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DIHYDROTESTOSTERONE ACTIVATES CREB SIGNALING IN CUTURED HIPPOCAMPAL NEURONS

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Abstract

Although androgens induce numerous actions in brain, relatively little is known about which cell signaling pathways androgens activate in neurons. Recent work in our laboratory showed that the androgens testosterone and dihydrotestosterone (DHT) activate androgen receptor (AR)-dependent mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling. Since the transcription factor cyclic AMP response element binding protein (CREB) is a downstream effector of MAPK/ERK and androgens activate and CREB in non-neuronal cells, we investigated whether androgens activate CREB signaling in neurons. First, we observed that DHT rapidly activates CREB in cultured hippocampal neurons, as evidenced by CREB phosphorylation. Further, we observed that DHT-induced CREB phosphorylation is AR-dependent, as it occurs in PC12 cells stably transfected with AR but in neither wild-type nor empty vector-transfected cells. Next, we sought to identify the signal transduction pathways upstream of CREB phosphorylation using pharmacological inhibitors. DHT-induced CREB phosphorylation in neurons was found to be dependent upon protein kinase C (PKC) signaling but independent of MAPK/ERK, phosphatidylinositol 3-kinase, protein kinase A, and Ca^{2+}/calmodulin-dependent protein kinase IV. These results demonstrate that DHT induces PKC-dependent CREB signaling, which may contribute to androgen-mediated neural functions.

Keywords

Androgen receptor; dihydrotestosterone; protein kinase C; signal transduction; testosterone

1. INTRODUCTION

A large body of data documents numerous androgen actions in brain [14,81,96]. As the nervous system develops, testosterone, either directly through androgen-specific pathways and or indirectly (via aromatization) through estrogen-specific pathways, regulates apoptosis in sexually dimorphic regions of brain [51,76,103]. In addition, testosterone exerts a range of neurotrophic effects including cell differentiation [129], neurite growth [59,65], hippocampal excitability [89], neurogenesis [104,128], and development of hippocampal [75], motor [42,

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49,67], and autonomic [51] neurons. Further, testosterone can regulate glial activity [20,78] in ways that promote neuron survival.

Increasing evidence indicates a key role of testosterone and related androgens in regulation of neuron loss due to disease-related insults [88]. In some cases, androgens can promote neuron loss [74,124,125]. In addition, androgens can also promote neuron survival against cell death induced by β-amyloid protein (Aβ) [72,86,130], serum withdrawal [36], pro-oxidants [3], and excitotoxins [91]. Androgen neuroprotection is dependent at least in part on androgen receptors (AR), as evidenced by inhibition of protective effects by AR antagonists [3,36,130]. Consistent with these findings, we observed that androgens reduce cell death in cultured hippocampal neurons challenged with Aβ toxicity [72], an apoptotic insult with relevance to Alzheimer's disease. Furthermore, we found androgen neuroprotection in a PC12 cell line stably transfected with AR but in neither wild-type nor empty vector-transfected cells [72].

Downstream of AR, the mechanisms underlying the neurotrophic actions of androgens are unclear. We reported that androgen neuroprotection involves AR-dependent activation of a mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), leading to activation of p90 ribosomal S6 kinase (Rsk) and subsequent inactivation of Bcl-2 associated death protein (Bad) [72]. An important downstream transcriptional effector of MAPK/ERK is the cyclic AMP response element binding protein (CREB) [23,31,112,114].

CREB activation is known to regulate a variety of neurotrophic effects, including neuroprotection [18,29,52]. Interestingly, androgens activate CREB signaling in non-neural cells [30,111,116]. Further, androgen-induced, AR-dependent MAPK/ERK-CREB signaling in prostate cancer cells attenuates apoptosis [56]. In addition to MAPK/ERK, several other cell signaling pathways modulate CREB activity, including phosphatidylinositol 3-kinase (PI3K)/Akt [82], protein kinase A (PKA) [43,113], Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV [21,93], and protein kinase C (PKC) [94,131]. In this study, we investigated the ability of the potent endogenous androgen dihydrotestosterone to activate CREB in neurons, and identified upstream signaling pathways regulating this action.

2. RESULTS

Dihydrotestosterone induces CREB phosphorylation in cultured hippocampal neurons

To investigate whether androgens activate CREB signaling in neurons, we tested the ability of dihydrotestosterone (DHT), a non-aromatizable metabolite of testosterone, to phosphorylate CREB in cultured hippocampal neurons. Cultures were treated for increasing amounts of time between 0 to 24 h with 10 nM DHT, a concentration previously determined to exert significant neuroprotective activity in this culture system [86], and then processed for western blot using phospho-specific and total CREB antibodies. In hippocampal neuron cultures treated with DHT, CREB phosphorylation significantly increased within 15 min, peaked by 60 min, decreased at 4 h to 8 h after DHT, and returned to basal levels by 24 h (Fig. 1) in comparison to vehicle-treated control conditions. Reprobing the immunoblot with a pan CREB antibody showed that total CREB levels were maintained across conditions, verifying an effect on CREB phosphorylation state rather than expression levels. Thus, androgen activation of CREB occurs rapidly and in a time-dependent manner.

DHT-induced CREB phosphorylation is AR-dependent

To determine if androgen-induced CREB activation requires AR, we compared DHT effects on levels of CREB phosphorylation in wild-type PC12 cells, pcDNA3-ctl (empty vector) cells, and pcDNA3-AR (AR-transfected) cells. PC12 cell cultures were switched to serum-free medium for 24 h, treated with 10 nM DHT ranging in times from 0 to 24 h, and then processed

for western blot using phospho-specific and total CREB antibodies. DHT treatment did not significantly affect CREB phosphorylation in wild-type PC12 (Fig. 2A) or pcDNA3-ctl (Fig. 2B) cells, which lack AR expression [72]. However, in AR-transfected pcDNA-AR cells, DHT resulted in levels of CREB phosphorylation that were significantly increased within 15 min of treatment, remained elevated through 8 h, and returned to basal levels by 24 h (Fig. 2C).

