Activated 3',5'-Cyclic AMP-dependent Protein Kinase Is Sufficient to Induce Neuroendocrine-like Differentiation of the LNCaP Prostate Tumor Cell Line*

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Neuroendocrine (NE) differentiation within prostate tumors is proposed to be a contributing factor in disease progression. However, the cellular origin and molecular mechanism controlling differentiation of prostatic NE cells are unresolved. The prostate tumor cell line, LNCaP, can reversibly acquire many NE characteristics in response to treatment with β-adrenergic receptor agonists and activators of adenylyl cyclase. In this study, we demonstrate that these treatments induce protein kinase A (PKA) activation in LNCaP cells and that ectopic expression of a constitutively activated form of the PKA catalytic subunit, Cαo, results in acquisition of NE characteristics, including the extension of neuritic processes, cessation of mitotic activity, and production of neuron-specific enolase. Forskolin-, epinephrine-, and isoproterenol-dependent NE differentiation of LNCaP cells was significantly inhibited by expressing a dominant negative mutant of the PKA regulatory subunit, RIK. These results demonstrate that prostatic NE differentiation in response to these agents depends on PKA activation, and this signaling pathway may provide a therapeutic target for treating advanced forms of prostate cancer.

Efforts to develop novel therapeutic strategies for treating advanced prostate cancer have resulted in a considerable interest in the potential role of prostatic neuroendocrine (NE) cells in disease progression (reviewed in Refs. 1 and 2). It has become evident that NE cells are a normal component of both the developing and mature prostatic epithelium and are postulated to be derived from stem cells common to both the exocrine and basal cell populations (3, 4). NE cells produce a variety of neurosecretory products that exhibit growth-promoting activities, including parathyroid hormone-related peptides, neurotensin, serotonin, calcitonin, and bombesin-related peptides (5–9), suggesting that these cells function through endocrine/paracrine mechanisms to regulate the normal development and secretory activity of the prostate.

Virtually all prostatic adenocarcinomas contain foci of cells with NE characteristics (10). The presence of extensive multifocal NE features in tumors is an indication of increased aggressiveness and androgen independence (11–13). While the prognostic value of tumor NE status remains controversial, a strong link between NE status and long term, disease-specific survival has been reported (14). Tumor cell populations become enriched for NE cells following long term anti-androgen therapy (15), and although the NE cells appear to be nonmitotic, the carcinoma cells adjacent to these NE foci have been noted to exhibit increased proliferative activity (16, 17). These studies suggest that NE cells that develop within prostate tumors may produce neurosecretory factors that contribute to increased malignancy and decreased responsiveness to androgen ablation therapy.

The androgen-responsive prostate tumor cell line, LNCaP, has emerged as a useful model for testing the development of a NE phenotype in adenocarcinoma cells with an exocrine phenotype. LNCaP cells have been shown to acquire NE characteristics in response to increased intracellular cAMP levels (18, 19), long term androgen ablation (20), and stimulation with the cytokines interleukin-1β and -6 (21, 22). Treating LNCaP cells with the β-adrenergic receptor agonist epinephrine (Epi) and isoproterenol (Isop) or with the adenylyl cyclase activator, forskolin (Fsk), induces the rapid accumulation of intracellular cAMP and acquisition of numerous NE characteristics, including development of neuritic processes; cessation of mitotic activity; increased expression of neuron-specific enolase (NSE) and the biogenic amine serotonin; and the release of parathyroid hormone-related peptide and neurotensin into the culture medium (19). However, maintenance of the NE phenotype is lost upon withdrawal of differentiation-inducing agents, resulting in retraction of neuritic processes, reinitiation of mitotic activity, and loss of neuronal marker expression (19). These findings suggest that the phenotype of tumor cells may be dynamic and determined in part by the balance of differentiative and mitogenic factors in the local environment.

