Figure 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 hr post-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 4 independent experiments. * $p < 0.05$ compared to PKCδ transfection alone (One-sample t test).

Figure 4. Dexras1 and PKC kinase activity. HEK-D_{2L} cells were transiently tranfected with PKCδ in the absence and presence of Dexras1. At 48 hr post-transfection cells were prepared for an *in vitro* PKC kinase assay as described in Materials and Methods. PKC specific activity was obtained by substracting nonspecific activity from total catalytic activity. The data are expressed

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as mean ± S.E.M. of 5 independent experiments performed in duplicate. * $p < 0.05$ versus untransfected control cells (Repeated measures ANOVA with Bonferroni posttest). Inset, immunoblot analysis of phospho-PKCδ and total PKCδ from the eluates following DEAE chromatography. Immunoblots shown are representative of two independent experiments.

Figure 5. Dexras1 and PKCδ interactions in intact cells. HEK-D_{2L} cells were co-transfected with PKCδ and either Dexras1 or pcDNA3 control. At 48 hr post-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 in 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 in 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 co-immunoprecipitation experiments.

Figure 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 hr post-transfection cells were serum starved overnight. Subsequently, 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 x g for 30 min at 4°C to separate the membrane and cytosolic fractions. PKCδ translocation was examined

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by immunoblot analysis using a phospho-specific PKCδ antibody as described in Materials and Methods. Shown is a representative immunoblot from three independent experiments.

Figure 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 hr post-transfection 10 μM FTI-277 was added to the culture media. At 48 hr post-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 phospho-specific 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 3 independent experiments. * $p < 0.05$ compared with PKCδ transfection alone (One-sample t test).

Figure 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 Dexras1C277S. At 48 hr post-transfection cells were scraped from the plates and centrifuged at 100 000 x 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 in Materials and Methods. Shown is a representative blot from three independent experiments.

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Figure 9. Dexras1, Dexras1C277S, and PKCδ autophosphorylation. (A) HEK-D_{2L} cells were co-transfected with PKCδ and either wild-type Dexras1 or Dexras1C277S as indicated. At 48 hr post-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 in 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 4 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 Dexras1C277S and examined for Dexras1 expression by immunoblot analysis as described in Materials and Methods using an anti-Dexras1 antibody. Shown is a representative blot from 3 independent experiments.

Figure 10. Dexras1, Dexras1C277S, and AC2 activity. HEK-D_{2L} cells were co-transfected with PKCδ and either wild-type Dexras1 or Dexras1C277S as indicated. At 48 hr post-transfection cells were stimulated with 300 nM PMA for 15 min at 37°C in the presence of IBMX. Cyclic AMP accumulation was subsequently measured as described in Materials and Methods. The data are presented as mean ± S.E.M. of 3 independent experiments performed in duplicate (Repeated measures ANOVA with Bonferroni posttest).