DHT acts as a potent agonist of AR but is also metabolized into androgens that act independently of AR. DHT is converted in brain by 3β-hydroxysteroid dehydrogenase into the androgen 5α-androstan-3β,17β-diol (3β-diol), which can activate estrogen receptor β (ERβ) [62,77,119,120]. Because ER activation can induce CREB phosphorylation in neurons [1,11, 100,109,132], we investigated the possibility that DHT-induced CREB activation may result from conversion to 3β-diol and subsequent activation of ERβ. First, cultured hippocampal neurons were pretreated for 1 h with 10 μM trilostane, which effectively inhibits 3β-hydroxysteroid dehydrogenase activity at this concentration [6,101]. Following trilostane pretreatment, cultures were exposed to 10 nM DHT for 2 h, and then probed by western blot for levels of CREB phosphorylation. Trilostane treatment had no effect on basal levels of CREB phosphorylation and did not significantly alter the DHT-induced increase in CREB phosphorylation (Fig. 2D). In these experiments, we also evaluated the effects of 1 μM ICI 182,780, an ER antagonist [115] previously demonstrated to block ER actions in neuron cultures at this concentration [127]. We found that ICI 182,780 altered neither basal levels nor the DHT-induced increase in CREB phosphorylation (Fig. 2D).

DHT-induced CREB phosphorylation is mediated by neither MAPK/ERK, PI3K/Akt, PKA, nor CaMKIV signaling pathways

Next, we evaluated cell signaling pathways that may contribute to the observed AR-dependent CREB activation. One key upstream regulator of CREB activation is MAPK/ERK [10,11], which we previously found to be activated by androgens in neurons [72]. To determine if MAPK/ERK signaling mediates the activation of CREB in our neuronal paradigm, we compared CREB phosphorylation in the presence and absence of MEK inhibitors PD98059 and U0126 [19], which interrupt the MAPK/ERK pathway at a point just upstream of ERK. Hippocampal neuron cultures were treated with 50 μM PD98059 [19,24,79] or 10 μM U0126 [19,22,27] for 2 h, followed by exposure to DHT for 2 h, and then collected for western blot. Though both MEK inhibitors blocked the DHT-induced increases in ERK, Rsk, and Bad phosphorylation [72], they did not block the androgen-induced increase in CREB phosphorylation (Fig. 3A). Thus, inhibiting upstream MEK does not prevent androgen-induced CREB activation.

We then evaluated alternative upstream effectors of CREB activation, including PI3K/Akt, which androgens activate in non-neuronal cells [7,50,54], PKA, and CaMKIV. To determine if these signaling pathways underlie androgen-induced CREB activation, we used the specific kinase inhibitors LY294002 (PI3K/Akt) [12,45,126], H89 (PKA) [15,19,28], and KN93 (CaMKIV) [26,60,64], and assessed their effects on CREB phosphorylation. We treated hippocampal neuron cultures with 10 μM LY294002, 1 μM H89, or 10 μM KN93 for 2 h, followed by exposure to DHT. Similar to findings with MEK inhibitors, the pharmacological inhibitors of PI3K/Akt, PKA, and CaMKIV did not block the DHT-induced CREB phosphorylation (Fig. 3B). Thus, inhibiting PI3K/Akt, PKA, or CaMKIV signaling does not prevent the androgen activation of CREB.

PKC contributes to DHT-induced CREB phosphorylation

Emerging data suggest a role for PKC in regulation of CREB activity [94,131]. To test whether PKC mediates androgen-induced CREB activation, we first evaluated the efficacies of specific PKC inhibitors GF109203X (2 μM) and Gö6983 (2 μM) on CREB phosphorylation. At the

concentration used, Gö6983 inhibits activities of all PKC isoforms except PKCμ [34,107, 117], whereas GF109203X has high affinity for conventional (α, β, γ) and novel (ζ, ε) PKC isoforms [33,55,110]. We found that 2 h pretreatment of hippocampal neuron cultures with GF109203X or Gö6983 resulted in complete attenuation of DHT-induced CREB phosphorylation (Fig. 4A). In contrast, bisindolylmaleimide V (2 μM, 2 h), an inactive structural analog of GF109203X and Gö6983 [63,102], had no effect on CREB phosphorylation (Fig. 4A). These findings provide pharmacological evidence of PKC involvement in androgen activation of CREB.

One unique property of phorbol esters such as PMA is that when applied chronically, they down-regulate expression of conventional and novel PKC isoforms [4,16,108]. Using this approach of chronic PMA exposure, we assessed androgen-induced CREB activation in PKC-depleted hippocampal neuron cultures. Similar to findings with specific PKC inhibitors (Fig. 4A), we observed that a 24 h exposure to 100 ng/ml PMA reduced the DHT-induced increase in CREB phosphorylation (Fig. 4C). In contrast, the same concentration of an inactive structural analog of PMA, 4α-phorbol, did not affect phosphorylation of CREB protein (Fig. 4C). As a control, we verified that chronic PMA exposure depleted PKC by stripping and reprobing the immunoblot for conventional PKCα. While chronic treatment with PMA down-regulated PKCα expression, the inactive analog 4α-phorbol had no effect on PKCα levels (Fig. 4B). Neither PMA nor 4α-phorbol had a significant effect on total CREB levels (Fig. 4C).