Although cAMP-dependent protein kinase (PKA) is often considered essential for mediating the wide range of physiological effects initiated by cAMP, direct evidence for its role is often limited. The recent identification of cAMP-responsive guanine nucleotide exchange factors for Rap1 (23, 24) highlights the existence of PKA-independent cAMP signaling pathways. Additionally, treatment of LNCaP cells with a butyrate analogue was shown to be sufficient to induce cell cycle arrest (25), suggesting the possibility that dibutyryl cyclic AMP (Bt2cAMP) may have antiproliferative effects due to the butyrate component of the reagent and not due to activation of...
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Dr. L. W. K. Chung (University of Virginia, Charlottesville, VA) and maintained in T-Media (Life Technologies, Inc.) containing 5% fetal bovine serum (FBS; Life Technologies, Inc.), unless otherwise indicated, at 37 °C in a humidified 5% CO₂ environment (26). Phenol red-free RPMI 1640 medium and charcoal-stripped FBS were from Life Technologies, Inc. FBS, Epi, Iso, Bt-CAMP, and epidermal growth factor (EGF) were from Sigma. Interleukin-6 (IL-6) was from Calbiochem, and the PKA inhibitor, H89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isquinoline-sulfonamide), was from Seikagaku Co. (Toyko, Japan). LNCaP cells were transfected using Lipofectin (Life Technologies, Inc.) as described by the manufacturer. The green fluorescent protein (GFP)-expressing reporter plasmid, pGreen Lantern™, was from Life Technologies, Inc. Plasmids encoding the wild type and mutant isoforms of the PKA catalytic subunit, CIₐ, and the dominant-negative mutant of the PKA regulatory subunit, RII, were gifts from Dr. G. S. McKnight (University of Washington, Seattle, WA) (27, 28). CIₐ and RII subunits were amino-terminally FLAG-tagged by polymerase chain reaction using PflII polymerase (Stratagene, La Jolla, CA) to encode the peptide, MDYKD-DDDGP, and placed under the transcriptional control of a Rous Sarcoma virus long terminal repeat promoter in the plasmid, pOPRSV1 (Stratagene). The dominant-negative RII subunit, dNIₐ, was provided in a murine metallothionein I promoter-driven expression vector (pMDmRId; Ref. 28). Expression of dNIₐ was induced by treating cells with 1.5 μM CdCl₂ and 150 μM ZnSO₄ (Cd/Zn). FLAG-tagged extracellular regulated kinase 2 expression plasmid was a gift from Drs. S. Eblin and M. J. Weber (University of Virginia, Charlottesville, VA).

Immunoblotting—After treatment, cells were washed with phosphate-buffered saline (50 mM sodium phosphate, 150 mM NaCl, pH 7.4), lysed in HO buffer (50 mM HEPES, 100 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM sodium vanadate, 40 mM β-nitrophenyl phosphate, 2 μg/ml microcin, pH 7.2) on ice, and processed for SDS-polyacrylamide gel electrophoresis and electro- phoretic transfer to nitrocellulose (Schleicher and Schuell). As described (29) Immunoblotting for FLAG-tagged kinases, NSE, and PKA was performed using the anti-FLAG epitope antibody, M5 (Eastman Kodak Co.), a polyclonal anti-NSE antiserum (DAKO, Carpinteria, CA), and a polyclonal anti-PKA CIₐ catalytic subunit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively. Immunoblotting, as visualized by ECL using horseradish peroxidase-conjugated sheep anti-mouse IgG or Protein A as appropriate (Amersham Pharmacia Biotech). Relative band intensities were determined by densitometry using ImageQuant software.

Kinase Assays—PKA kinase activity was assessed by immune complex kinase assay derived from previously published protocols (28, 30). Immunoprecipitations were performed from 500 μg of lysate protein using 1 μg of rabbit polyclonal anti-PKA CIₐ catalytic subunit antibody (Santa Cruz Biotechnology) collected on Protein A-Sepharose (Sigma) or 10 μl of M2 anti-FLAG epitope antibody conjugated to agarose beads (Kodak). Immune complexes were washed 3 times with HO lysis buffer and once with kinase buffer (50 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 0.014% Tween 20) and resuspended in 40 μl of kinase buffer. Kinase reactions were started by adding 10 μl of kinase buffer containing 28 μM synthetic PKA peptide substrate, malantide (Sigma), and 1 mM (1 μCi/μmol) γ³²P]ATP (NEN Life Science Products) and incubated at 30 °C for 10 min. Reactions were terminated by adding 10 μl 1 M HCl, and 35 μl of the reaction was spotted onto one 1-cm² strip of phosphocellulose (P81; Whatman). The P81 strips were washed four times for 10 min each in 75 mM H₂PO₄, washed once in methanol, dried, and counted by Cherenkov radiation. Malantide is a synthetic dodecapeptide corresponding to the PKA phosphorylation site of phospho- rylose kinase β-subunit and is efficiently phosphorylated by PKA in vitro (Kₘ = 15 μM; Vₘax = 23 μmol of phosphate transferred min⁻¹ mg⁻¹ (28)). Relative PKA-specific activity was determined as pmol of phosphate incorporated into malantide per min per unit of immunoprecipitated PKA CIₐ (Fig. 1) or FLAG-tagged kinase (Fig. 3) as measured by immunoblotting as described above.

