CaM Kinase Control of AKT and LNCaP Cell Survival

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ABSTRACT

AKT and its substrate BAD have been shown to promote prostate cancer cell survival. Agonists, such as carbachol, and hormones that increase intracellular calcium concentration can activate AKT leading to cancer cell survival. The LNCaP prostate cancer cells express the carbachol-sensitive M_3-subtype of G protein-coupled receptors that cause increases in intracellular calcium and activate the family of Ca^{2+}/calmodulin-dependent protein kinases (CaM Ks). One type of CaM Kinase, CaM Kinase Kinase (CaM KK), phosphorylates several substrates including AKT on threonine 308. AKT phosphorylation and activation enhances cell survival through phosphorylation of BAD protein and the subsequent blockade of caspase activation. Our goals were to examine the mechanism of carbachol activation of AKT and BAD in LNCaP prostate cancer cells and evaluate whether CaM KK may be mediating carbachol's activation of AKT and cell survival. Our results suggest that carbachol treatment of LNCaP cells promoted cell survival through CaM KK and its phosphorylation of AKT. The bacterial toxin anisomycin triggered caspase-3 activation in LNCaP cells that was blocked by carbachol in a CaM KK- and AKT-dependent manner. AKT and BAD phosphorylation were blocked by the selective CaM KK inhibitor, STO-609, as well as siRNA directed against CaM KK. BAD phosphorylation was also blocked by treating cells with the AKT inhibitor, AKT-X, as well as siRNA to AKT. Additionally, epinephrine promoted LNCaP cell survival through activation of AKT that was insensitive to STO-609. Taken together these data suggest a survival role for CaM KK operating through AKT and BAD in LNCaP prostate cancer cells. J. Cell. Biochem. 113: 1514–1526, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: AKT; PROTEIN KINASE B; PKA; CAMKK; CaM KINASE KINASE; CALMODULIN; CARBACHOL; LNCaP; EPINEPHRINE

Prostate cancer is the second most common cause of death among men in the United States [Schwartz and Hanchette, 2006; Colli and Amling, 2008]. The cell signaling pathways that regulate prostate cancer cell behaviors are an important area of current cancer research. Several cellular molecules have been implicated in promoting prostate cancer cell survival and metastasis including p53, Bcl-xL, PI3-K, BAD, PKA, and the phosphatase PTEN [Moul, 1999; Assinder et al., 2009]. PTEN has been characterized as a tumor suppressor that is frequently mutated in prostate cancer resulting in the activation of various cellular enzymes including protein kinase B also known as AKT [Assinder et al., 2009]. AKT is activated in numerous cells by various agonists and hormones including insulin, epinephrine, epidermal growth factor, and isoproterenol. AKT has been implicated in promoting cell survival or anti-apoptotic (death) signals within certain cells including the lymphnode carcinoma of the prostate (LNCaP) cell model system [de Souza et al., 2009; Goc et al., 2011]. AKT can be phosphorylated on threonine 308 and activated by several kinases including PDK1, mTOR, CaM Kinase Kinase, and PKA [Yano et al., 1998; Chan et al.,

1999; Sastry et al., 2007; Assinder et al., 2009]. AKT has the ability to phosphorylate numerous substrates involved in cell survival, such as MDM2, BAD, and XIAP [Cheng et al., 2005, 2008]. Consequently, the ability of a cell to carefully regulate AKT activity and its downstream targets is imperative for managing whether the cell may undergo apoptosis or survival.

AKT has been shown to promote cell survival through phosphorylation of the protein BAD in several cell types including NG108 neuroblastoma and LNCaP cells [Yano et al., 1998; Sastry et al., 2007]. The phosphorylation of BAD at serine 136 promotes its binding to the cytoplasmic protein, 14-3-3, and BAD sequestration away from mitochondria [Rapp et al., 2007]. BAD is a proapoptotic molecule that enhances cell death when it binds to mitochondrial Bcl-2 or Bcl-xL proteins and causes the release of cytochrome c. Increases in cytoplasmic cytochrome c concentration promote the activation of the cell death-inducing caspases including caspase-3 [Condorelli et al., 2001]. Caspase-3 and its downstream targets directly and exponentially execute cellular apoptosis. Consequently, BAD phosphorylation and association with 14-3-3 prevents the

activation of caspases and may promote cell survival. Interestingly, recently this has been shown that PKA activation of AKT in LNCaP cells leads to their survival [Sastry et al., 2007]; however, the role of intracellular calcium signaling molecules upstream of AKT in LNCaP cells has not been carefully evaluated.

Recent epidemiological data suggest that calcium may play an important role in prostate cancer progression in certain populations [Butler et al., 2010; Raimondi et al., 2010]. Hormones and agonists (i.e., carbachol) that increase the concentration of intracellular calcium can activate several intracellular molecules including calmodulin, calmodulin-dependent protein kinases (CaM Kinases), PKC, phospholipase A, and CalDAG-GEFs [Means, 2008; Stefanini et al., 2009; van Rossum and Patterson, 2009]. The LNCaP cells express an array of cellular receptors that may trigger cellular growth, survival, and intracellular calcium signaling pathways. G protein-coupled receptors (GPCRs) have been implicated in activating AKT and also give rise to an increase of calcium inside the cell [Rayford et al., 1997; Nagmani et al., 2003; Sastry et al., 2007]. Specifically, the M_3-subtype of GPCRs are expressed in LNCaP cells and promote their growth in response to carbachol treatment however the mechanism of carbachol- and calcium-dependent signaling in LNCaP cells was not evaluated [Rayford et al., 1997].

Intracellular calcium binds calmodulin and activates the family of calcium/CaM Kinases that includes CaM KII (multiple isoforms) and CaM KKα/β isoforms as well as its downstream targets CaM KI (multiple isoforms) and the predominantly nuclear CaM KIV [Means, 2008; Wayman et al., 2008]. In addition, CaM KK can directly phosphorylate and activate AKT through phosphorylation of threonine 308 and AMPK at threonine 172 [Yano et al., 1998; Hurley et al., 2005]. CaM KK activation of AKT has been implicated in neuronal survival [Yano et al., 1998]. CaM KK signaling through CaM KI has recently been shown to promote ERK activation and breast cancer cell growth downstream of carbachol and estrogen signaling [Schmitt et al., 2010]. Interestingly, contemporary work by Frigo et al. [2011] has demonstrated that CaM KK is highly expressed in prostate cancer cells and mediates androgen-stimulated LNCaP cell migration through AMPK; however, the role for CaM KK activation of AKT and LNCaP survival was not examined.

Given that the activation of AKT and its subsequent modulation of downstream targets promotes cancer cell survival we examined, if CaM Kinases, specifically CaM KK, regulates AKT activation in LNCaP cells. Additionally, we sought to evaluate if CaM KK-AKT activation downstream of calcium signaling controls BAD phosphorylation and caspase-3 activity in prostate cancer cells. To accomplish these goals we utilized molecular, biochemical, and pharmacological approaches to elucidate the important role for CaM Kinases and AKT inside of LNCaP cells. Our data suggest that a novel calcium signaling pathway exists in LNCaP cells and may provide valuable cellular targets in prostate cancer disease treatment.

MATERIALS AND METHODS

MATERIALS

The following materials were purchased from Sigma–Aldrich; carbachol, epinephrine, EGTA, and MTT reagent. KN-93, STO-609, anisomycin, ionomycin, and 4-DAMP were from Tocris Biosciences. The AKT inhibitor, AKT-X was from EMD Biosciences. The chemiluminescent reagent that is a substrate for HRP was purchased from Thermo Scientific (Pierce Biotechnology). Antibodies that bind to phosphorylated AKT at Threonine 308 (p308) and Serine 473 (p473), phosphorylated BAD at Ser136 (pBAD), cleaved caspase-3 (Asp175), and β-actin were purchased from Cell Signaling Technology. Antibodies that recognize CaM KKα, CaM KIγ, CaM KIα, phospho-CaM KI, AKT, BAD, HA as well as HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. siRNAs directed against human AKT1/2 (sc-43609), human CaM KIα (sc-38947), or scrambled control siRNAs were also purchased Santa Cruz Biotechnology. The caspase-3 colorimetric assay was purchased from Biovision Inc. The Guava Nexin Reagent was purchased from Guava Technologies/Millipore (Hayward, CA).

