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ERK Activation and Cell Growth Require CaM Kinases in MCF-7 Breast Cancer Cells

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Abstract Previous studies on MCF-7 breast cancer cells have shown that the G-protein coupled receptor (GPCR) agonist carbachol increases intracellular calcium levels and the activation of extracellular signal-regulated kinase (ERK). Calcium and calmodulin regulate the calcium/calmodulin-dependent kinase (CaM kinase) family of proteins that have been proposed to regulate ERK and gene transcription. Our results suggest that both estrogen (E2) and carbachol treatment of MCF-7 breast cancer cells trigger phosphorylation of ERK1/2 and the transcription factor Elk-1. Carbachol and estrogen triggered nearly a four- to sixfold increase in MCF-7 cell proliferation by 96 h, respectively. Carbachol-stimulated ERK activation and cell growth was completely blocked by the Muscarinic M_3-subtype GPCR inhibitor, 4-DAMP, and siRNA against the M_3-subtype GPCR. Interestingly, blockade of CaM KK with the selective inhibitor STO-609 prevented carbachol activation CaM KI, ERK, Elk-1, and cell growth. Consistent with these observations, knockdown of CaM KKα and CaM KIγ with shRNA-containing plasmids blocked ERK activation by carbachol. In addition, Elk-1 phosphorylation and luciferase activity in response to carbachol treatment was also dependent upon CaM kinases and was inhibited by U0126, STO-609, and siRNA knockdown of CaM kinases and ERK2. Finally, blockade of either CaM KK (with STO-609) or ERK (with U0126) activities resulted in the inhibition of carbachol- and estrogen-mediated cyclin D1 expression and MCF-7 cell growth. Taken together, our results suggest that carbachol treatment of MCF-7 cells activates CaM KI, ERK, the transcription factor Elk-1, cyclin D1, and cell growth through CaM KK.

Keywords ERK · CaM kinase · Carbachol · Elk-1 · Estrogen · Cell growth · Cyclin D1

Abbreviations

MAPK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinase
CaM	Calmodulin
AKT	Protein kinase B
EGF	Epidermal growth factor
β-Estradiol	Estrogen (E2)
CaM kinase	Calcium/calmodulin-dependent protein kinase

Introduction

The Extracellular Signal-Regulated Kinase (ERK) is ubiquitously expressed throughout the body, including neurons, osteoblasts, myocytes, epithelial, and endothelial cells [1–3]. ERK activation and signaling have been shown to be involved in numerous processes like neuronal memory and development, cardiac hypertrophy, apoptosis, and cancer [1]. The ERK signaling pathway is under investigation in cancer cells because of its regulation of several transcription and translation factors, and its ability to promote cell growth and proliferation.

Previous studies have shown that the ERK pathway is activated in the breast cancer cell line, MCF-7, by various

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stimuli including epidermal growth factor (EGF), prolactin, angiotensin, estrogen, and carbachol [4–9]. Carbachol has been suggested to initiate activation of the ERK signaling pathway through the M_3-subtype of muscarinic G-protein coupled receptors (GPCRs) in MCF-7 cells [4]. Agonist binding to muscarinic GPCRs triggers the activation of heterotrimeric G proteins of the G_q-subtype and the release of intracellular calcium into the cytosol of the cell. Interestingly, hormones such as ghrelin, estrogen, and epinephrine may also cause intracellular calcium levels to rise inside of certain cells including MCF-7 cells [7, 10–13]. Calcium ions have a variety of intracellular targets including Pyk2, PKC, CalDAG-GEFs, glycogen phosphorylase kinase, troponin, and calmodulin [14, 15]. Calcium binding to calmodulin creates a molecular complex that in turn binds and activates the calcium/calmodulin-dependant protein kinase family of enzymes (CaM kinases) [16]. The CaM kinase family of proteins includes the serine/threonine enzymes CaM KK, CaM KII, CaM KIV, and CaM-KI as well as their various isoforms and splice variants. Active CaM KK phosphorylates and activates AMP-dependent kinase (AMPK), CaM KI, and CaM KIV as well as the pro-survival serine/threonine protein kinase, AKT [15]. The physiological roles for CaM KK proteins have been an area of active investigation, and CaM KK has recently been linked to dendritic cell survival, autophagy, energy homeostasis, and neuronal development [10, 17–20]. Studies in neurons have demonstrated that CaM KK and CaM KI activate ERK through Ras, Raf-1, and MEK [19, 21]. ERK activation in neurons has been shown to regulate both protein synthesis as well as gene expression [22, 23]. Interestingly, MCF-7 cells do not express endogenous CaM KIV; however, CaM KK working through CaM KI has been implicated in signaling pathways that promote MCF-7 cell growth and proliferation [23, 24]. It is currently unknown whether the CaM KK and CaM KI utilize ERK or its substrates to control MCF-7 cell growth and proliferation.