To further investigate the role of PKC in androgen-induced CREB activation, we used a specific peptide inhibitor of conventional and novel PKC isoforms (iPKCc/n) [25,37,118] and a specific inhibitor for atypical PKCζ (iPKCζ) [105,106]. These inhibitors differ from GF109203X and Gö6983 in that they are cell-permeable peptides that specifically bind to the pseudosubstrate region of PKCα and PKCβ (iPKCc/n) or PKCζ (iPKCζ), thereby blocking entry to enzyme substrate [25,37]. We found that the DHT-induced increase in CREB phosphorylation in hippocampal neuron cultures was significantly attenuated by 2 h pretreatment with 1 μM iPKCc/n but not with 1 μM iPKCζ (Fig. 4D), suggesting involvement of conventional and or novel PKC but not atypical PKCζ in androgen activation of CREB.

3. DISCUSSION

Our investigation of androgen cell signaling yields several novel findings. We found that the androgen DHT rapidly increases CREB phosphorylation in neurons. This DHT-induced CREB activation is AR-dependent. In addition, pharmacological inhibition of upstream MAPK/ERK, PI3K/Akt, PKA, or CaMKIV did not prevent DHT-induced phosphorylation of CREB, while inhibition or depletion of PKC largely blocked the phosphorylation. Taken together, these results identify an AR- and PKC-dependent pathway of DHT-induced CREB activation that may contribute to androgen actions in neurons.

The primary male androgen testosterone mediates its effects not only by directly activating AR but also by functioning as a prohormone that is metabolized to other active hormones. In particular, testosterone is converted by *i*) aromatase to the estrogen 17β-estradiol, which acts on estrogen receptors ERα and ERβ, and *ii*) 5α-reductase to the androgen DHT, which is a potent agonist for AR [14]. DHT is a substrate for additional metabolizing enzymes in brain, including 3α-hydroxysteroid dehydrogenase that reversibly forms 5α-androstan-3α,17β-diol and 3β-hydroxysteroid dehydrogenase that irreversibly yields 3β-diol [66]. Recent evidence demonstrated that 3β-diol is an agonist for ERβ [119,120] and that some DHT actions in brain are mediated by conversion to 3β-diol and subsequent ERβ activation [62,77]. Our observations are not consistent with a 3β-diol/ERβ pathway since DHT-induced CREB phosphorylation was inhibited by neither ER antagonism nor pharmacological inhibition of 3β-hydroxysteroid

dehydrogenase. Rather, our data with AR-transfected PC12 cells suggests that CREB phosphorylation is dependent on AR activation.

Our observation of androgen-mediated CREB phosphorylation in neurons is consistent with the actions of sex steroid hormones in neurons and other cell types. For example, estrogen [1,11,100,109,132] and in some circumstances progesterone [35] increase CREB phosphorylation in neurons. Although an effect of androgens on neuronal CREB activation has not been previously demonstrated, neonatal male rat pups exhibit higher levels of CREB phosphorylation than female pups in several brain regions including hippocampus [5]. Also consistent with our observations are findings in non-neural cells of a similarly rapid and AR-dependent increase in CREB phosphorylation in prostate cancer cells [111] and Sertoli cells [30,116]. Delayed CREB activity has also been observed 1–4 days after androgen treatment in rat granular convoluted tubules/submandibular gland [53].

The initial goal of our study was to elucidate non-classical, indirect genomic mechanisms of androgen signaling downstream of MAPK/ERK activity [2]. In previous work, we identified a non-genomic mechanism of androgen protection against Aβ toxicity involving AR-dependent ERK-Rsk-Bad signaling in hippocampal neurons [72]. The MAPK/ERK cascade not only mediates phosphorylation-dependent, non-genomic changes in protein activity [47,98], but also regulates gene transcription [39,112]. The transcription factor CREB was among the first documented downstream effectors of MAPK/ERK signaling [10,31,70]. MAPK/ERK activation of CREB regulates many neuronal actions, including cell proliferation, differentiation, long-term potentiation, and survival [31]. For example, Bonni and colleagues [10] found that suppression of ERK-CREB activity (and synthesis of viability proteins) facilitates apoptosis in cerebellar granule cells, while ERK-induced inactivation of Bad (a death protein) facilitates cell survival against apoptosis. Despite *in vivo* [70,80] and *in vitro* [10,97] evidence that links the MAPK/ERK pathway to CREB, in our paradigm, androgen-induced CREB activation appears to be independent of MAPK/ERK activity since it is not reduced by MEK inhibitors. These data are consistent with reports that CREB can function independently of ERK signaling in neuronal paradigms of differentiation [103], synapse activity [44], and gene regulation [48].

CREB transcriptional regulation underlies many gene-dependent neuronal functions induced by several signaling pathways [29,83]. Four well-characterized signaling pathways in neurons apart from MAPK/ERK [10,112,123] are PI3K/Akt [82], PKA [43,113], CaMKIV [21,93], and PKC [94,131]. These kinases can localize to the nucleus to activate CREB and other transcription factors [58,68,69]. Studies indicate that CREB at serine 133 may be activated specifically by one pathway (e.g., PI3K/Akt) [41,58], two converging pathways (e.g., PKA and PKC) [94], or irrespective of pathway (e.g., MAPK/ERK or CaMKIV) [57]. There is significant crosstalk between pathways, which can vary according to stimulus intensity (e.g., toxic or non-toxic), and pattern of kinase activation (i.e., rapid and transient or delayed and prolonged) [40,57,97]. Since MAPK/ERK is not involved in our androgen-induced CREB activation, we assessed the contribution of other protein kinases. We found that androgen-induced CREB activation is independent of PI3K/Akt, PKA, and CaMKIV, as inhibitors of these kinases do not reduce the DHT-induced increase in CREB phosphorylation. To a certain extent, our results are surprising, as androgens activate upstream signaling kinases such as PI3K/Akt in prostate cancer cells [54,84], osteoblasts [50], and epithelial cells [7], with direct relevance to inhibition of apoptosis [54].