CELL CULTURE

The LNCaP cells were a generous gift from Dr. Thomasz Beer at OHSU (Portland, OR). LNCaP cells were cultured in RPMI and 5% CO_2 according to ATCC guidelines. LNCaP cells were serum-starved in RPMI overnight, pretreated as indicated, and then stimulated for Western blotting, caspase activity, or the cell survival assay. Cells were pretreated with the following inhibitors for 30 min prior to stimulation, 4-DAMP (2 μM), KN-93 (5 μM), EGTA (2.5 mM), AKT-X (5 μM), or STO-609 (5 μM), and then treated with ionomycin (1 μM), carbachol (10 μM), or epinephrine (100 nM) as indicated. Anisomycin (5 μM) was incubated with cells for 6 h prior to stimulation as indicated.

WESTERN BLOTTING AND IMMUNOPRECIPITATION

LNCaP cells were stimulated, lysed, and equivalent protein amounts were blotted onto PVDF membranes and examined by Western blotting. Prior to Western blotting, protein concentrations were determined using the Bradford protein assay and a microplate reader (Bio-Rad Model 680). PVDF membranes were probed using the indicated antibodies, scanned, and densitized using a molecular imaging system (Kodak Image Station, 2000R) and evaluated for significant differences among treatments. In all cases, phosphorylated or cleaved protein levels were normalized to total protein levels and controls for final analysis and presented as fold over basal. For transfection experiments, 500 μg of whole-cell lysates were precipitated overnight with the indicated antibodies at 4°. Precipitates were washed 2× in ice-cold lysis buffer, run on SDS–PAGE gels, and analyzed by Western blotting.

TRANSFECTION EXPERIMENTS

LNCaP cells were co-transfected with 2.5 μg HA-AKT along with 2.5 μg shCaMKKα, 2.5 μg shCaMKIγ (all three were a generous gift from Dr. Tom Soderling at the Vollum Institute, OHSU), siRNA directed against CaM KIα (Santa Cruz Biotechnology), scrambled control siRNA, or the control pcDNA3 plasmid as indicated. The shCaMKIγ and shCaM KKα sequences and vectors have been previously described in detail [Wayman et al., 2006; Saneyoshi et al., 2008; Wayman et al., 2011]. LNCaP cells were also transfected with siRNA directed against CaM KIα, AKT1/2, or scrambled control siRNAs according to the manufacturer's guidelines (Santa Cruz

Biotechnology). Transfections were done at approximately 50–60% confluence for ∼6 h using Lipofectamine 2000 (Invitrogen, Corporation). Following transfection, cell media was replaced with complete media and cells were allowed to recover ∼24 h. Transfected cells were then serum-starved overnight prior to treatment with carbachol (10 μM), lysis, and Western blot analysis.

CASPASE-3 ASSAY

Caspase-3 activity was examined from LNCaP cell extracts based on its ability to recognize and cleave its substrate, DEVD-*p*NA. Caspase activity was assayed according to the manufacturer's guidelines (BioVision Research Products). Equivalent LNCaP cell numbers were placed on 96-well plates in RPMI, induced into quiescence for 24 h, and pretreated with inhibitors for 30 min and then anisomycin, carbachol, epinephrine, or the indicated combinations for 6 h, lysed, and equivalent protein amounts (200 μg) were incubated with DEVD-pNA substrate for 2 h according to the manufacturer's guidelines. Samples were then read using a microplate reader (Bio-Rad Model 680) with the 405 nm filter. Caspase-3 activity was normalized to untreated control cells and presented as fold over basal.

CELL SURVIVAL ASSAY

Equivalent LNCaP cell numbers were placed on 96-well plates in RPMI, induced into quiescence for 24 h, and pretreated with inhibitors for 30 min and then anisomycin, carbachol, epinephrine, or the indicated combination for 6 h. Twenty microliters of MTT Reagent (Thiazolyl blue tetrazolium bromide) was added to the cells and incubated for 2 h at 37°C. The MTT-treated cells were carefully homogenized by the addition of SDS-containing detergent and the plate was read using a microplate reader (Bio-Rad Model 680) with the 595 and 655 nm filters.

APOPTOSIS ASSAY

Equivalent LNCaP cell numbers were placed on 3.5 cm plates in RPMI, induced into quiescence for 24 h, and pretreated with inhibitors for 30 min and then anisomycin, carbachol, epinephrine, or the indicated combination for 6 h. Approximately 300,000 cells were analyzed for each treatment from four independent experiments. Following the treatments, LNCaP cells were mechanically dislodged and incubated with Guava Nexin Reagent (that contains dye-labeled Annexin V and 7-AAD) in the dark according to the manufacturer's guidelines (Guava Technologies/Millipore). Cells were then placed into 96-well plates and immediately read and analyzed using a Guava Flow Cytometry System (Guava Technologies). Cellular apoptosis was determined based on dye-labeled Annexin V cell surface binding to LNCaPs and presented as the percentage of apoptotic (Annexin V positive) over non-apoptotic (Annexin V negative). The apoptosis assay was performed in the laboratory of Dr. Brian Druker in the Knight Cancer Institute at OHSU.

STATISTICS

To determine if significant differences exist among treatments in our system statistical analysis was performed on quantified data. Data from each treatment was averaged, the standard error was determined, and statistical significance (*P*-value) analyzed using a Student's *t*-test. In all cases, "*" and "**" represented as *P*-value of ≤0.05 and ≤0.01, respectively.

RESULTS

CARBACHOL BLOCKS APOPTOSIS IN LNCAP CELLS VIA CAM KK

LNCaP cells express muscarinic acetylcholine receptors that increase intracellular calcium concentrations and promote cell growth upon carbachol stimulation [Rayford et al., 1997]. Carbachol and calcium-elevating agents are also sufficient to activate CaM Kinases and AKT that in turn promote cell growth and survival [Yano et al., 1998; Jimenez and Montiel, 2005; Schmitt et al., 2010]. To examine the physiological significance of calcium and CaM Kinases in promoting LNCaP cell survival, we utilized the MTT assay to quantify cell viability under various stimulatory conditions. Furthermore, we utilized the toxin anisomycin at a dose and duration that has previously been shown to promote caspase-3 activation and prostate cancer cell death [Curtin and Cotter, 2002; Sah et al., 2003; Hori et al., 2008]. As can be seen in Figure 1A, treatment of LNCaPs with anisomycin triggered a substantial drop in cell survival that was reversed by stimulation with carbachol. To examine the role for calcium and CaM Kinases in LNCaP survival we began by using the compound EGTA that functions as a chelator of calcium ions and KN-93 that selectively inhibits the CaM Kinase family of enzymes. Carbachol's ability to promote survival was significantly blocked by EGTA, and the broad CaM Kinase inhibitor, KN-93 (Fig. 1A). To evaluate which CaM Kinase may be mediating carbachol's effects we used the selective inhibitor of CaM KK, STO-609, as well as the AKT inhibitor AKT-X. Carbachol's ability to enhance cell survival was blocked by both STO-609 and AKT-X (Fig. 1B). Epinephrine promotes PKA-dependent AKT phosphorylation and LNCaP cell survival [Sastry et al., 2007], therefore, to confirm the specificity of the STO-609 compound in our system, we stimulated cells with epinephrine in the presence or absence of STO-609. Epinephrine treatment of LNCaPs also promoted cell survival and reversed anisomycin's toxic effects (Fig. 1C). Interestingly, STO-609 did not affect epinephrine signaling however inhibition of AKT with AKT-X blocked epinephrine's survival effect on LNCaPs (Fig. 1C).