Extracellular signal-regulated kinase promotes a number of cellular functions such as increased gene transcription and protein translation, increased cell cycle progression, cell adhesion, blockade, or promotion of apoptosis and cell growth [1, 25]. ERK has several cytoplasmic and nuclear targets that it utilizes to control cellular function. For example, ERK is able to trigger phosphorylation of the transcription factor CREB leading to the increased expression of Wnt-2 genes in neurons [19]. ERK can also phosphorylate p90Rsk, MSK1/2, MNK1/2, c-Fos, and the transcription factor Elk-1, among others [26–34]. Elk-1 is an Ets family transcription factor that dimerizes with serum response factor (SRF), binds DNA, and increases transcription. Elk-1 typically binds DNA upstream of the transcription start site in consensus regions called serum response elements (SREs) [35–39]. ERK can directly phosphorylate Elk-1 on serine 383 to enhance SRE binding, gene transcription, and cell proliferation [5, 37]. Interestingly, estrogen has been shown to increase Elk-1 phosphorylation and SRE activity via ERK in MCF-7 cells; however, the mechanism of ERK activation downstream of estrogen remains unknown [29]. Moreover, the ability of CaM kinases to specifically mediate calcium's effects on ERK and Elk-1 downstream of estrogen or GPCR agonists such as carbachol is an area of interest.

Several genes have been demonstrated to be activated by hormones and agonists in breast cancer cells. For example, estrogen has been shown to increase expression of the early growth response-1 (Egr-1) and *c-fos* genes through ERK and Elk-1 in MCF-7 cells [5, 6, 40]. In addition, recent data suggests that prolactin treatment of MCF-7 cells increased synthesis of the transcription factor, activating protein 1 (AP-1) [9]. The cell cycle regulatory protein, cyclin D1, has also been proposed to be over-expressed in nearly 50% of all breast cancer cases [41–43]. Interestingly, a recent study by Rodriquez-Mora et al. suggested that CaM kinases may play a role in regulating cyclin D1 expression in MCF-7 cells [24]. The precise role of ERK and Elk-1 downstream of CaM kinases in cyclin D1 expression was not examined. Cyclin D1 is also regulated by several hormones including estrogen; however, the CaM kinase- and ERK-dependent promotion of cyclin D1 expression have not been investigated in MCF-7 cells. Furthermore, we are currently unaware of a mechanism by which carbachol that activates ERK and enhances MCF-7 cell growth, triggers cyclin D1 expression in MCF-7 cells. Our aim was to carefully evaluate the roles of CaM kinases in calcium's activation of ERK and Elk-1 and the expression of cyclin D1 in MCF-7 cells. We have utilized molecular biochemical as well as pharmacological approaches to assess the ability of these molecules to act as mediators of MCF-7 cell growth and proliferation.

Experimental procedures

Materials

4-DAMP and STO-609 were purchased from Tocris (Ellisville, MO). EGTA, Carbachol, DMSO, β-estradiol (Estrogen, E2), EGF, Flag (M2) antibody, Flag (M2)-conjugated agarose, and MTT reagent (Thiazolyl blue tetrazolium bromide) were purchased from Sigma. KN-93, ionomycin, and U0126 were purchased from EMD Biosciences (Calbiochem, Riverside, CA). Antibodies specific to phosphorylated and activated ERK (pERK1/2) that bind phosphorylated ERK1 and ERK2 at residues threonine 202 and tyrosine 204 were purchased from Cell Signaling