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Figure 1

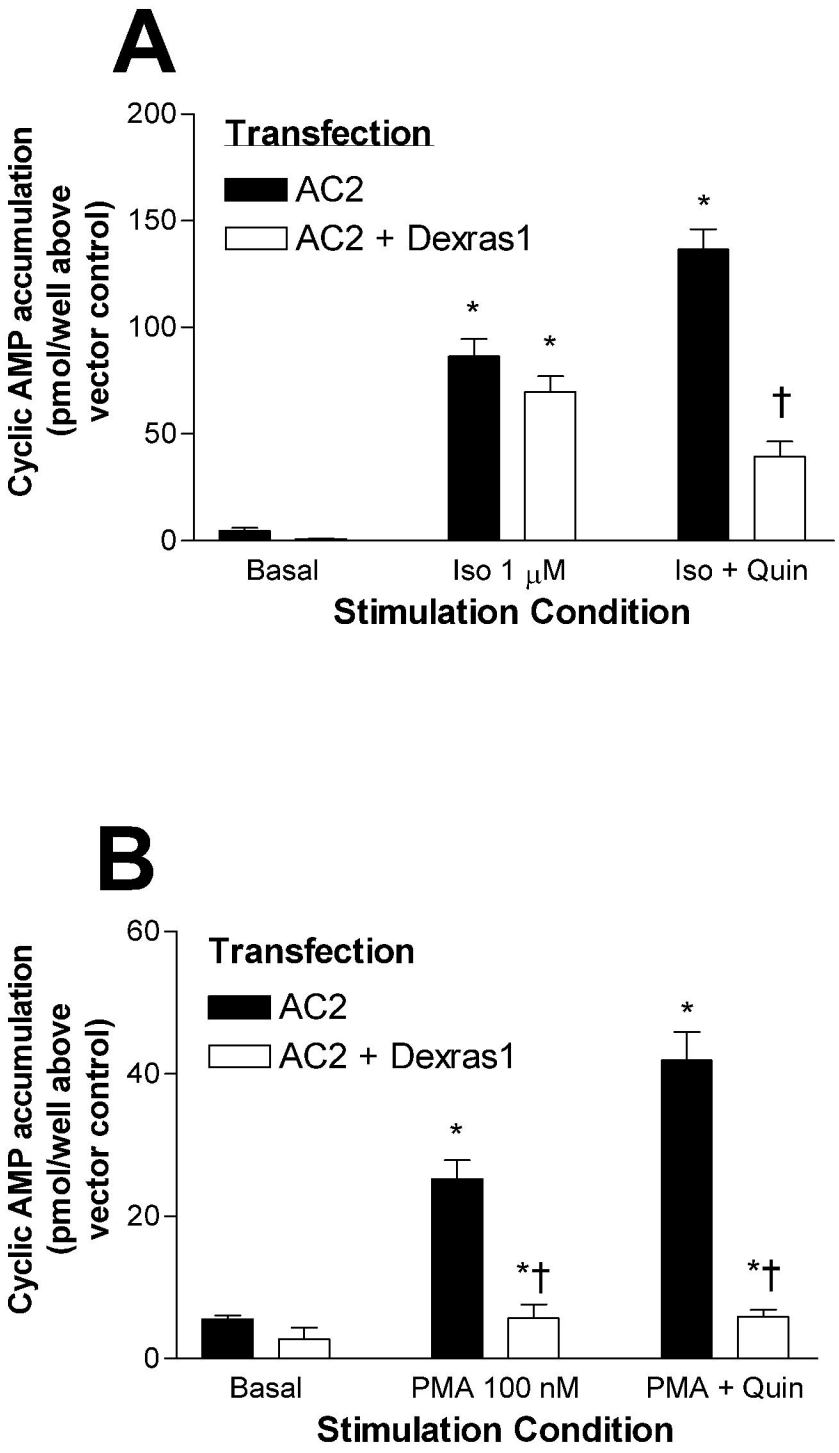

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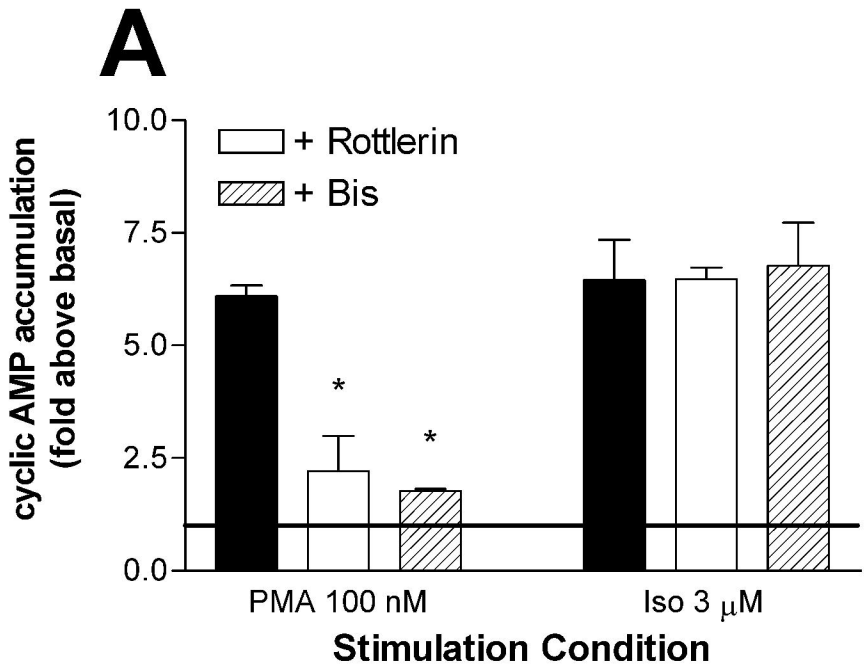
Figure 2