Cellular apoptosis can also be evaluated based on the ability of apoptotic cells to bind fluorescently labeled Annexin V on their plasma membrane followed by flow cytometry analysis. To directly assess the ability of anisomycin to trigger cellular apoptosis LNCaPs were treated as in Figure 1 and then incubated with Annexin V (Guava Nexin Reagent) and analyzed by flow cytometry on a Guava Flow System. Consistent with the data from Figure 1, anisomycin potently enhanced LNCaP apoptosis that was blocked by carbachol treatment of cells (Fig. 2A). Carbachol's survival effects were significantly inhibited by pretreatment with EGTA and KN-93 (Fig. 2A) as well as STO-609 and AKT-X (Fig. 2B). To further confirm the protective effects of epinephrine and the selectivity of STO-609 in our system, LNCaP cells were stimulated with epinephrine and analyzed for Annexin V binding by flow cytometry. Epinephrine inhibited anisomycin-induced apoptosis in a manner that was AKT-dependent (blocked by AKT-X) but insensitive to STO-609 (Fig. 2C).
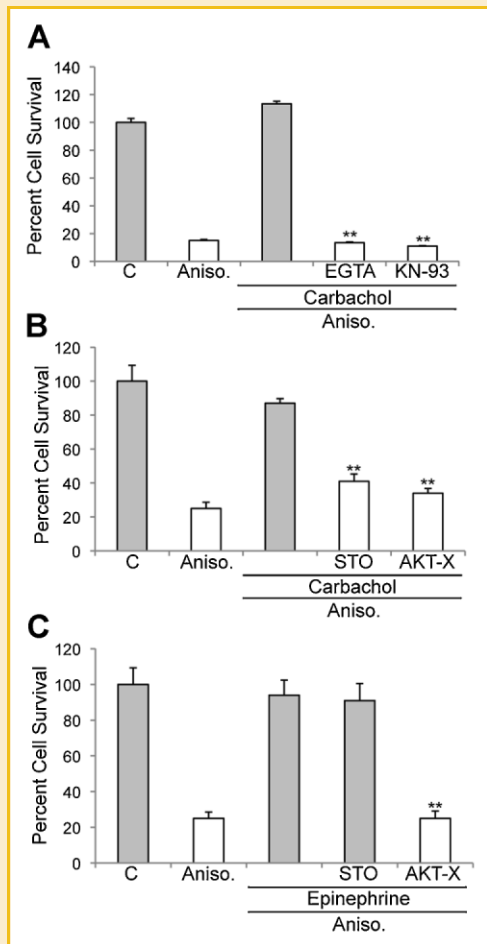
Fig. 1. Carbachol promotes LNCaP survival through CaM KK and AKT. A: Calcium and CaM Kinases mediate carbachol-stimulated cell survival. LNCaP cells received no pretreatment or were pretreated with the CaM Kinase inhibitor KN-93 (5 μM) or the calcium chelator EGTA (2.5 mM) as indicated and then with anisomycin (5 μM), carbachol (10 μM), or the combination for 6 h. In all figures "C" represents the control non-stimulated cells. Cell survival was then analyzed using the MTT assay as described in the Materials and Methods Section (N = 4, ±SE, **P ≤ 0.01). B: STO-609 and AKT-X block calcium-stimulated cell survival. LNCaP cells received no pretreatment or were pretreated with the CaM KK inhibitor, STO-609 (5 μM) or the AKT inhibitor, AKT-X (5 μM), and then stimulated similarly to panel A and analyzed with the MTT assay (N = 4, ±SE). C: AKT but not CaM KK regulates epinephrine-stimulated cell survival. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM) or AKT-X (5 μM), and then stimulated with anisomycin (5 μM), epinephrine (100 mM), or the combination for 6 h and analyzed with the MTT assay (N = 4, ±SE).
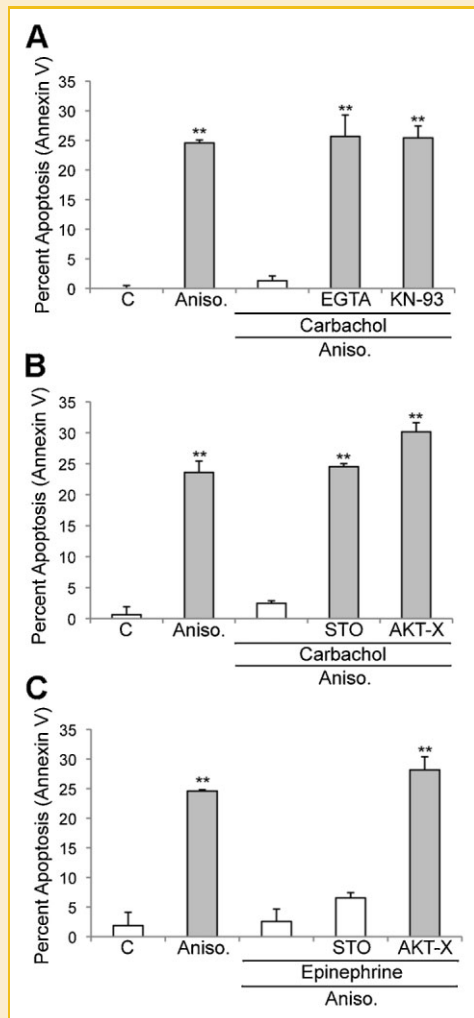

Fig. 2. Carbachol blocks apoptosis through CaM KK and AKT. A: Carbachol inhibits apoptosis through calcium and CaM Kinases. LNCaP cells received no pretreatment or were pretreated with EGTA (2.5 mM) or KN-93 (5 μM), and then with anisomycin (5 μM), carbachol (10 μM), or the combination for 6 h. Cells were then incubated with Guava Nexin Reagent (Annexin V) and analyzed as described in the Materials and Methods Section. Cellular apoptosis was then analyzed using flow cytometry with a Guava Flow System as described in the Materials and Methods Section (N = 4, ±SE). B: CaM KK and AKT block anisomycin-induced apoptosis. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM) or AKT-X (5 μM), and then stimulated similarly to panel A and analyzed with the Guava System assay (N = 4, ±SE). C: Epinephrine inhibits apoptosis through AKT but not CaM KK. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM) or AKT-X (5 μM), and then stimulated with anisomycin (5 μM), epinephrine (100 mM), or the combination for 6 h, and analyzed with the Guava System assay (N = 4, ±SE).

Taken together the above results suggest that carbachol specifically utilizes calcium, CaM KK, and AKT to promote LNCaP survival and block apoptosis. In addition, the hormone epinephrine also promotes cell survival in a CaM KK-independent manner but appears to utilize AKT.