(Beverly, MA). Phosphorylation-specific antibodies to activated Elk-1 (phospho-serine 383) and total Elk-1 were also purchased from Cell Signaling (Beverly, MA). Antibodies to cyclin D1 and ATP-citrate lyase were purchased from Cell Signaling (Beverly, MA). Antibodies to total ERK proteins (ERK1/2), the M_3-subtype muscarinic receptor (H-210), and total CaM KI were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Monoclonal antibodies to phosphorylated and activated CaM KI (pCaM KI, threonine 177) were generated from immunized mice and a gift of the Soderling laboratory at OHSU (Portland, OR). HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNA to the M_3 receptor and control siRNA-a were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Enhanced luminal reagent was purchased from Pierce Biotechnology (Rockford, IL). The scrambled control and p42 ERK siRNAs were purchased from Cell Signaling (Beverly, MA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). The luciferase assay kit was purchased from Stratagene (La Jolla, CA).

Cell culture and treatments

The MCF-7 breast adenocarcinoma cell line was purchased from ATCC and cultured in Eagle's Minimal Essential medium (EMEM) plus 10% fetal bovine serum, penicillin/streptomycin, Earle's BSS and L-glutamine at 37°C in 5% CO_2. Prior to cell stimulations, MCF-7 cells were serum starved overnight in EMEM at 37°C in 5% CO_2 for western blotting. MCF-7 cells were pretreated with the inhibitors KN-93 (5 μM), U0126 (10 μM), 4-DAMP (2 μM), STO-609 (5 μM), or 2 mM EGTA for 30 min prior to treatment. Where indicated, cells were stimulated with ionomycin (1 μM), EGF (300 ng/ml), E2 (10 nM), H_2O_2 (10 nM), and carbachol (10 μM) for the indicated times. Treated and stimulated MCF-7 cells were analyzed by either SDS-PAGE, western blotting, luciferase activity, or the MTT assay as indicated.

Western blotting

Immediately after MCF-7 cell stimulation the EMEM was aspirated and equivalent amounts of ice-cold lysis buffer (10% glycerol, 1% NP40, 50 mM Tris–HCl pH 7.4, 200 mM NaCl, 2 mM $MgCl_2$) plus freshly added inhibitors (1 mM PMSF, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM sodium orthovanadate) were added to each plate of cells. On ice, plates were scraped and cellular proteins placed in ice-cold microcentrifuge tubes. Whole cell lysates were briefly spun at 8 K for 5 min at 4°C to pellet the cytoskeleton and nucleotides. Supernatants from each experimental treatment were then quantified by the Bradford protein assay on a 96-well plate along with bovine serum albumin standards. Protein standards and sample concentrations were read by a Bio-Rad 680 micro-plate reader. Equivalent amounts of MCF-7 cell extracts were run on PAGE-gels, transferred to PVDF membranes, and examined by western blotting. Membranes were probed using the indicated antibodies, incubated with luminol, scanned and densitized using a molecular imaging system (Kodak Image Station 2000R), and evaluated for significant differences among treatments. Band densities were normalized to untreated controls and then to the loading control and presented as fold phosphorylation as indicated.

siRNA/shRNA transfections

Fifty to sixty percent confluent cells were transfected with either the scrambled control siRNAs or siRNAs directed to either the M_3 receptor (Santa Cruz Biotechnology) or p42 ERK (Cell Signaling Technology), according to the manufacturer's guidelines (Cell Signaling Technology). Each plate received the same amount of siRNA (20 mM), following transfection cells were allowed to recover in complete media for 24 h. Cells were also co-transfected with Flag-ERK2 along with one of the following; siM_3, shCaM KKα, shCaM KIγ, shCaM KIV, constitutively active (ca) CaM KK, caCaM KI, caCaM KK/KI, 5Gal4E1B-luciferase, Gal4-Elk-1, control siRNA-a, or pcDNA3 as indicated using Lipofectamine 2000 for 6 h. These plasmids were a gift of the Soderling laboratory at OHSU (Portland, OR) and have been described previously [19–21, 44]. Each plate received the same total amount of plasmid (5 μg), following transfection cells were allowed to recover in complete media for 24 h. Cells were then serum starved overnight, treated as indicated and lysed in ice-cold lysis buffer. Cellular lysates were examined by western blot, immunoprecipitated, and then examined by western blot or luciferase activity as indicated.