Immunocytochemistry—For immunofluorescence analysis, LNCaP cells were plated onto glass coverslips and processed as described previously (31). FLAG-tagged CIₐ or Cpr expression was detected using anti-FLAG antibody, M5, and Texas Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Cells undergoing DNA synthesis were detected by bromodeoxyuridine (BrdU) labeling, performed by adding 100 μM BrdUrd to the culture medium of cells during the last 20 h of incubation. BrdUrd incorporation into DNA was assessed by immunofluorescence using anti-BrdUrd-fluorescein isothiocyanate antibody (Roche Molecular Biochemicals) as described by the manufacturer.

Statistical Analysis—Assessment of differences in response was performed using analysis of variance models fit using maximum likelihood techniques using single factor analysis of variance models for Figs. 2A and B and multifactor analysis of variance models for Figs. 1, 5B, and 8. Variance-stabilizing transformations were used when required to meet assumptions of a linear model (constant variance and normally distributed error terms). A natural logarithmic transformation was used when required for the purpose of obtaining a linear model. The data in Fig. 8 required an arc sine square root (sin⁻¹(√x/√n)) transformation for which there is no convenient interpretation of a pairwise difference. Therefore, only the relative magnitude and statistical significance of these results were of interest. Type III F-tests were used to determine the importance of effects and interactions. Pairwise comparisons were made at the highest level of complexity that was significant for this test at the 5% level. Confidence intervals for pairwise differences were generated by Fisher’s least significant difference method with a comparison type I error rate of 0.05 (32). A 95% confidence interval that does not contain 0 (or 1 in the case of fold-change) implies statistical significance at the 5% level. Representative photomicrographs and immunoblots are provided to demonstrate the primary data.

RESULTS

PKA Is Activated in LNCaP Cells Induced to Undergo NE Differentiation—Our previous studies demonstrated that LN-
CaP cells treated with Epi, Isop, or Fsk induced an increase in cytosolic cAMP levels and the reversible acquisition of a number of NE characteristics. We measured PKA-specific kinase activity from LNCaP cells acutely stimulated with these agents to assess their ability to mediate activation of PKA. PKA-specific kinase activity was significantly increased (−4-fold) in lysates from LNCaP cells treated with Fsk or Isop as compared with that of lysates from unstimulated cells or cells stimulated with IL-6 or EGF (Fig. 1). Additionally, the kinase activity immunoprecipitated from untreated cells could be significantly inhibited (−80%) by addition of the PKA inhibitor, H89, to the kinase assay. These results establish that PKA is activated in LNCaP cells under treatment conditions that induce the acquisition of NE characteristics.

**Transfection of PKA Catalytic Subunits into LNCaP Cells**—To directly assess whether activated PKA was sufficient to induce acquisition of NE characteristics by LNCaP cells, we employed cDNAs encoding amino-terminal FLAG-tagged wild type PKA catalytic subunit, CIα, or constitutively activated mutant, Cqr, which is unable to bind PKA regulatory subunit, RIα, in the absence of cAMP due to the point mutations, H87Q and W196R (27) (Fig. 2). LNCaP cells were transiently transfected with equal amounts of plasmids encoding FLAG-tagged CIα, Cqr, or extracellular signal-regulated kinase 2 and assessed for expression and PKA-specific kinase activity (Fig. 3). The CIα and Cqr subunits were readily detected in anti-CIα immunoblots of anti-FLAG immunoprecipitations from the appropriately transfected populations (Fig. 3B, upper panel), while similar levels of all three kinase constructs were detected in anti-FLAG epitope immunoblots of anti-FLAG immunoprecipitates (Fig. 3B, lower panel).

When normalized to the amount of FLAG-tagged kinase immunoprecipitated, the PKA kinase activity from Cqr-transfected cells was −2-fold greater than the anti-FLAG immunoprecipitated activity detected in CIα-transfected cells stimulated with Fsk and −5-fold greater than the activity of unstimulated CIα-transfected cells (Fig. 3A). Treatment of Cqr-transfected cells with Fsk had no discernible effect on FLAG-extracellular signal-regulated kinase 2-transfected LNCaP cells exhibited a basal level of malantide phosphorylating activity equivalent to one-tenth the activity detected from unstimulated, CIα-transfected cells. This basal activity was independent of Fsk stimulation. Each construct has been assessed three times with similar results, indicating that tagging and subcloning these constructs did not affect the previously reported differences in enzymatic activity attributed to these CIα isoforms (27).