Our data suggest that androgen-mediated CREB activation is dependent upon PKC. Prior studies have also reported PKC-dependent CREB phosphorylation in neurons [94,131]. However, to our knowledge, this study provides the first evidence of PKC involvement in androgen activation of CREB, in either neuronal or non-neuronal cells. Our observations

support previous findings by Peterziel and colleagues indicating that PKC contributes to androgen signaling in prostate cancer cells [84]. Involvement of PKC in androgen actions is consistent with prior observations that androgens can act via second messengers such as cyclic AMP [95], inositol trisphosphate [61], phospholipase C [9], diacylglycerol (DAG) [61], and calcium (Ca^{2+}) [122], which in turn can result in PKC-dependent CREB phosphorylation [13,38].

PKC isoforms belong to conventional (α, β_I, β_{II}, γ), novel (δ, ε, η/Δ, θ, μ), or atypical (ζ, λ/ι) classes [90]. Conventional isoforms need $Ca2^{+}$ and DAG for activation, while novel isoforms need only DAG [76]. Ca^{2+} and DAG are not necessary for activation of atypical isoforms [71]. Two lines of evidence implicate conventional and or novel PKC isoforms in androgen activation of CREB. First, we observed that CREB activation is blocked by chronic exposure to PMA, which acts on the DAG binding region of conventional and novel PKC isoforms [73]. Second, both general (Gö6983) and conventional/novel-specific (GF109203X) PKC inhibitors block activation. The same CREB inhibition appears using conventional/novel-specific (iPKCc/n) but not atypical (iPKCζ) PKC peptide inhibitors. Interestingly, our previous data implicates conventional PKC isoforms in the mechanism of estrogen neuroprotection [17].

To our knowledge, our observation that androgens induce CREB activation in primary hippocampal neuron cultures is the first demonstration of this androgen pathway in neurons. Prior studies have reported an androgen effect on CREB in non-neuronal prostate cancer cells [111], Sertoli cells [30,116], and granular convoluted tubule cells [53]. Interestingly, though these studies found that androgen-induced CREB activation occurs downstream of AR-dependent MAPK/ERK signaling, we did not observe such effect. Excluding other upstream effectors such as (PI3K)/Akt [82], PKA [43,113], and CaMKIV [21,93], we found that androgens induce CREB activation via regulation by PKC signaling. CREB activation is a mechanism of cell survival in neuron cultures [11,32,92,114] against insults such as ischemia/hypoxia [46,99], excitotoxicity [57], and Aβ [8], as well as underlying many beneficial brain actions, including proliferation [83], differentiation [5], axon growth [132], and synaptic plasticity [52,121]. Our observations that androgens induce CREB activation may be relevant to functions important for cell growth, maintenance, and resilience.

4. EXPERIMENTAL PROCEDURE

Materials

Dihydrotestosterone (DHT) and trilostane were purchased from Steraloids (Newport, RI). PD98059, U0126, LY294002, H89, KN93, phorbol-12-myristate-13-acetate (PMA), 4α-phorbol-12, 13-didecanoate (4α-phorbol), GF109203X (bisindolylmaleimide I), Gö6983, bisindolylmaleimide V, cell-permeable (myristoylated) pseudosubstrate peptide inhibitor of conventional and novel protein kinase C (iPKCc/n), and myristoylated PKCζ (iPKCζ) pseudosubstrate peptide inhibitor were obtained from Calbiochem-Novabiochem (La Jolla, CA). ICI 182,780 was purchased from Tocris (Ellisville, MO). Mouse monoclonal phosphorylated cyclic AMP response element binding protein (CREB; serine 133) and rabbit polyclonal total CREB antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse monoclonal PKCα was acquired from Transduction Laboratories (Lexington, KY). Horseradish peroxidase (HRP)-conjugated anti-mouse was from Jackson ImmunoResearch Laboratories (West Grove, PA), and HRP-conjugated anti-rabbit was acquired from Pierce Chemical (Rockford, IL).

Neuron culture

Hippocampal neurons were obtained from embryonic day 18 Sprague-Dawley rats ($n \geq 6$ per preparation) and cultured according to a previously described technique that yields cultures that are ~95% neuronal based upon differential immunoreactivity with neuron-specific (NeuN, catalog #MAB377; Chemicon, Billerica, MA) and astrocyte-specific (GFAP, catalog #Z0334; Dako, Carpinteria, CA) antibodies [72,87]. In brief, we dissected the hippocampi of pups, dissociated cells enzymatically with 0.125% trypsin-EDTA for 10 min at 37°C and mechanically with a flamed-tipped glass pipette, and then filtered the cell solution through a 40 μm strainer (Falcon, Franklin Lakes, NJ). We diluted the single cell solution in serum-free Dulbecco's modified Eagle medium with 20 mM HEPES added, and supplemented the medium with 100 μg/ml transferrin, 5 μg/ml insulin, 100 μM putrescine, and 30 nM selenium. Cells were then plated onto poly-L-lysine-coated 12-well plates at densities of 1×10^5 cells/cm^2. For 3–4 days, cell cultures were stored in a humidified incubator (room air/5% CO_2, 37°C) before experimentation.