Immunoprecipitations and western blotting

MCF-7 cells were transfected, stimulated, lysed, and equivalent protein amounts were used for either immunoprecipitation or for SDS-PAGE and blotted onto PVDF membranes and examined by western blotting as previously described [21]. For transfected lysates, ~500 μg of protein was precipitated overnight at 4°C using the indicated antibody. Precipitates were washed 2X in ice-cold lysis buffer, run on SDS-PAGE gels, and analyzed by western blotting. Membranes were probed using the indicated antibodies, scanned, densitized using a molecular imaging system (Kodak Image Station 2000R), and evaluated for significant differences among treatments. Band

densities were normalized to untreated controls and then to the loading control and presented as fold phosphorylation or expression as indicated.

Luciferase assay

MCF-7 cells were transfected with 5Gal4E1B-luciferase, Gal4-Elk-1, and pretreated with inhibitors or co-transfected with pcDNA3 or shRNAs as indicated. Stimulated cells were lysed using luciferase assay lysis buffer and analyzed according to the manufacturer's protocol (Stratagene, La Jolla, CA). Immediately following cell lysis, equivalent protein amounts were assayed for luciferase activity by incubation with luciferase substrate every 8 s for 1 min. The average light units per treatment were calculated and normalized to untreated controls and presented as fold luciferase activity as indicated.

Cell growth assay

In order to measure cell growth and proliferation, MCF-7 cells were seeded at low-density ($\sim$500 cells/well) on 96-well plates with complete EMEM media for 24 h to allow them to adhere. MCF-7 cells were then serum starved for 24 h and then pretreated with appropriate inhibitors for 30 min. Carbachol (10 μM), E2, EGF, or controls compounds were then added to each well as indicated for up to 96 h. Next, 20 μl of MTT reagent (Thiazolyl blue tetrazolium bromide) was added and incubated for 3 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 as previously described [45]. Absorbance values were normalized to untreated controls and presented as fold cell growth as indicated.

Statistics

In order to evaluate whether significant differences existed between experimental treatments, we performed a Student *t* test on the data with significance levels set at 0.05. Significant values (*P* values) are indicated in the figures as either a single asterisk where $P \leq 0.05$ or a double asterisk where $P \leq 0.01$.

Results

Carbachol, estrogen, and EGF have previously been shown to increase ERK1/2 phosphorylation and activation in MCF-7 cells [4, 7]. We investigated the ability of these agonists to trigger ERK1/2 phosphorylation and activation within 5 min of stimulation in our MCF-7 model system. 10 μM carbachol treatment of MCF-7 cells triggered ERK 1/2 phosphorylation (pERK1/2) within 5 min as measured by western blotting for the activated form of ERK1/2 (Fig. 1a). Similarly, treatment of cells with either β-estradiol (E2) or EGF promoted ERK1/2 phosphorylation within 5 min, whereas neither DMSO nor H_2O_2 activated ERK1/2. Carbachol and E2 have been proposed to increase intracellular calcium concentrations as a means to activate ERK1/2 (ERK). Ionomycin is an ionophore capable of stimulating increases in intracellular calcium and is a useful mimic to study intracellular calcium signaling pathways. Interestingly, treatment of MCF-7 cells with ionomycin, potently stimulated ERK phosphorylation within 5 min (Fig. 1b). In order to evaluate the physiological effects of these agonists on MCF-7 growth and proliferation cells were stimulated as indicated and analyzed after 96 h. E2 and EGF triggered a six- and sevenfold increase in cell growth by 96 h, respectively. Moreover, carbachol stimulated a fourfold increase in cell growth, whereas DMSO and a sub-lethal dose of H_2O_2 had no effect on cell growth at this time point (Fig. 1c). Interestingly, pre-treatment with EGTA, a calcium chelator, completely blocked carbachol activation of ERK (Fig. 1d). Taken together, the above data suggest that agonists that elevate intracellular calcium are capable of activating ERK and promoting MCF-7 cell growth.