CARBACHOL INHIBITS CASPASE-3 ACTIVITY

The caspase family of enzymes regulate cell survival and provide an important role in controlling the fate of a cell. Caspases can be activated through a variety of means including cell stresses that cause BAD association with mitochondria and subsequent release of cytochrome c from the organelle [Condorelli et al., 2001]. Caspase-3 activity is an established means to promote cell death therefore we evaluated the ability of carbachol and CaM KK to regulate caspase-3 activity in anisomycin-treated LNCaP cells. We utilized the toxin anisomycin at a dose and duration similar to Figures 1 and 2.
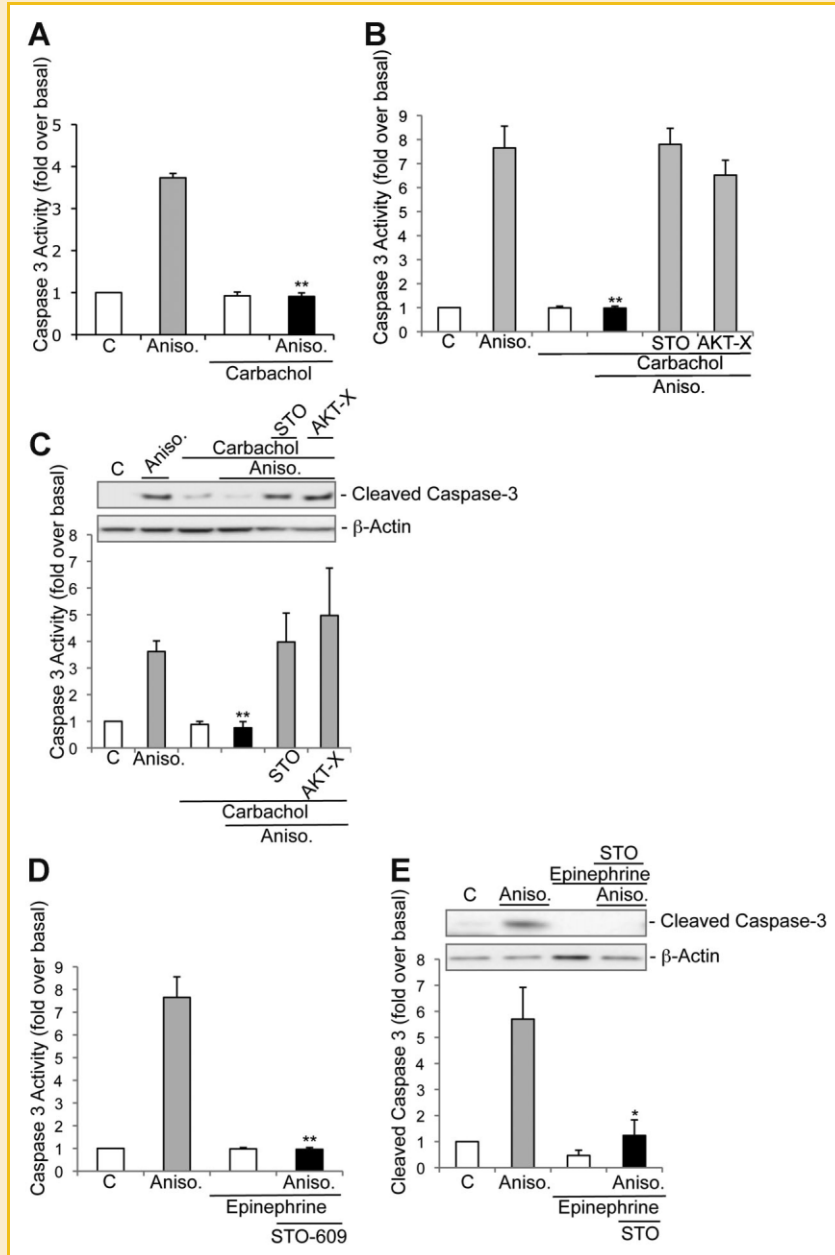
Fig. 3. CaM KK and AKT mediate carbachol's blockade of caspase-3 activity. A: Carbachol blocks caspase-3 activity in LNCaP cells. LNCaP cells received no pretreatment or were treated with anisomycin (5 μM), carbachol, or the combination for 6 h. Caspase-3 activity was then analyzed as described in the Materials and Methods Section (N = 4, ±SE). B: Carbachol inhibits caspase-3 activity through CaM KK and AKT. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM) or AKT-X (5 μM), and then incubated with anisomycin (5 μM), carbachol, or the combination for 6 h as in panel A. Caspase-3 activity was then analyzed (N = 4, ±SE). C: Carbachol blocks caspase-3 activation and cleavage. LNCaP cells were treated identically to panel B and endogenous cleaved caspase-3 was measured by Western blotting and the bands were densitized, normalized to total actin, and quantitated (N = 4, ±SE). D: Epinephrine blocks caspase activation independent of CaM KK. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM), and then incubated with anisomycin (5 μM), epinephrine (100 nM), or the combination for 6 h. Caspase-3 activity was then analyzed (N = 4, ±SE). E: Epinephrine prevents caspase-3 activation and cleavage. LNCaP cells were treated identically to panel D and endogenous cleaved caspase-3 was measured by Western blotting and the bands were densitized, normalized to total actin, and quantitated (N = 4, ±SE, *P ≤ 0.05).

Caspase-3 activity was quantified using PARP cleavage and as can be seen in Figure 3A, the bacterial toxin, anisomycin, promotes a fourfold increase in caspase-3 activity. Carbachol treatment of LNCaP cells did not activate caspase-3 and significantly attenuated anisomycin's ability to promote caspase activity (Fig. 3A). To determine if CaM KK and AKT mediate carbachol's inhibitory effect on caspase-3, cells were pretreated with either STO-609 or the AKT compound, AKT-X in the presence or absence of anisomycin and carbachol as indicated. Consistent with the previous data, anisomycin promoted caspase-3 activity that was blocked by

carbachol; however, both STO-609 and AKT-X reversed carbachol's inhibitory effects on caspase-3 activity (Fig. 3B). As an additional means to directly evaluate caspase-3 activity in our system, we evaluated the cleaved and active form of caspase-3 in response to anisomycin treatment. The immunoblots presented in Figure 3C demonstrate that anisomycin promotes caspase-3 activation that is blocked by carbachol treatment of cells. Similar to the preceding data, carbachol's inhibition of caspase activity was inhibited by either STO-609 or AKT-X (Fig. 3C). Epinephrine promotes LNCaP cell survival and consistent with these data, it also blocked the ability of anisomycin to promote caspase-3 activation (Fig. 3D). To confirm the selectivity of STO-609 in our system, epinephrine-treated cells were also evaluated with the CaM KK inhibitor. Epinephrine was capable of blocking caspase-3 activity in the presence of STO-609 (Fig. 3D). The epinephrine effect on caspase-3 activity was also evaluated by Western blotting for the active form of caspase-3. Epinephrine treatment of LNCaPs inhibited caspase-3 cleavage in a manner that was insensitive to STO-609 (Fig. 3E). Taken together, these results suggest that carbachol and epinephrine treatment of LNCaPs block caspase-3 cleavage and activity. Furthermore, these data also purport that CaM KK specifically mediates carbachol's effects on caspase activity in our system.

CARBACHOL AND CAM KINASES REGULATE AKT ACTIVITY

LNCaP cells express endogenous muscarinic receptors and carbachol may work through the M_3-subtype muscarinic receptor to activate CaM Kinases in cancer cells [Rayford et al., 1997; Jimenez and Montiel, 2005; Schmitt et al., 2010]. CaM KK has been shown to directly phosphorylate and activate the survival protein AKT through its phosphorylation of threonine 308 [Yano et al., 1998], therefore we sought to examine the ability of carbachol to trigger AKT phosphorylation in LNCaP cells. The selective M_3-subtype muscarinic receptor antagonist, 4-DAMP, blocks carbachol activation of CaM Kinases and is a useful tool for evaluating carbachol signaling in cancer cells [Schmitt et al., 2010]. Carbachol treatment of LNCaP cells triggered AKT phosphorylation at both threonine 308 (p308) and serine 473 (p473) within 5 min (Fig. 4A). AKT phosphorylation at both sites was completely blocked by inhibition of the M_3 muscarinic receptor with the 4-DAMP compound (Fig. 4A). To determine if calcium and its downstream targets, the CaM Kinases, are involved in AKT activation we pretreated LNCaP cells with either KN-93 or EGTA. As can be seen in Figure 4B, carbachol stimulates AKT phosphorylation and full activation in a manner that is dependent upon CaM Kinases and calcium as it is blocked by KN-93 and EGTA, respectively. Previous research has shown that epinephrine triggers the cAMP and PKA-dependent phosphorylation of AKT [Sastry et al., 2007]. As an important control for the specificity of our compounds we pretreated cells with either KN-93 or 4-DAMP and then stimulated them with epinephrine. Epinephrine potently stimulated AKT phosphorylation in a manner that was not affected by either inhibitor (Fig. 4C).

Based on the observation that STO-609 blocked carbachol's survival effects and that CaM KK has previously been shown to activate AKT through direct phosphorylation at threonine 308 we

evaluated LNCaP cells for the presence of endogenous CaM KK. Western blotting of cellular extracts revealed the presence of CaM KKα (alpha) in LNCaP cells (Fig. 4D). Given that LNCaP cells express endogenous CaM KK and that KN-93 inhibits AKT activation we wanted to determine if the selective inhibitor of CaM KK, STO-609, affected carbachol's activation of AKT. Carbachol induced a potent phosphorylation and activation of AKT that was significantly inhibited by treating cells with STO-609 (Fig. 4E). To evaluate whether CaM KK mediates other calcium signaling pathways, cells were also stimulated with ionomycin to increase intracellular calcium following a pretreatment with STO-609. Ionomycin promoted nearly a fourfold increase in AKT phosphorylation that was dependent upon CaM KK as it was completely blocked by STO-609 (Fig. 4F). Since epinephrine promotes PKA-dependent AKT phosphorylation [Sastry et al., 2007], we next sought to confirm the specificity of the STO-609 compound in our system by stimulating cells with epinephrine in the presence or absence of STO-609. Consistent with previous work, epinephrine enhanced AKT phosphorylation at both sites however STO-609 had no effect on epinephrine's activation of AKT (Fig. 4G). Taken together these data suggest that CaM KK mediates calcium-dependent phosphorylation and activation of AKT in LNCaP cells.