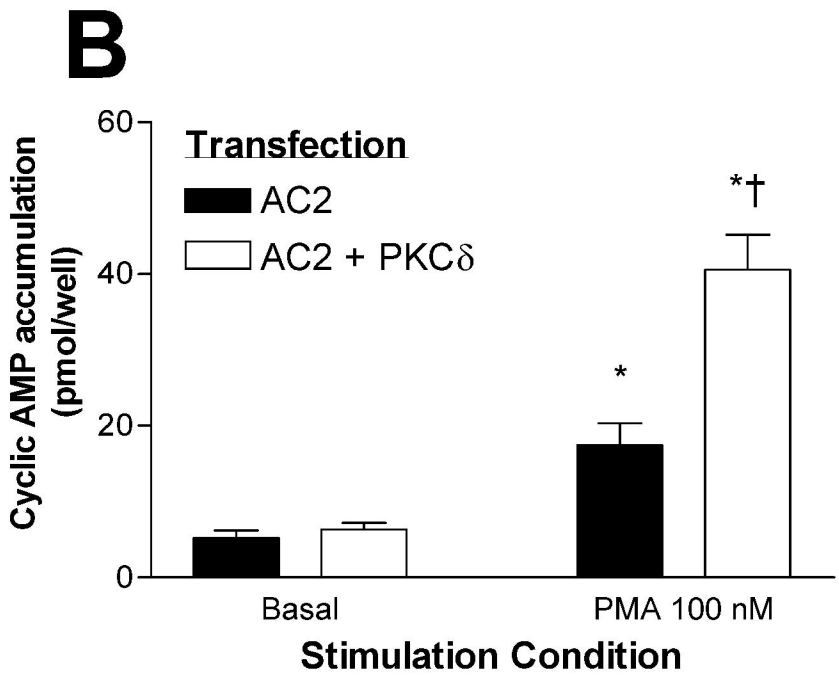


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Figure 3

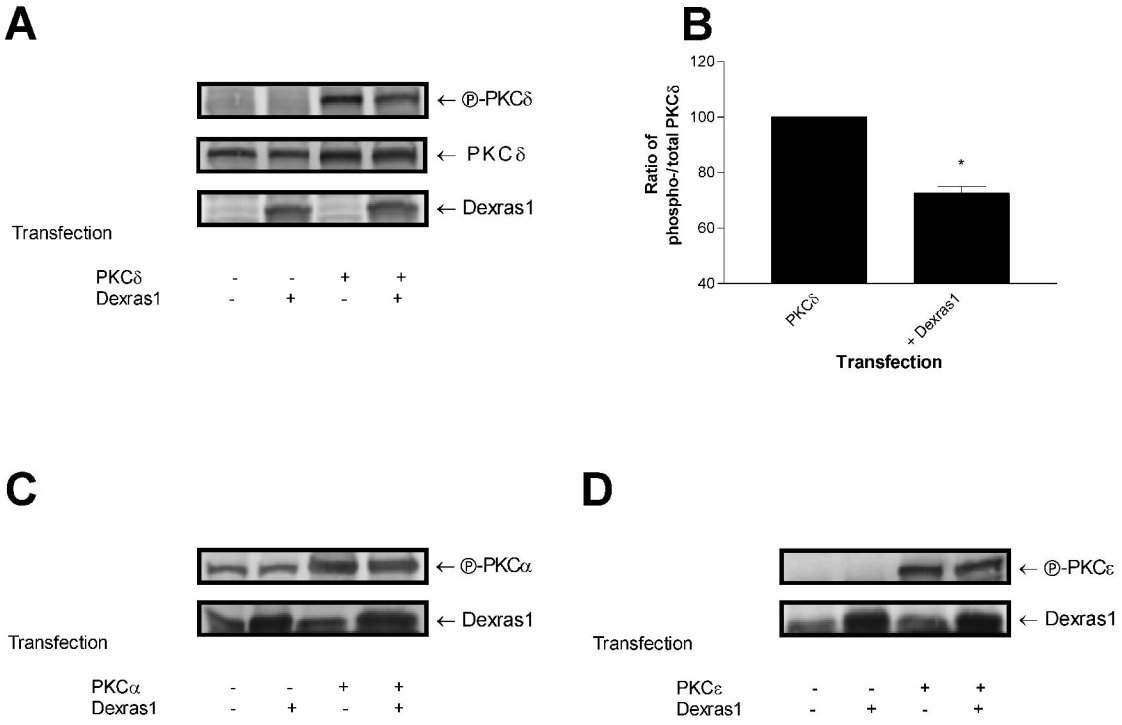

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Figure 4