**PKA Activation Is Sufficient to Induce Morphological Differentiation of LNCaP Cells**—We have established that treatment of LNCaP cells with β-adrenergic receptor agonists or Fsk increases intracellular cAMP levels (19) and PKA activity (Fig. 1). To determine whether expression of activated PKA catalytic subunit was sufficient to induce acquisition of NE characteristics in LNCaP cells, we compared the morphology of cells cotransfected with empty vector or CIα- or Cqr-expressing plasmids along with the GFP reporter plasmid. Three days after transfection, cells were fixed and stained for the FLAG epitope. When either the Cqr- or CIα-containing plasmid was transfected into LNCaP cells along with the GFP reporter plasmid at a 5:1 molar ratio, greater than 95% of the GFP-positive cells were also positive for the FLAG epitope. Transient transfection of LNCaP cells routinely resulted in ~30% of the cells in the
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Fig. 4. Expression of constitutively activated PKA catalytic subunit is sufficient to induce NE differentiation. LNCaP cells were cotransfected with expression plasmids at a 5:1 ratio of the FLAG-tagged wild type (pFLAG-CIα; A–C) or constitutively activated (pFLAG-Cqr; D–I) PKA catalytic subunit to GFP (pGreen Lantern™). Cells were maintained under normal culture conditions for 3 days. Expression of the constructs was visualized by indirect immunofluorescence microscopy for GFP-positive cells (A, D, G), CIα expression using M5 anti-FLAG tag monoclonal antibody (B, E, H), and phase microscopy (C, F, I) of the identical fields, at ×20 (A–F) or ×40 (G–I) magnification.

The identical fields, at 3 was significantly greater than the less than 20% of the CI (pFLAG-CIα or pFLAG-Cqr), PKA catalytic subunit is sufficient to induce NE differentiation morphology in LNCaP cells. We previously showed that Fsk- and Epi-induced activation of PKA alone is sufficient to induce the NE morphological characteristics (Fig. 5, A–C), while the surrounding untransfected cells and cells transfected with CIα (Fig. 4, A–C, F, and I) exhibited morphologies typical of untreated LNCaP cells. Greater than 80% of the Cqr-transfected cells exhibited a NE morphology (cells with processes greater than two cell bodies (40 µm) in length). This was significantly greater than the less than 20% of the CIα or empty vector-transfected cells determined to exhibit such morphological characteristics (Fig. 5A). These results indicate that activation of PKA alone is sufficient to induce the NE differentiation morphology in LNCaP cells.

PKA Activation Is Sufficient to Induce Mitotic Arrest in LNCaP Cells—We previously showed that Fsk- and Epi-induced activation of NE characteristics included the cessation of mitotic activity. We therefore assessed the mitotic activity of the PKA catalytic subunit—transfected cells cultured for 3 days in T-Media containing 5% FBS, serum-free and phenol red-free RPMI 1640 (S-F), or phenol red-free RPMI 1640 containing 5% charcoal-stripped FBS (CS-FBS) to characterize the extent to which the different transfections affected the LNCaP phenotype. Under all conditions tested, the rate of BrdUrd incorporation observed in Cqr—transfected cells was significantly reduced as compared with that of the CIα-transfected cells (Fig. 5B) with the exception of the case discussed below. Furthermore, the rate of BrdUrd incorporation observed in CIα—transfected cells was indistinguishable from that of the untransfected cells (data not shown). Approximately 48% of the CIα—transfected cells grown in FBS were positive for BrdUrd at the end of the 20-h labeling indicating a doubling time of about 2 days. This is in excellent agreement with the previously reported doubling time for LNCaP cells (26, 33) and indicates that the doubling time of transfected cells had not been influenced by expression of the wild type CIα subunit.