CAM KK SPECIFICALLY MEDIATES AKT ACTIVATION

Since LNCaP cells express endogenous CaM KK and the phosphorylation of AKT is blocked by KN-93 and STO-609 we wanted to directly evaluate if CaM KK is activated in our system as well as inhibited by our compounds. CaM KI is directly phosphorylated and activated by CaM KK on threonine 177 and functions as a means to monitor CaM KK activity [Wayman et al., 2011]. Stimulation of LNCaP cells with carbachol triggered a threefold increase in CaM KI phosphorylation in a manner that was blocked by either KN-93 or STO-609 suggesting that CaM KK is active in our system (Fig. 5A). To determine whether CaM KK is required for AKT activation in LNCaP cells, we transfected cells with shRNA plasmids designed to knockdown expression of CaM KKα. The specificity of the CaM Kinase shRNA plasmids have previously been described and characterized in several cell types including cancer cells [Wayman et al., 2006; Saneyoshi et al., 2008; Davare et al., 2010; Schmitt et al., 2010; Wayman et al., 2011]. Transfection of LNCaPs with shCaM KKα plasmids significantly reduced target protein expression, whereas the control pcDNA3 plasmid had no effect (Fig. 5B). We next sought to determine if carbachol's stimulation of AKT phosphorylation specifically utilized CaM KKα. As can be seen in Figure 5C, carbachol treatment of pcDNA3/HA-AKT-transfected cells triggered HA-AKT phosphorylation, an effect that was reversed by blocking CaM KKα expression. CaM KK has numerous substrates including AMPK, CaM KIV, AKT, and the various isoforms of CaM KI including CaM KIγ and CaM KIα. We have previously shown that CaM KIγ works downstream of CaM KKα in breast cancer cells to promote their growth [Schmitt et al., 2010], whereas CaM KIα may play a role in neuronal development [Saneyoshi et al., 2008]; therefore, we sought to examine if LNCaP cells express either CaM KI isoform. As can be seen in Figure 5D, LNCaP cells appear to express both isoforms of CaM KI and CaM KIγ appears to be relatively more abundant that CaM KIα in LNCaP cells. To control for the specificity
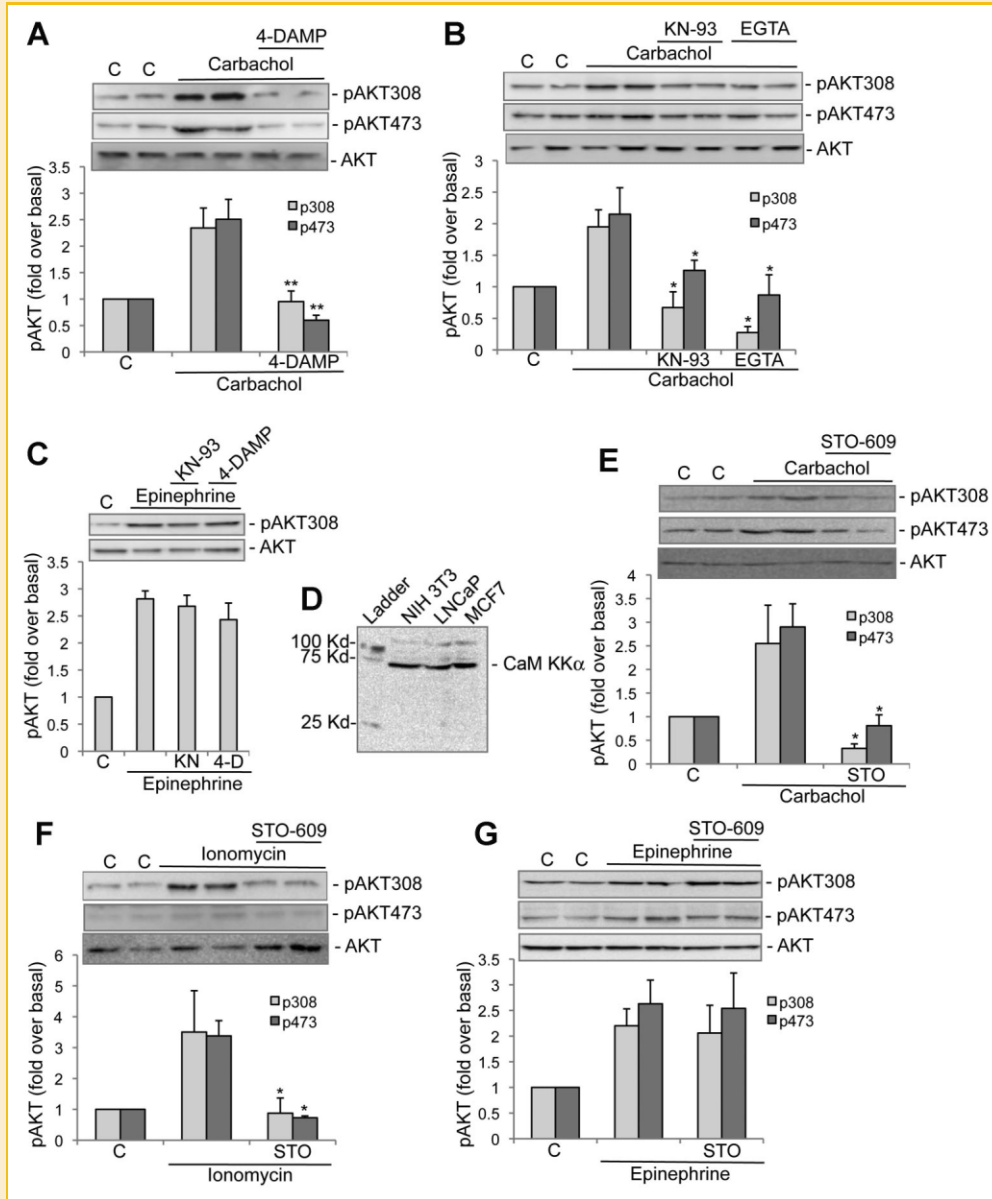
Fig. 4. AKT activation in LNCaP cells. A: Carbachol activates AKT through muscarinic receptors in LNCaP cells. LNCaP cells were stimulated with carbachol (10 μM) for 5 min in the presence or absence of the 4-DAMP (2 μM) compound as described in the Materials and Methods Section. Cell lysates were analyzed and endogenous AKT activation was measured by Western blotting for phosphorylated-AKT (pAKT) at both threonine 308 (p308) and serine 473 (p473). In all figures "p308" and "p473" represent phosphorylation at each site. The lower panel is a Western blot of total AKT proteins (AKT), demonstrating equal loading of AKT used for pAKT assays. Data are presented as fold AKT phosphorylation over control (N = 8, ±SE). B: AKT activation utilizes CaM Kinases and calcium. LNCaP cells were stimulated with carbachol for 5 min or pretreated with the CaM Kinase inhibitor KN-93 (5 μM) or EGTA (2.5 mM) as indicated and AKT activation was measured by Western blotting as in panel A (N = 4, ±SE). C: KN-93 and 4-DAMP do not effect epinephrine signaling in LNCaP cells. LNCaP cells received no pretreatment or were pretreated with KN-93 (5 μM) or 4-DAMP (2 μM), and then stimulated with epinephrine (100 nM) for 5 min. Endogenous AKT activation was analyzed by Western blotting (N = 4, ±SE). D: LNCaP cells express endogenous CaM KK alpha (α). Lysates of NIH3T3, LNCaP, and MCF-7 cells were analyzed by Western blotting for endogenous CaM KKα. E: AKT activation by carbachol is CaM KK-dependent. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM) and then stimulated with carbachol (10 μM) for 5 min as in panel A (N = 4, ±SE). F: Ionomycin activates AKT via CaM KK. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM), and then stimulated with ionomycin (1 μM) for 5 min (N = 6, ±SE). G: Epinephrine activation of AKT is CaM KK-independent. LNCaP cells received no pretreatment or were pretreated with STO-609 (5 μM) and then stimulated with epinephrine (100 nM) for 5 min (N = 6, ±SE).