PC12 culture

Wild-type PC12 cells and clones stably transfected with full-length rat AR (pcDNA3-AR) or empty vector (pcDNA-ctl) have been described previously [72]. Wild-type and cell lines were grown in poly-L-lysine (0.05 mg/mL)-treated 75 cm^2 flasks (Fisher Scientific, Tustin, CA) containing RPMI 1640, 20 mM HEPES, 10 ml/L penicillin-streptomycin, 10% horse serum/5% fetal bovine serum, and 100 μg/ml G418 (except for wild-type). Cells were plated at a density of 5×10^4 cells/cm^2 in 12-well plates. After 24 h, cultures were switched to serum-free medium, and maintained at 37°C in a humidified incubator with room air/5% CO_2 for another 24 h before experimentation.

Experimental treatment of cultures

Cultures were treated with 10 nM DHT or ethanol vehicle for 15 min, 60 min, 4 h, 8 h, or 24 h. DHT was solubilized in 100% ethanol and diluted in culture medium to a final ethanol concentration of ≤ 0.1%; vehicle controls for DHT consisted of 0.1% ethanol. In experiments with an ER antagonist (1 μM ICI 182,780) and specific active or inactive inhibitors of MAPK/ERK kinase (MEK; 50 μM PD98059 or 10 μM U0126), PI3K/Akt (10 μM LY294002), PKA (1 μM H89), CaMKIV (10 μM KN93), PKC (2 μM GF109203X/bisindolylmaleimide I, 2 μM Gö6983, 2 μM bisindolylmaleimide V, 1 μM iPKCc/n, or 1 μM iPKCζ), and 3β-hydroxysteroid dehydrogenase (10 μM trilostane), the inhibitor or an equal amount of the final concentration of dimethyl sulfoxide (DMSO) vehicle was added 1 or 2 h before steroid treatment. Chronic PMA exposure consisted of 100 ng/ml PMA, inactive 4α-phorbol, or an equal concentration of 100% ethanol vehicle added 24 h before steroid treatment. All of the inhibitors, except for PKC peptide inhibitors (dissolved in sterile double deionized water), were solubilized in DMSO and diluted in culture medium to a final DMSO concentration of ≤ 0.1%. PMA was solubilized in 100% ethanol, and diluted in culture medium to a final ethanol concentration of ≤ 0.1%.

Western blot

Cell lysates were processed for phospho-specific and total levels of CREB or PKCα proteins using a standard western blot protocol [72,85]. In brief, protein samples were loaded into 12% polyacrylamide gels, electrophoresed for 2 h at constant 120 V, and then transferred onto PVDF membranes (Millipore, Bedford, MA) for 1 h at constant 100 V. After blocking (10 nM Tris, 100 nM NaCl, 0.1% Tween, 3% bovine serum albumin), immunoblots were incubated with phospho-CREB (1:1000) or PKCα (1:1000) antibodies overnight at 2–6°C, and then visualized with chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ). To assure equal amounts of total proteins across conditions, immunoblots were stripped (15 min in 100

mM glycine, pH 2.5, followed by 15 min in 2% SDS, 0.7% 2β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.7, 60°C) and reprobed with a pan antibody for CREB (1:1000). Blots were quantified by band densitometry of scanned films using NIH Image 1.61 software. Phosphorylation of CREB is expressed as a ratio of phosphorylated to total proteins. Raw data of the ratios of phosphorylated to total proteins were statistically analyzed using a split-plot ANOVA design, followed by pairwise comparisons using the Student's t test (significance indicated by $p < 0.05$). Three independent preparations were included for each experiment. For graphical presentation, phosphorylated CREB or PKCα is expressed as a percentage of control values with denotation of significance obtained from statistical analyses of raw data.

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Abbreviations

AR	Androgen receptor
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CREB	cyclic AMP response element binding protein
DHT	dihydrotestosterone
ERK	extracellular signal-regulated kinase
MAPK	mitogen-activated protein kinase
PMA	phorbol 12-myristate 13-acetate
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C

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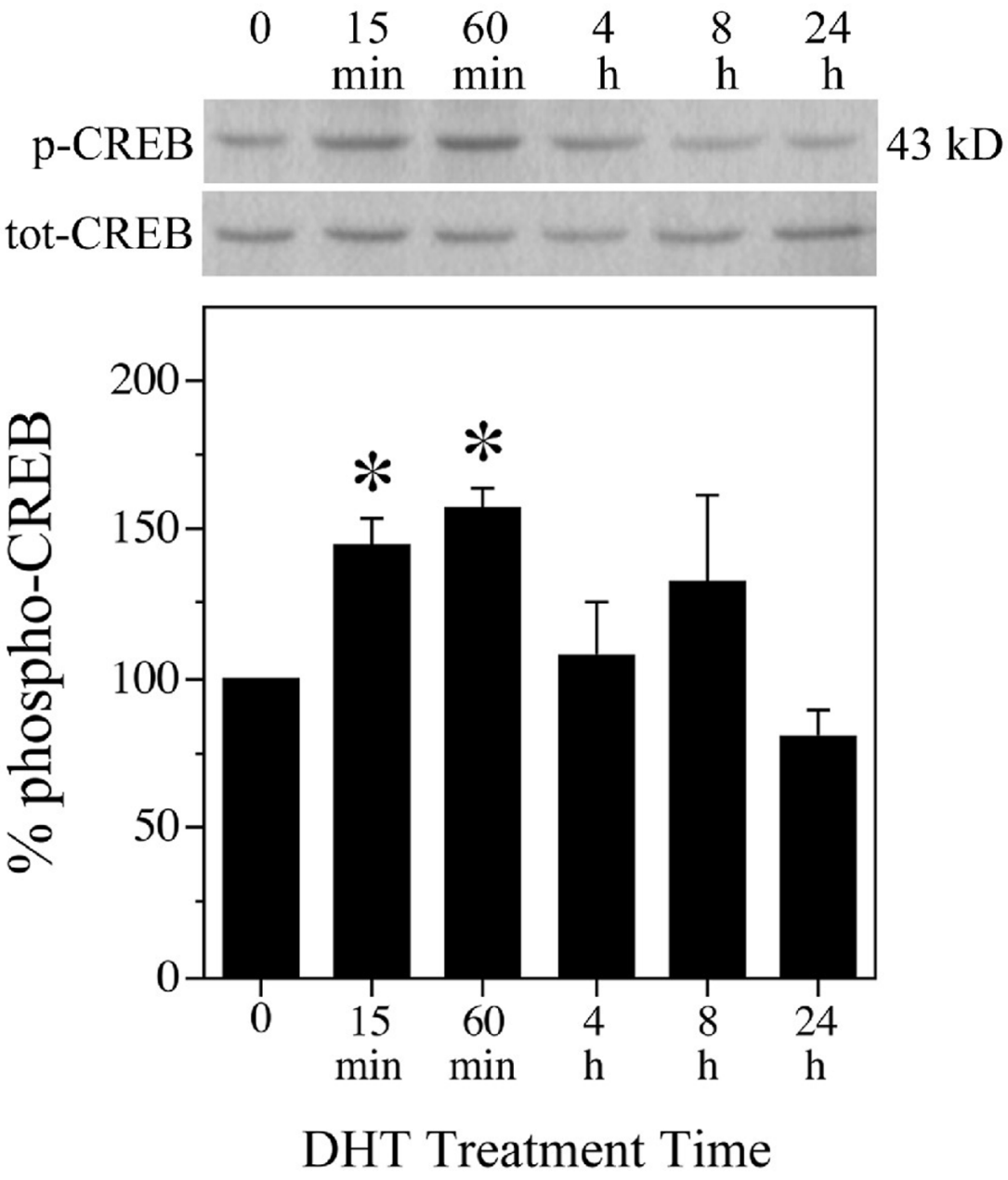