The level of BrdUrd incorporation observed in Cqr—transfected cells cultured in FBS was comparable with the level of inhibition of [3H]thymidine incorporation observed in LNCaP cells treated with Bt2cAMP, Fsk, or Epi (19) and indistinguishable from that observed in CIα—transfected cells cultured under S-F conditions (Fig. 5B). This latter condition has been reported to induce growth arrest and eventual acquisition of NE characteristics in LNCaP cells (20). These results indicate that, even under optimal growth conditions, expression of Cqr is sufficient to reduce the mitotic activity level of transfected cells that to observed for untransfected or CIα—transfected cells under S-F conditions. Additionally, while the rate of BrdUrd incorporation observed in CIα—expressing cells under charcoal-stripped FBS conditions was significantly greater than that of cells under S-F conditions, the BrdUrd incorporation rate of the Cqr—expressing cells under the same conditions were indistinguishable (Fig. 5B). These results indicate that while androgen withdrawal had a modest impact on the mitotic activity of LNCaP cells, it significantly increased the susceptibility of LNCaP cells to PKA activation by initiating cell cycle arrest.

PKA Activation Is Sufficient to Induce NSE Expression in LNCaP Cells—To further characterize the extent to which activation of PKA affected the LNCaP phenotype, we assessed the expression of NSE in cells transfected with the FLAG-tagged PKA catalytic subunit vectors (Fig. 6). For comparison, NSE levels were measured from lysates of LNCaP cells transfected with a GFP expression plasmid and cultured for 2 days in the presence or absence of Bt2cAMP and from lysates of bovine adrenomedullary chromaffin cells. As compared with untreated GFP—transfected cells, NSE expression was ∼5-fold higher in CIα—transfected cells and ∼60-fold higher in Cqr—transfected cells. NSE expression in Cqr—transfected cells was ∼35% of that detected in Bt2cAMP—treated GFP—transfected cells. To compare the NSE levels in cells transfected with CIα or Cqr, the NSE/FLAG ratio of CIα—transfected cells was nor-
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PKA Activation Is Required for β-Adrenergic Receptor- and Fsk-mediated Acquisition of NE Characteristics—To determine if PKA activation is required for Fsk or β-adrenergic receptor agonists to mediate acquisition of NE characteristics, LNCaP cells were transfected with pMTdnRIα or pMTd-α-transfected, Fsk-stimulated LNCaP cells retained the typically fusiform LNCaP morphology, while the surrounding cells acquired a NE morphology (Fig. 7, A and B). However, in the presence of Cd/Zn, pMTdnRIα-transfected, Fsk-stimulated cells retained the typically fusiform LNCaP morphology, while the surrounding cells acquired a NE morphology (Fig. 7, C–F).

The effect of RIα expression on acquisition of a NE phenotype in response to induction by Fsk, Epi, and Isop was quantitatively assessed for three independent sets of transfected and treated cells (Fig. 8). Because of the relatively high endogenous levels of PKA regulatory subunit in LNCaP cells, we were unable to determine the fold induction of RIα expression in the transfected cells. However, treatment of RIα-transfected cells with Cd/Zn significantly inhibited the ability of Isop-, Epi- or Fsk-stimulated cells to acquire a NE morphology. These results suggest that PKA signaling is required for LNCaP cells to acquire NE characteristics in response to β-adrenergic agonists and Fsk, further supporting the idea that activated PKA is involved in controlling cAMP-mediated NE differentiation in prostatic adenocarcinomas.

DISCUSSION

NE cells are found in essentially all prostatic adenocarcinomas, and since these cells produce factors capable of affecting the proliferative and metastatic state of prostate tumor cells, the potential role of NE differentiation in prostatic cancer progression is of interest. However, the lack of a consensus concerning the prognostic value of tumor NE status clearly indicates that a more accurate assessment of the potential significance of NE differentiation in the progression of prostate

Fig. 6. PKA expression is sufficient to induce NSE expression. LNCaP cells were transfected with plasmids encoding FLAG-tagged PKA catalytic subunits (Clα, lane 1; F-Cqr, lane 2) or GFP either left untreated (lane 3) or treated with 0.1 mM Bt2cAMP for 2 days (lane 4). Whole cell lysates (100 μg/ lane) were subjected to immunoblot analysis using an anti-NSE antibody (upper panel of immunoblot and histogram) or M5 anti-FLAG epitope antibody (lower panel of immunoblot). Bovine adrenomedullary chromaffin cell lysate (Chrom., lane 5; 100 μg) was utilized as a positive control for NSE expression. Statistical significance of the pairwise difference for the ratio of NSE expressed per unit of FLAG-tagged PKA catalytic subunit expressed from Clα to Cqr was averaged from five independent experiments and is described under "Results."
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The use of LNCaP cell lines expressing wild type and mutant isoforms of components of the PKA signaling pathway should just such a model. The use of LNCaP cell lines expressing wild type and mutant isoforms of components of the PKA signaling pathway should provide systems to critically evaluate the outstanding issues concerning control of NE differentiation and the possible influence NE cells have on prostate cancer progression.