of our shRNA to CaM KKα as well as ensure that CaM KK is not using either CaM KI isoform to trigger AKT phosphorylation we ablated the expression of CaM KIγ and CaM KIα in LNCaP cells through siRNA knockdown. Expression of pcDNA3 had no effect on CaM KIγ

protein levels, however shCaM KIγ expression potently inhibited CaM KIγ production in our system (Fig. 5E). To confirm that CaM KKα does not utilize CaM KIγ to activate AKT, we co-transfected cells with the indicated constructs and then stimulated them with
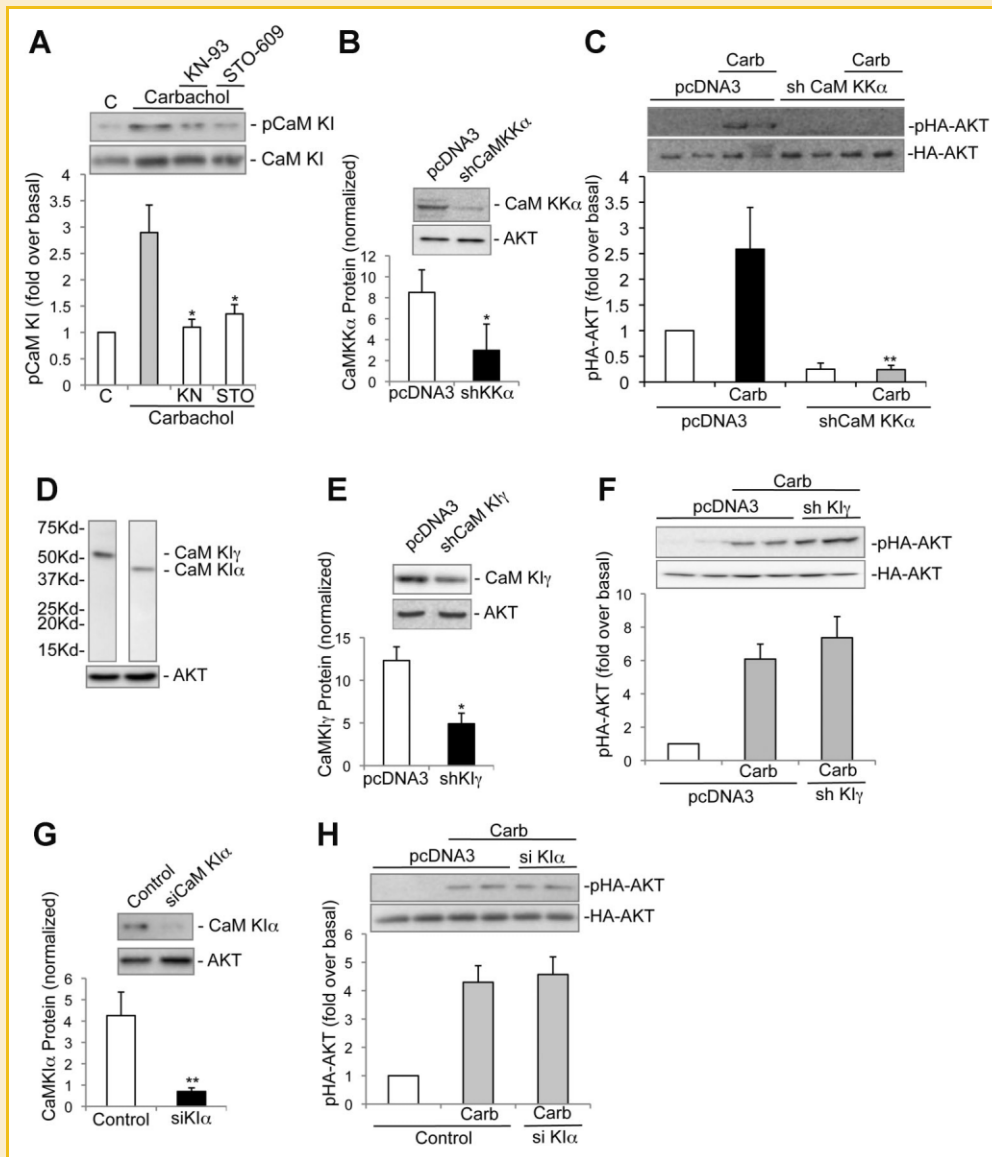
Fig. 5. AKT activation requires CaM KK but not CaM KI. A: Carbachol activates CaM KK in LNCaP cells. LNCaP cells received no pretreatment or were pretreated with KN-93 (5 μM) or STO-609 (5 μM), and then stimulated with carbachol (10 μM) for 5 min as in Figure 4. Cell lysates were analyzed and endogenous CaM KK activation was measured by Western blotting for phosphorylated-CaM KI (pCaM KI) at threonine 177 (N = 4, ±SE). B: shRNA directed against CaM KKα reduces its expression. LNCaP cells were transfected with either pcDNA3 or shRNA as described in the Materials and Methods Section. Endogenous CaM KKα expression was measured by Western blotting and the bands were densitized, normalized to total AKT, and quantitated. Data are presented as fold CaM KKα expression over AKT (N = 8, ±SE). C: Carbachol activation of AKT requires CaM KKα. LNCaP cells were co-transfected with HA-AKT along with pcDNA3 or shCaM KKα, stimulated with carbachol (10 μM) for 5 min, and HA-AKT phosphorylation was analyzed as indicated in the Materials and Methods Section (N = 6, ±SE, $^{**}P \leq 0.01$). D: LNCaP cells express endogenous CaM KI gamma (γ) and CaM KI alpha (α). Lysates of LNCaP cells were analyzed by Western blotting for endogenous CaM KI isoforms. The lower panel is a Western blot of total AKT proteins (AKT), demonstrating similar loading of AKT. E: shRNA to CaM KIγ blocks its expression in LNCaP cells. Cells were transfected as in panel A with either pcDNA3 or shRNA against CaM KIγ. Endogenous CaM KIγ expression was analyzed and presented as fold over AKT (N = 8, ±SE). F: AKT activation is CaM KIγ-independent in LNCaP cells. LNCaP cells were transfected as in panel E, stimulated with carbachol (10 μM) for 5 min, and HA-AKT phosphorylation was analyzed (N = 6, ±SE). G: siCaM KIα blocks CaM KIα expression. LNCaP cells were transfected with scrambled siRNA or siRNA directed against CaM KIα, CaM KIα expression was analyzed by Western blot, and normalized to total AKT protein (N = 8, ±SE). H: CaM KIα does not effect carbachol activation of AKT. LNCaP cells were transfected as in panel G, stimulated with carbachol (10 μM) for 5 min, and HA-AKT phosphorylation was analyzed (N = 6, ±SE).

carbachol. Carbachol treatment of LNCaP cells promoted AKT phosphorylation in the presence or absence of shCaM KIγ (Fig. 5F). Transfection of LNCaP cells with siRNA against CaM KIα also blocked CaM KIα expression (Fig. 5G); however in cells co-transfected with HA-AKT/siCaM KIα, AKT phosphorylation by carbachol was not inhibited (Fig. 5H). Taken together, these results suggest that carbachol's phosphorylation of AKT on threonine 308 utilizes CaM KKα but not CaM KIγ or CaM KIα.