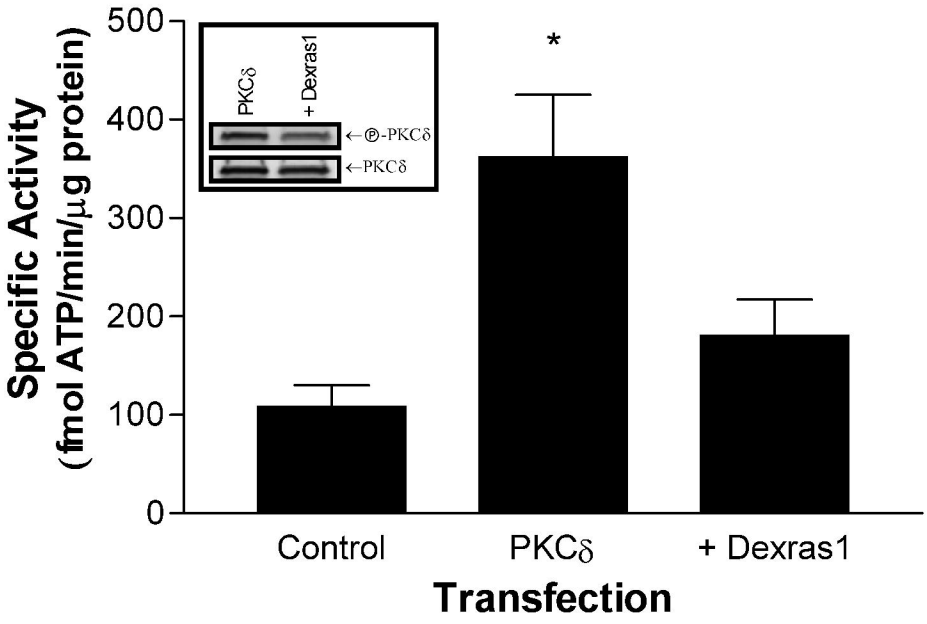

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Figure 5

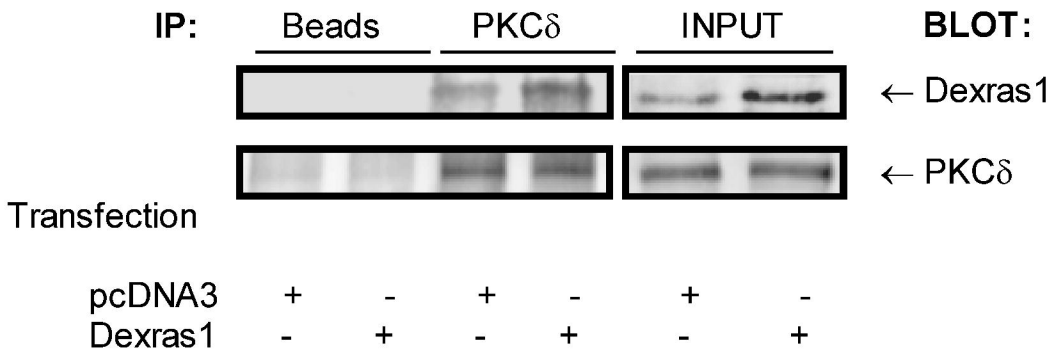

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Figure 6