MCF-7 cells express the M_3-subtype of muscarinic GPCRs that couple to elevation of intracellular calcium following carbachol stimulation [4]. In order to examine the role of M_3 receptors in ERK activation, we pre-treated MCF-7 cells with 4-DAMP, a selective M_3 receptor antagonist that completely blocked carbachol's activation of ERK (Fig. 2a). In order to ensure the specificity of 4-DAMP in our system, MCF-7 cells were pre-treated with either 4-DAMP followed by stimulation with EGF. ERK activation by EGF was not effected by pre-treatment with 4-DAMP (Fig. 2b). In addition to the pharmacological approach of inhibiting the M_3 receptor, we also employed siRNA transfection of MCF-7 cells as a means to further evaluate the requirement of the M_3-subtype GPCR in ERK activation. Transfection of MCF-7 cells with siRNA directed against the M_3 receptor knocked down its expression by 37% as compared to control transfected cells (Fig. 2c). The knockdown of the M_3 receptor was specific, because total ERK levels were unchanged in either transfection groups (Fig. 2c). Consistent with the previous results, co-transfection of cells with siRNA directed at the M_3 GPCR along with Flag-ERK2 significantly inhibited carbachol activation of ERK2 as compared to control cells transfected with scrambled siRNA (Fig. 2d). Consistent with its effects on ERK activation, inhibition of the M_3 receptor using 4-DAMP also blocked carbachol's stimulation of cell growth (Fig. 2e).