CARBACHOL REGULATES THE APOPTOTIC PROTEIN BAD

The cellular protein, BAD, has several regulatory phosphorylation sites that promote its association with 14-3-3, sequestration from
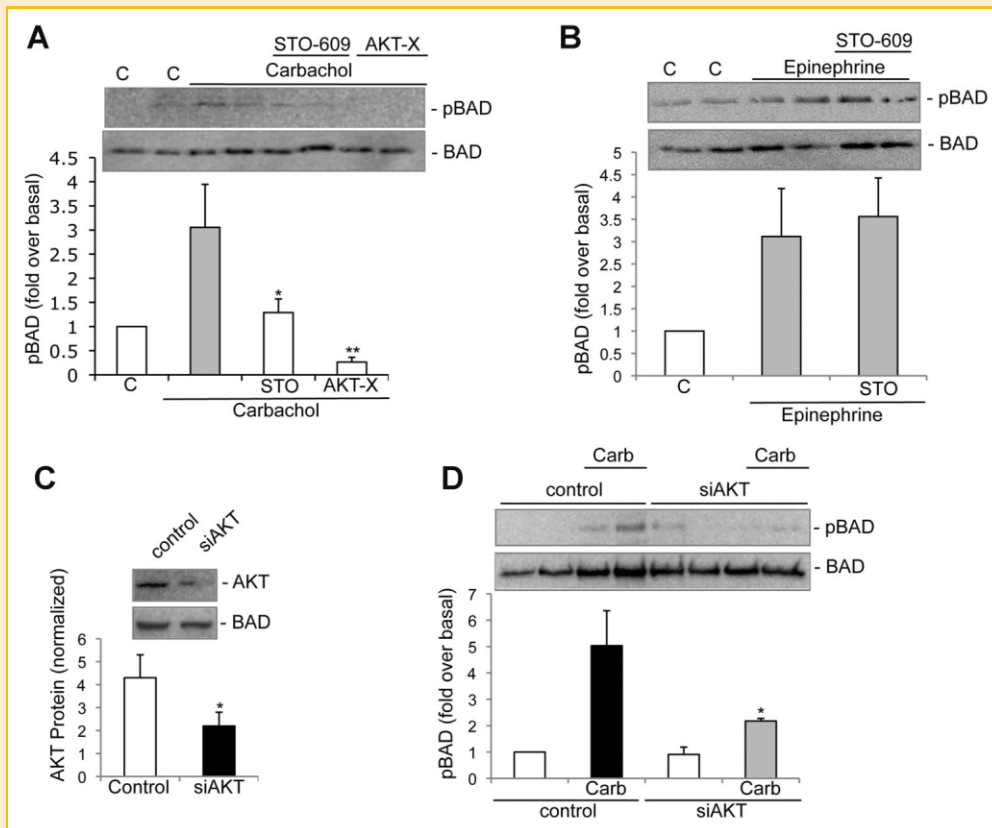
Fig. 6. Carbachol stimulates BAD phosphorylation through CaM KK and AKT. A: STO-609 and AKT-X block carbachol activation of BAD. LNCaP cells received no pretreatment or were pretreated with either STO-609 (5 μM) or AKT-X (5 μM), and then stimulated with carbachol (10 μM) for 5 min. Endogenous BAD phosphorylation was measured by Western blotting for phosphorylated-BAD (pBAD). Phospho-BAD bands were densitized, normalized to BAD, and quantitated. Data are presented as fold BAD phosphorylation over control (N = 6, ±SE). B: Epinephrine activation of BAD in LNCaP cells is insensitive to STO-609. LNCaP cells were pretreated similarly to panel A and then stimulated with epinephrine (100 nM) for 5 min. Endogenous BAD phosphorylation was measured and analyzed as in panel A (N = 6, ±SE). C: siAKT blocks AKT expression. LNCaP cells were transfected with scrambled siRNA or siRNA directed against AKT, AKT expression was analyzed by Western blot, and normalized to total BAD protein (N = 8, ±SE). D: siAKT blocks carbachol-stimulated phosphorylation of BAD. LNCaP cells were transfected as in panel C and then stimulated with carbachol (10 μM) for 5 min. Endogenous BAD phosphorylation was measured and analyzed (N = 8, ±SE).

the mitochondria, and pro-survival role including on serine 136 [Datta et al., 1997; Pastorino et al., 1999; Tan et al., 2000; Zhou et al., 2000; Li et al., 2004]. Interestingly, carbachol stimulation of LNCaP cells enhanced BAD phosphorylation at serine 136 an effect that was blocked by pretreatment with STO-609 or the AKT inhibitor, AKT-X (Fig. 6A). Previous studies have shown that epinephrine utilizes PKA to promote BAD phosphorylation and LNCaP survival [Sastry et al., 2007]. To examine the specificity of STO-609 in our system we treated cells with epinephrine in the presence or absence of the CaM KK inhibitor. Consistent with previous work, epinephrine triggered BAD phosphorylation, however CaM KK inhibition had no effect on this pathway (Fig. 6B). To evaluate the requirement for AKT operating downstream of carbachol in our pathway, we transfected cells with either control scrambled siRNA or siAKT to inhibit AKT expression. Expression of siAKT significantly blocked AKT expression (Fig. 6C) and carbachol-stimulated phosphorylation of BAD (Fig. 6D). Taken together, these data suggest that carbachol stimulates BAD phosphorylation in a CaM KK- and AKT-dependent manner in LNCaP cells.

DISCUSSION

The cell signaling second messenger, calcium, plays a pivotal role in a variety of cellular processes including growth, survival, metabolism, contraction, and development [Borodinsky and Spitzer, 2006; Endo, 2006]. In the present study, we have demonstrated that calcium and CaM KK appear to promote LNCaP cell survival through direct phosphorylation and activation of AKT and its downstream target BAD (see model Fig. 7). The muscarinic receptor agonist, carbachol, working through the M₃ GPCR utilizes calcium and CaM KK to enhance prostate cancer cell resistance to apoptosis. LNCaP cells express endogenous CaM Kinases including the CaM KKα (alpha) isoform. Using siRNA and pharmacological inhibitors, our data reveal that CaM KK but not CaM KIγ or CaM KIα mediates calcium activation of AKT in our system (Fig. 7). AKT regulation of BAD appears to prevent caspase-3 activation and apoptosis in LNCaP cells. Our data also suggest that epinephrine activates AKT and blocks apoptosis independent of CaM KK signaling (Fig. 7).
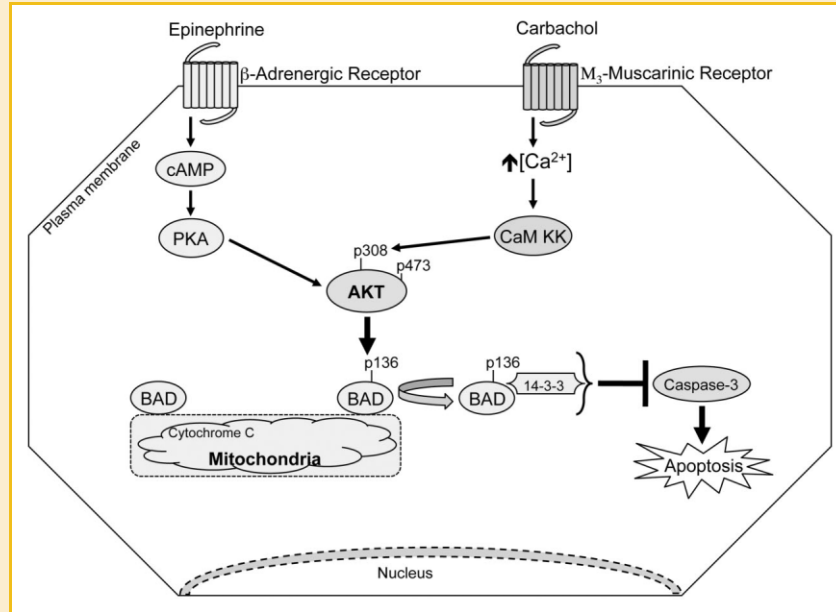
Fig. 7. Proposed model for PKA- and CaM KK-mediated LNCaP survival. Epinephrine treatment of LNCaP cells stimulates β-adrenergic receptors and an increase in intracellular cAMP. cAMP activates PKA leading to the phosphorylation and activation AKT. Carbachol activates the M_3-subtype of muscarinic receptors leading to increases in intracellular calcium levels and the subsequent activation of CaM KK. CaM KK directly phosphorylates AKT on threonine 308 leading to full AKT activation and dual phosphorylation. AKT phosphorylates the survival protein, BAD, leading to its association with 14-3-3 and subsequent blockade of caspase-3 activity and cellular apoptosis.

Several hormones and agonists have been shown to regulate LNCaP cell signaling and their behaviors. For example, recent work has demonstrated that vasoactive intestinal peptide (VIP) promotes prostate cancer cell growth through activation of PKA, Rap1, and ERK [Xie et al., 2007]. Similarly, PMA, interleukin-6 and Forskolin enhance ERK activation and increase LNCaP cell proliferation [Carson et al., 1999; Chen et al., 1999; Gioeli et al., 1999]. Indeed ERK activation correlates with prostate cancer disease progression [Gioeli et al., 1999] and previous work has suggested that calcium elevating agents also increase ERK phosphorylation in LNCaP cells [Chen et al., 1999], although the role for CaM Kinases in those studies were not examined. Recent work from the Weber laboratory has also elucidated how the hormone epinephrine working through PKA and AKT promotes LNCaP cell survival [Sastry et al., 2007]. Our results are consistent with these findings on epinephrine and expand them also to include the calcium signaling agonists, carbachol and ionomycin and may provide significant insight into our understanding of LNCaP cell survival. Based on our data, it appears as though calcium requires CaM KK to directly activate AKT although the ability of CaM KK to crosstalk with cAMP and PKA was not specifically examined in our studies.