Fig. 1.

Androgens activate CREB in primary hippocampal neurons. Cultures were treated with 10 nM DHT or vehicle for the indicated times, and then examined by western blot using phosphorylated (p-CREB) and total (tot-CREB) CREB (43 kDa) antibodies. Percent phospho-CREB is expressed as a ratio of phosphorylated to total CREB, normalized to the vehicle-treated control condition (bottom panel). Immunoblots pictured are of representative experiments and data shown are means (± SEM) of the combined experiments (n = 3), [F (5,11) = 4.5; P = 0.018]. * $p < 0.05$ relative to the vehicle-treated control condition.

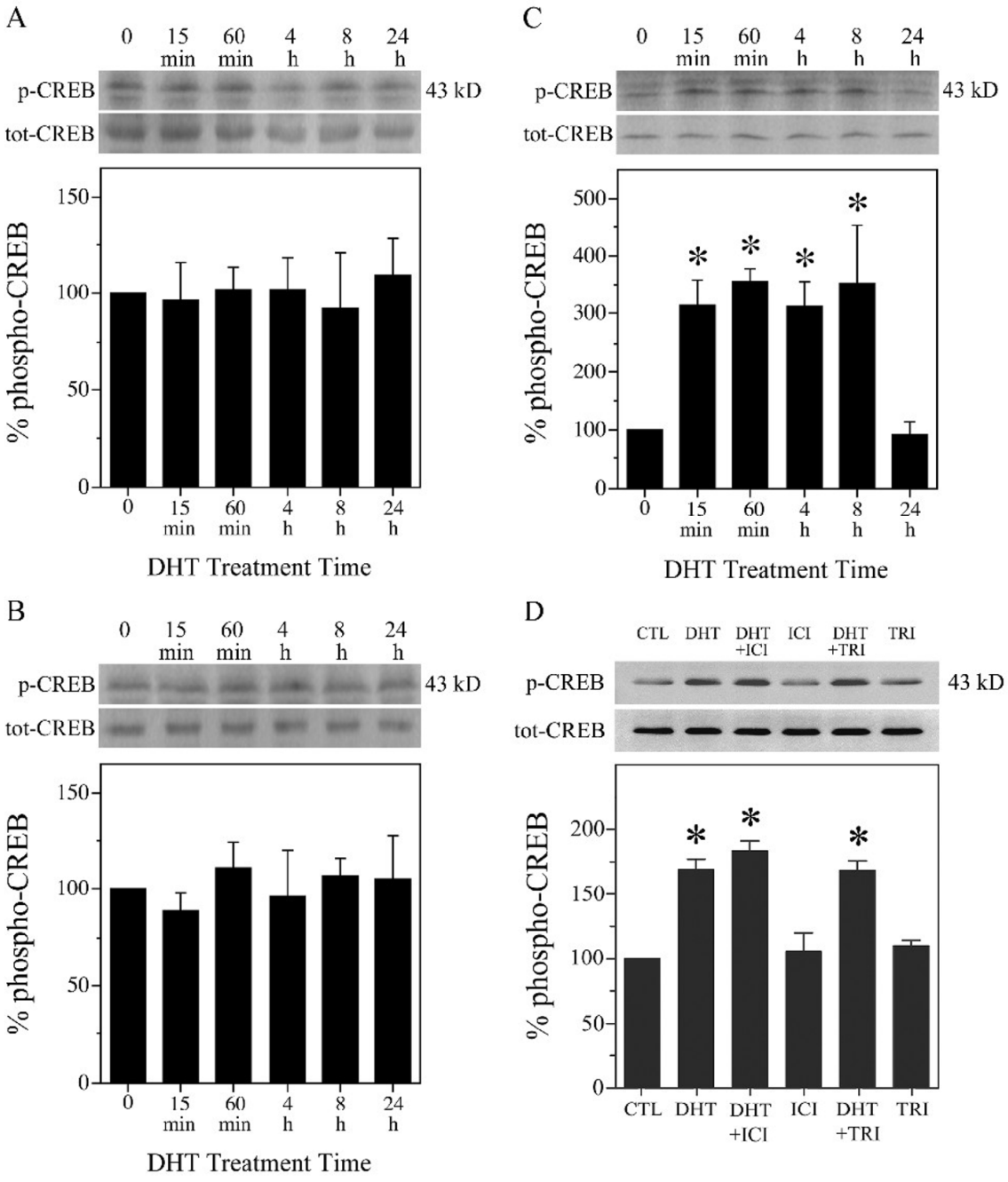

Fig. 2.