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disease is needed.

In this report, we used the LNCaP cell line to demonstrate directly that activated PKA plays an essential role in mediating the acquisition of NE characteristics induced by β-adrenergic receptor agonists and activators of adenylate cyclase. This conclusion is based on our demonstration 1) that these agents induce activation of PKA kinase activity, 2) that ectopic expression of an activated isoform of PKA catalytic subunit is sufficient to initiate acquisition of NE characteristics by these cells, and 3) that inhibition of PKA signaling by expressing a dominant-negative isoform of the PKA regulatory subunit is capable of inhibiting the ability of β-adrenergic receptor agonists and activators of adenylate cyclase to induce NE differentiation. We have measured acquisition of a neuritic morphology and inhibition of mitotic activity because these were the most readily quantifiable measures of NE differentiation applicable to the single-cell assays utilized here, and because these changes in morphology and mitotic activity were completely coincident with the expression of the NE markers, NSE (Fig. 6), serotonin, parathyroid hormone-related peptide, and neurotensin (19).

These results, coupled with our previous report (19) indicating that cAMP-mediated acquisition of NE characteristics in LNCaP cells is completely reversible, such that maintenance of the NE phenotype requires the continual presence of differentiating agents, implicate PKA as a central player in mediating NE differentiation in adenocarcinomas.

The prostate has been demonstrated to be a rich source of α2- and β2-adrenergic receptors with both adrenergic and cholinergic innervation into the glandular epithelia (34). Moreover, β-adrenergic receptor agonists have been implicated in the maintenance of prostate homeostasis and as mitogens for prostatic stromal cells (35). Perhaps the requirement for continual cAMP signaling for the maintenance of a NE phenotype in LNCaP cells reflects a normal requirement for β-adrenergic signaling for proper prostatic function.

A potentially strong selective advantage is provided to tumors that, in response to factors either present (such as catecholamines) or absent (such as androgens) in their environment, can reversibly generate cells that serve as endocrine/ paracrine sources and are themselves nonproliferative and androgen-independent. Our observations indicating that PKA activation initiates acquisition of NE characteristics and that these effects are accentuated under conditions of androgen withdrawal (Fig. 5B) suggest that the LNCaP cell line provides just such a model.

The use of LNCaP cell lines expressing wild type and mutant isoforms of components of the PKA signaling pathway should provide systems to critically evaluate the outstanding issues concerning control of NE differentiation and the possible influence NE cells have on prostate cancer progression.

Fig. 7. Dominant-negative PKA RIαa inhibits forskolin-induced NE differentiation. LNCaP cells were cotransfected with a 5:1 ratio of plasmids encoding the dominant negative PKA regulatory subunit (pMTdnRIαa) and GFP (pGreen Lantern™). Cells were treated with 5 μM Fsk with (C–F) or without Cd/Zn (A, B) for 3 days. Representative photographs are shown of GFP immunofluorescence (GFP; A, C, and E) and phase contrast (Phase; E, D, and F) of the corresponding fields at × 20 magnification.

Fig. 8. Expression of dominant-negative PKA regulatory subunit, RIαa, inhibits Epi-, Isop-, and Fsk-induced acquisition of a NE morphology. LNCaP Cells were transfected with pGreen Lantern™ and pMTdnRIαa (GFP/RIαa) or pGreen Lantern™ and cDNA3 as a control plasmid (GFP/cont.). Cells were left unstimulated (U) or stimulated with 10 μM Epi (E), Isop (I), or Fsk (F) and either not treated (No Trt.) or treated with Cd/Zn. For each treatment, the morphology of 50–100 GFP-positive cells was determined from at least five random fields. GFP-positive cells were scored for the NE phenotype as defined by cells with neuritic processes at least 2 times the length of the cell body (% Neurite-Bearing Cells). Statistical significance of pairwise differences for each stimulation condition of both transfectants in the presence or absence of Cd/Zn was performed as detailed under "Experimental Procedures." †, p < 0.05 for the indicated treatment pairs. †† indicates those treatment pairs for which acquisition of NE morphology was significantly different.

0 % Neurite-Bearing Cells

Transfection: GFP/pcDNA3  GFP/RIαa

Stimulation: U I E F U I E F

No Trt. Cd/Zn

Fsk

GFP

Phase

-171720

1714–1720

100

80

60

40

20

0
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