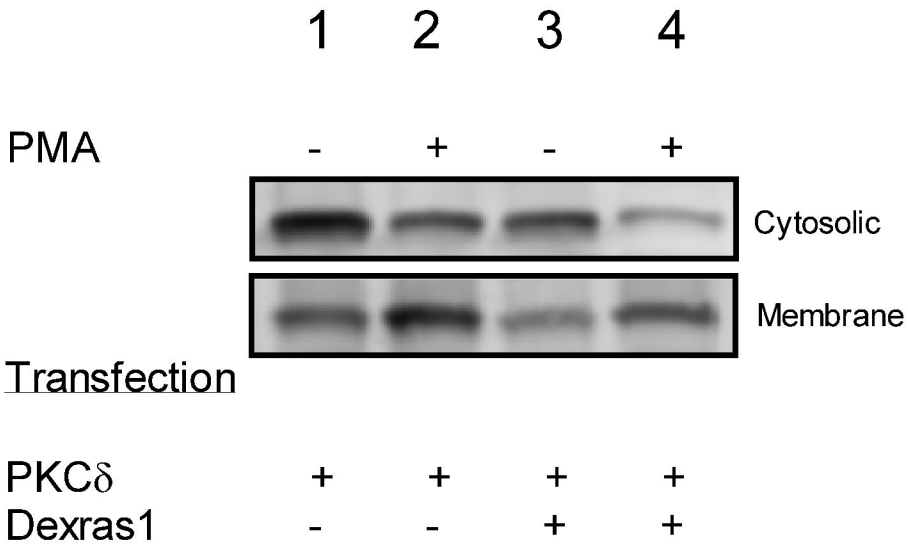

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Figure 7

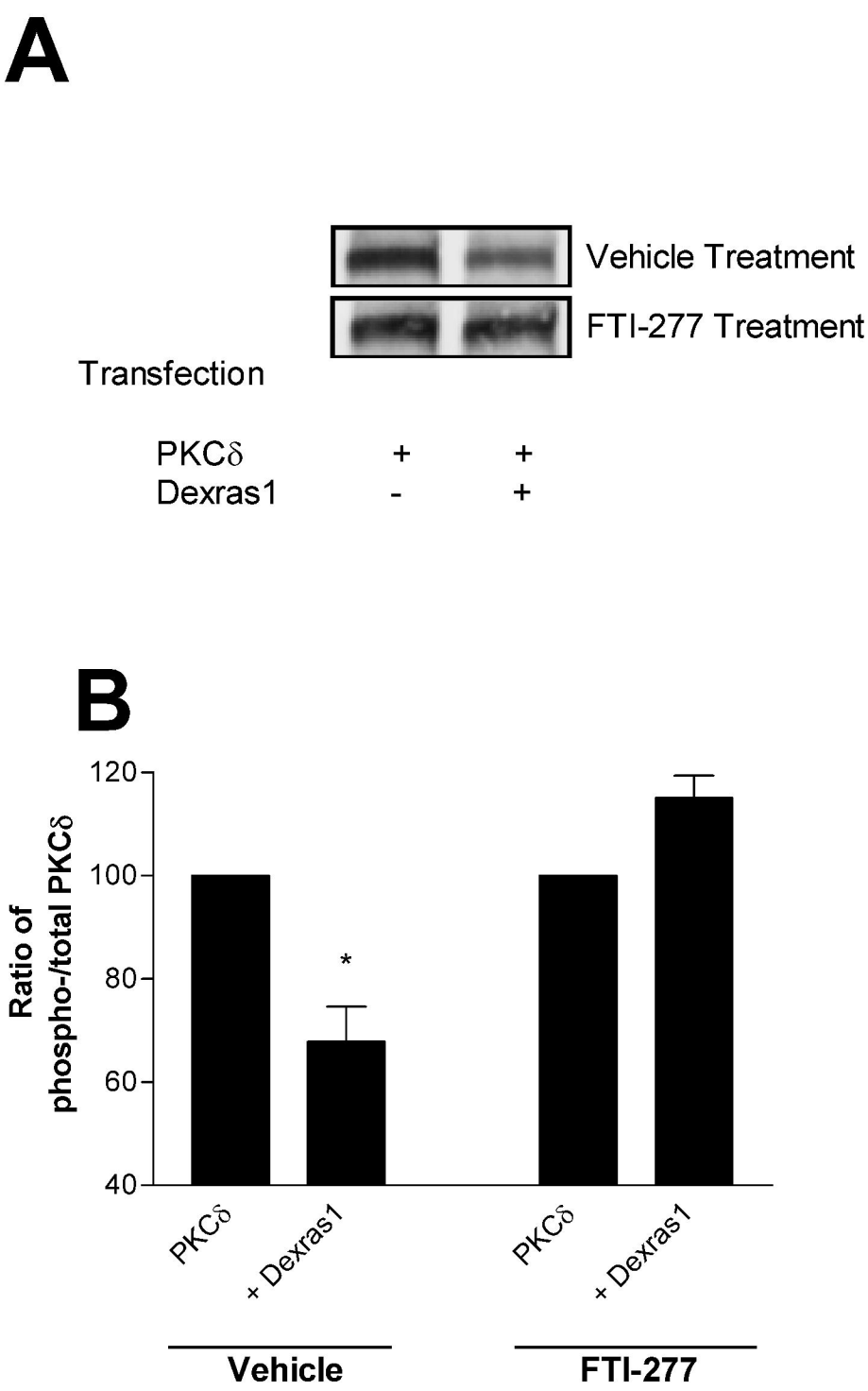

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Figure 8