Human prostate cells express a myriad of receptor types including those for estrogen, androgens, acetylcholine, α-/β-adrenergics, and glucocorticoids [Nagmani et al., 2003; Sydorenko et al., 2003; Van Coppenolle et al., 2004; Culig et al., 2005; Kung and Evans, 2009; Fowler et al., 2010]. LNCaP cells have also been shown to possess a number of functional endogenous receptors that may couple to calcium signaling and consequently regulate their growth, survival, and differentiation. Previous work has shown that androgens promote LNCaP survival independent of PI3-K yet they may simultaneously increase intracellular calcium concentration [Carson et al., 1999]. Interestingly, CaM KK also appears to mediate androgen-stimulated migration of prostate cancer cells [Frigo et al., 2011]; however the role of AKT was not evaluated in those studies. LNCaP cells also express multiple muscarinic GPCRs including the M_3-subtype that increase intracellular calcium levels and cell proliferation in response to carbachol [Rayford et al., 1997]. To the best of our knowledge, the ability of the M_3 GPCR and calcium to activate CaM Kinases in LNCaP cells has not been examined. Our data now demonstrate a unique signaling pathway for the M_3 receptor to activate CaM KK leading to its phosphorylation of AKT and the subsequent enhancement of LNCaP survival. This novel signaling mechanism through M_3 receptors and CaM KK in LNCaPs suggests that CaM KK inhibition may be a relevant therapeutic target. In contrast, prostate cancer cell survival that is triggered by adrenergic receptors may be blocked by PKA or AKT inhibitors but not CaM KK compounds.

The CaM Kinase family of enzymes have an established role in brain development and metabolism and recent work from numerous groups has also begun to elucidate their involvement in cancer. CaM KII appears to play a role in blood cell cancer differentiation and proliferation [Si et al., 2007; Si and Collins, 2008]. CaM KII may participate in prostate cancer cell signaling however LNCaP cells do not express the *alpha* isoform of CaM KII [Mamaeva et al., 2009]. Interestingly, the broad compound inhibitor of all CaM Kinases, KN-93, blocked prostate cancer cell proliferation and induced cell death [Rokhlin et al., 2010], therefore the role for additional CaM Kinases in prostate cancer is possible. CaM KK and its various substrates also have an emerging role in breast, prostate, and neuronal cancer cells. CaM KK directly activates and phosphorylates AKT on threonine 308

to promote neuroblastoma cell survival, an observation that is consistent with our data [Yano et al., 1998]. CaM KK working through CaM KI also appears to mediate estrogen-stimulated medulloblastoma cell migration [Davare et al., 2010]. In addition, CaM KK and CaM KI promote breast cancer gene expression and cell proliferation through ERK activation [Rodriguez-Mora et al., 2005; Schmitt et al., 2010]. We knocked down CaM KIγ and CaM KIα in our system without inhibiting AKT phosphorylation that supports the role for CaM KK control of AKT and BAD in LNCaP cells. CaM KK also phosphorylates AMPK [Hurley et al., 2005] that has recently been implicated in prostate cancer cell survival and appears to be activated in human prostate cancers. Frigo et al. [2011] have also shown that androgen treatment of LNCaP cells promotes a potent upregulation of CaM KK expression as well as phosphorylation of CaM KI and AMPK. CaM KK working through AMPK appears to mediate prostate cancer cell migration and invasion but not proliferation. Interestingly, the M_3 receptor was recently reported to promote CaM KK activation of AMPK and glucose uptake in muscle cells [Merlin et al., 2010], although the role for AKT was not evaluated. Based on the current data, it is intriguing to hypothesize that calcium and CaM KK may be regulating two important parallel pathways in prostate cancer cells; one pathway that operates through AMPK to promote cell migration and a second pathway to enhance cell survival. In future studies, it will be important to determine the kinetics and localization of these unique signaling cassettes as well as to evaluate the types of hormones and agonists that trigger these calcium-mediated pathways.

The data presented in Figure 6 suggest that calcium and CaM KK are capable of promoting LNCaP survival through the phosphorylation of BAD on serine 136. Consistent with other reports, both AKT and BAD appear to contain some basal phosphorylation [Carson et al., 1999; Sastry et al., 2007], that is, subsequently upregulated by hormone treatment and BAD phosphorylation on serine 136 is strongly correlated with cell survival [Datta et al., 1997]. BAD is currently hypothesized to associate with the outer mitochondrial membrane and modulate cytochrome c release and the subsequent activation of the death inducing caspase enzymes (Fig. 7). Previous studies have utilized the toxin anisomycin at a dose and duration similar to that in our studies to trigger apoptosis in both prostate and lymphoma cells [Curtin and Cotter, 2002; Sah et al., 2003; Hori et al., 2008]. In our system both epinephrine and carbachol prevented anisomysin-induced death at various timepoints and may utilize an array of rapid antiapoptotic transcriptional mechanisms. For example, testosterone has been shown to upregulate numerous miRNAs that target calcium homeostasis and proliferation in prostate cancer cells [Wang et al., 2011]. Calcium and CaM Kinases may also target various prosurvival transcription factors and genes including CREB, Elk-1, Wnts, C/EBP, Egr-1, and Hes-1 among others [Mishra et al., 2005; Wayman et al., 2006; Mamaeva et al., 2009; Schmitt et al., 2010]. In addition to BAD, AKT also regulates numerous downstream survival targets most notably the Forkhead box transcription factors and p53 [Cheng et al., 2005, 2008]. Interestingly, LNCaP cells in which intracellular calcium levels have been increased may regulate CREB, ETS, transient receptor potential channels (TRPCs), as well as several genes involved in cell metabolism [Ben Aicha et al., 2007]. Evaluating the role of various transcription factors and their targets downstream of CaM KK and AKT in our system is an area of current interest.

The TRPCs are highly expressed in LNCaP cells and appear to play an important role in their growth and migration. TRPCs are calcium-permeable channels that are functionally responsive to phenylephrine and carbachol in LNCaP cells [Sydorenko et al., 2003]. The TRPC family of molecules include several members and TRPC1, TRPC3, TRPV2, and TRPV6 have all been implicated in playing a role in prostate cancer cell physiology and disease progression [Pigozzi et al., 2006; Monet et al., 2010]. The role for TRPCs upstream of CaM KK and CaM KI activity in hippocampal neurons has recently been shown [Davare et al., 2009]; however we are unaware of a published role for TRPC regulation of CaM Kinases in LNCaP cells. Similarly, the calcium channel Orai1 and its regulator STIM may also participate in intracellular calcium signaling events and appear to block prostate cancer cell death [Flourakis et al., 2010]. The possible role for TRPCs or Orai1 in promoting survival through CaM KK is consistent with our data suggesting that extracellular calcium is required for AKT activation downstream of carbachol stimulation of LNCaP cells. Evaluating a functional link between these calcium channels and CaM Kinases in cancer cells is an intriguing area of research.

In summary, we have proposed a unique signaling pathway that exists in LNCaP cells downstream of the GPCR agonist carbachol. Epinephrine activated AKT and promoted LNCaP cell survival that appears to be independent of CaM KK. Muscarinic receptor-stimulation of LNCaP cells elevated intracellular calcium and activated CaM KK leading to AKT and BAD phosphorylation as well as survival of prostate cancer cells (Fig. 7). The results from our study further support the importance of CaM Kinases in both health and disease. In particular, CaM KK and its various downstream targets including AKT, AMPK, and CaM KI appear to be significant enzymes involved in cancer. The ubiquitous expression of these molecules and the emerging role of CaM KK in cancer biology may provide important therapeutic targets in the treatment of prostate cancer.

ACKNOWLEDGMENTS

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