Androgen-induced CREB activation is AR-dependent. Western blots using phosphorylated (p-CREB) and total (tot-CREB) CREB (43 kDa) antibodies show that exposure to 10 nM DHT did not affect levels of phosphorylated CREB in (*A*) wild-type (wt) [F (5,11) = 0.02; P = 0.999] and (*B*) empty vector-transfected (pcDNA-ctl) [F (5,11) = 0.23; P = 0.942] PC12 cells. *C*, In PC12 cells stably transfected with AR (pcDNA-AR), 10 nM DHT significantly increased CREB phosphorylation [F (5,11) = 6.9; P = 0.004]. *D*, Hippocampal neuron cultures were pretreated for 2 h with ER antagonist 1 μM ICI 182,780 (ICI), 3β-hydroxysteroid dehydrogenase inhibitor 10 μM trilostane (TRI), or vehicle, followed by exposure to DHT for 2 h. DHT-induced CREB phosphorylation was not blocked by ICI 182,780 or trilostane [*F*

(5,11) = 30.5; $P < 0.0001$]. Percent phospho-CREB is expressed as a ratio of phosphorylated to total CREB, normalized to the vehicle-treated control condition (bottom panels). Immunoblots are from representative experiments and data show means (± SEM) of the combined experiments (n = 3). *$p < 0.05$ relative to the vehicle-treated control condition.

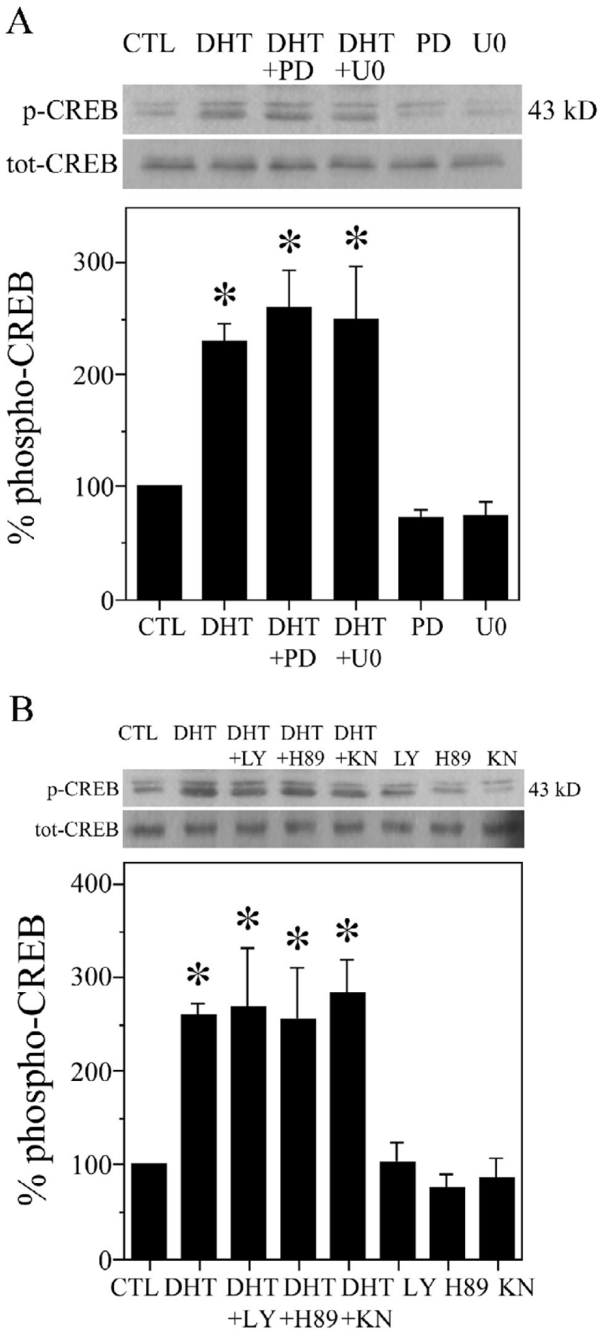

Fig. 3.

MAPK/ERK, PI3K/Akt, PKA, and CaMKIV do not contribute to androgen-induced CREB activation in hippocampal neuron cultures. DHT-induced CREB phosphorylation was significantly affected by neither (*A*) inhibitors of MAPK/ERK [F (5,11) = 5.3; P = 0.010] nor (*B*) inhibitors of PI3K/Akt, PKA, and CaMKIV [F (7,15) = 3.4; P = 0.023]. Cultures were pretreated for 2 h with kinase inhibitors 50 μM PD98059 (PD; MEK), 10 μM U0126 (U0; MEK), 10 μM LY294002 (LY; PI3K/Akt), 1 μM H89 (PKA), 10 μM KN93 (KN; CaMKIV), or vehicle, followed by exposure to 10 nM DHT for 2 h, and then were assessed by western blot using phosphorylated (p-CREB) and total (tot-CREB) CREB antibodies (43 kDa). Percent phospho-CREB is expressed as a ratio of phosphorylated to total CREB, normalized to the

vehicle-treated control condition (bottom panels). Immunoblots are from representative experiments and data show means (± SEM) of the combined experiments (n = 3). *$p < 0.05$ relative to the vehicle-treated control condition.