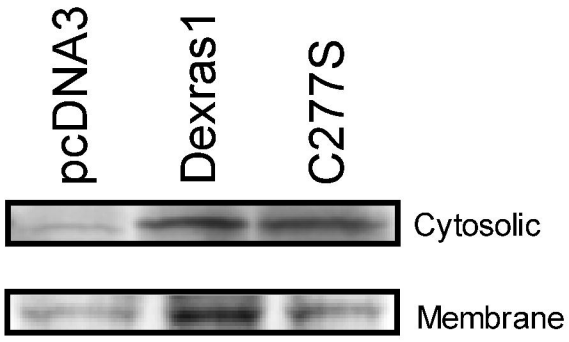

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Figure 9

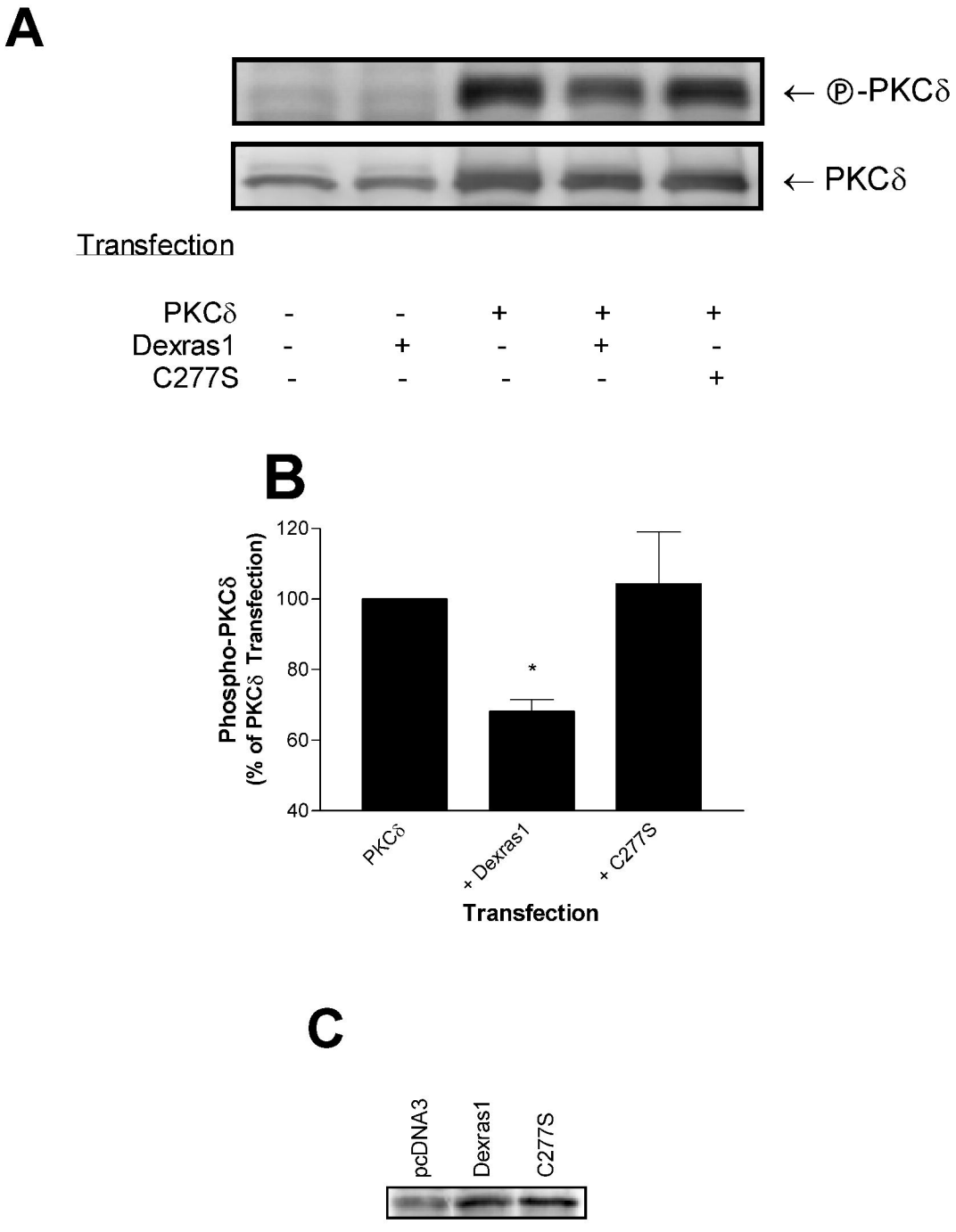

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Figure 10