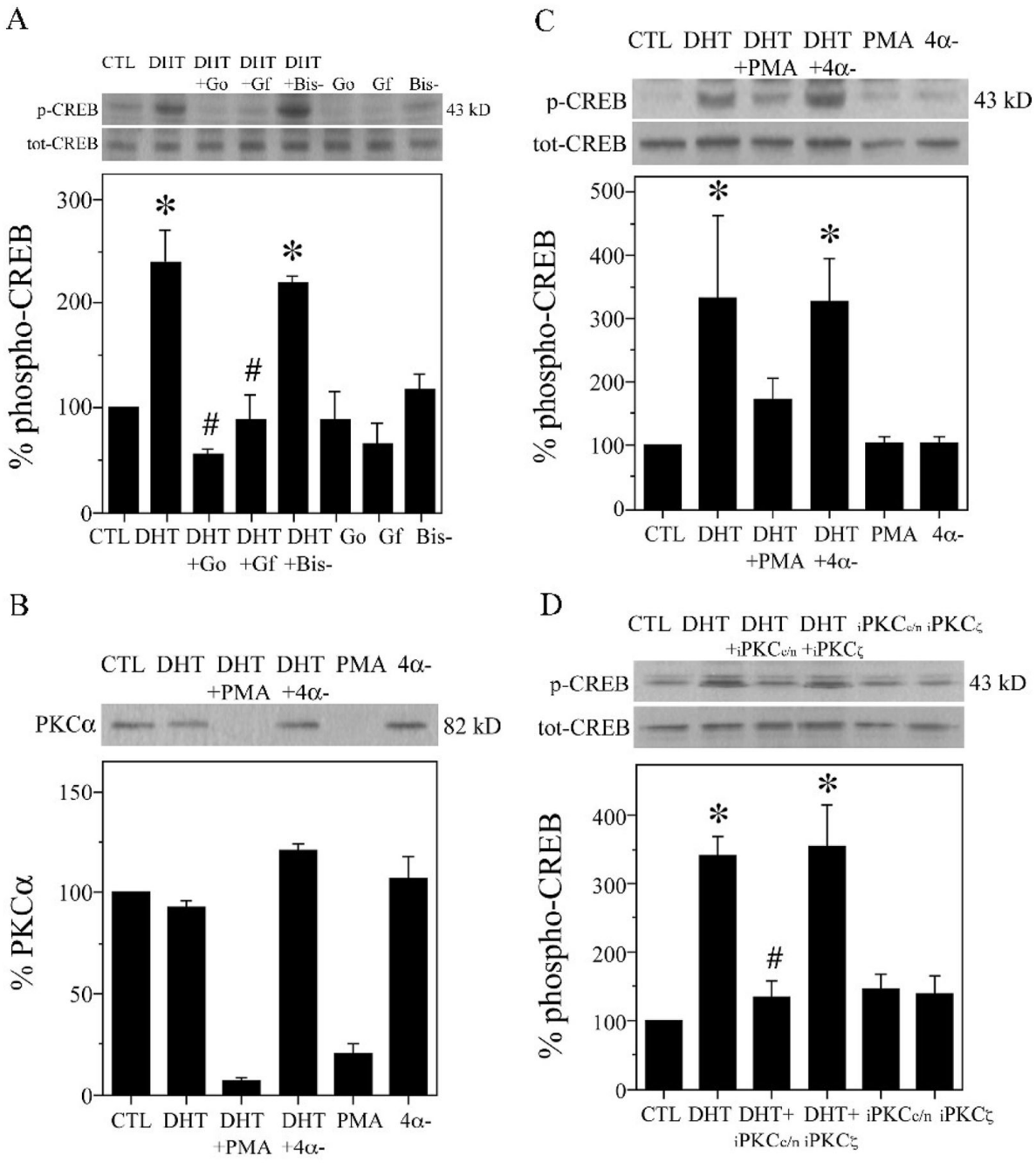

Fig. 4.

PKC contributes to androgen-induced CREB activation in hippocampal neuron cultures. *A*, Hippocampal neuron cultures were pretreated for 2 h with PKC inhibitor 2 μM GF109203X (GF), Gö6983 (Go), the inactive analog 2 μM bisindolylmaleimide V (Bis-), or vehicle, followed by 2 h exposure to 10 nM DHT, and then assessed by western blot using phosphorylated (p-CREB) and total (tot-CREB) CREB antibodies (43 kDa). Percent phospho-CREB is expressed as a ratio of phosphorylated to total CREB, normalized to the vehicle-treated control condition (bottom panels), [F (7,15) = 6.0; P = 0.002]. *B–C*, Hippocampal neuron cultures were pretreated with 100 ng/ml PMA, inactive 4α-phorbol (4α-), or vehicle, followed 24 h later by application of 10 nM DHT for 2 h, and then immunoblots were probed

for (*B*) PKCα (82 kDa) and (*C*) phosphorylated and total CREB [F (5,11) = 2.7; P = 0.075]. *D*, Neuron cultures were pretreated with 1 μM peptide inhibitors of conventional and novel (iPKCc/n) and atypical (iPKCα) PKC or vehicle for 2 h, and then exposed to 10 nM DHT for 2 h [F (5,11) = 3.6; P = 0.036]. Immunoblots are from representative experiments and data show means (± SEM) of the combined experiments (n = 3). *p < 0.05 relative to the vehicle-treated control condition. # p < relative to the paired DHT condition.