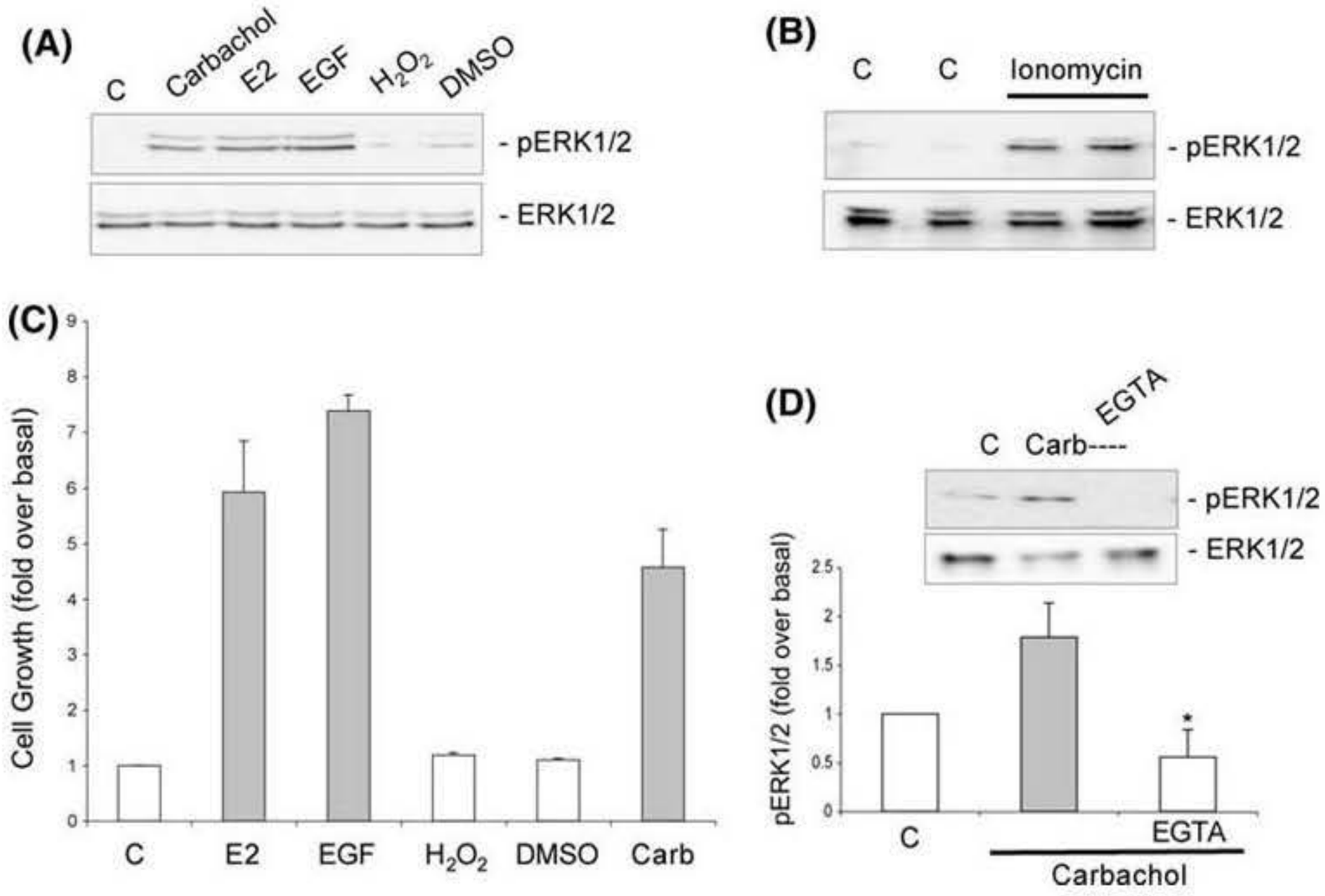

Fig. 1 Agonist activation of ERK and MCF-7 cell growth. **a** ERK activation by carbachol, estrogen, and EGF. Serum-starved MCF-7 cells were stimulated with carbachol (10 μM), β-estradiol (E2, 10nM), EGF (300 ng/ml), H_2O_2 (10nM), or DMSO for 5 min as described in "Experimental procedures" section. Untreated control cells are indicated by the letter "C". Cell lysates were prepared as indicated in "Experimental procedures" section. Endogenous ERK activation was measured by western blotting for phosphorylated ERK1/ERK2, p44 and p42, respectively (pERK1/2). The lower panel is a western blot of ERK1/2, demonstrating equal loading of ERK used for pERK1/2 assays. **b** Ionomycin activates ERK in MCF-7 cells. MCF-7 cells received no treatment or were stimulated with ionomycin (1 μM) for 5 min as described in "Experimental procedures" section. Endogenous ERK activation was measured by western blotting as in panel A. **c** Carbachol triggers MCF-7 cell growth. MCF-7 cells were serum starved and stimulated for 96 h with the indicated agonists as described in "Experimental procedures" section. Data are presented as cell growth over control ($N = 5$). **d** ERK activation by carbachol is calcium dependent. MCF-7 cells received no pretreatment or were pretreated with EGTA (2.5 mM) and then stimulated with carbachol (10 μM) for 5 min. Endogenous ERK activation was measured by western blotting as in panel A and the bands were densitized, normalized to ERK1/2, and quantitated. Data are presented as fold ERK1/2 phosphorylation over control ($N = 4 \pm$ SE, * $P \leq 0.05$)

Several cellular enzymes have been shown to be activated by increases in calcium ion concentration and to participate in ERK activation including the calcium/calmodulin-dependent protein kinase (CaM kinase) group of molecules. In order to investigate whether CaM kinases were involved in carbachol's activation of ERK, cells were pretreated with the selective CaM kinase family inhibitor compound, KN-93. Inhibition of the CaM kinase family of proteins with KN-93 blocked ERK activation (Fig. 3a). ERK activation was also dependent upon its immediate upstream activator MEK, as ERK phosphorylation by carbachol was completely blocked by the MEK inhibitor, U0126 (Fig. 3a). In order to further examine the role of CaM kinases downstream of calcium activation of ERK, MCF-7 cells were pre-treated with KN-93 and then stimulated with ionomycin. As can be seen in Fig. 3b, inhibition of CaM kinases blocked ionomycin-stimulated activation of ERK. In order to ensure that KN-93 was selectively inhibiting calcium/CaM kinase-dependent processes, cells were pretreated with KN-93 and then exposed to EGF. KN-93 had no effect on EGF-triggered calcium-independent activation of ERK (Fig. 3c). Estrogen and ionomycin are capable of increasing intracellular calcium concentration and ERK activation. In order to determine if these agonists activate ERK through its common activator MEK, cells were exposed to the MEK inhibitor U0126. Blockade of MEK by U0126 completely abolished ERK activation by ionomycin (Fig. 3d) and estrogen (Fig. 3e). The above data suggests that calcium-elevating agonists utilize the enzyme MEK and the CaM kinase family of proteins to activate ERK.

CaM KK and CaM KI have been suggested to promote MCF-7 cell growth and proliferation [24]. In order to determine if CaM KK mediates ERK activation in our system, MCF-7 cells were pretreated with the specific CaM KK inhibitory compound, STO-609 [23, 46]. Interestingly, both carbachol- and estrogen-stimulated activation of ERK were significantly blocked by inhibiting CaM KK with STO-609 (Fig. 4a, b). In order to ensure that the STO-609-mediated inhibition of ERK was due to specifically suppressing CaM KK, we stimulated MCF-7 cells with EGF in the presence of the inhibitor. CaM KK inhibition did not

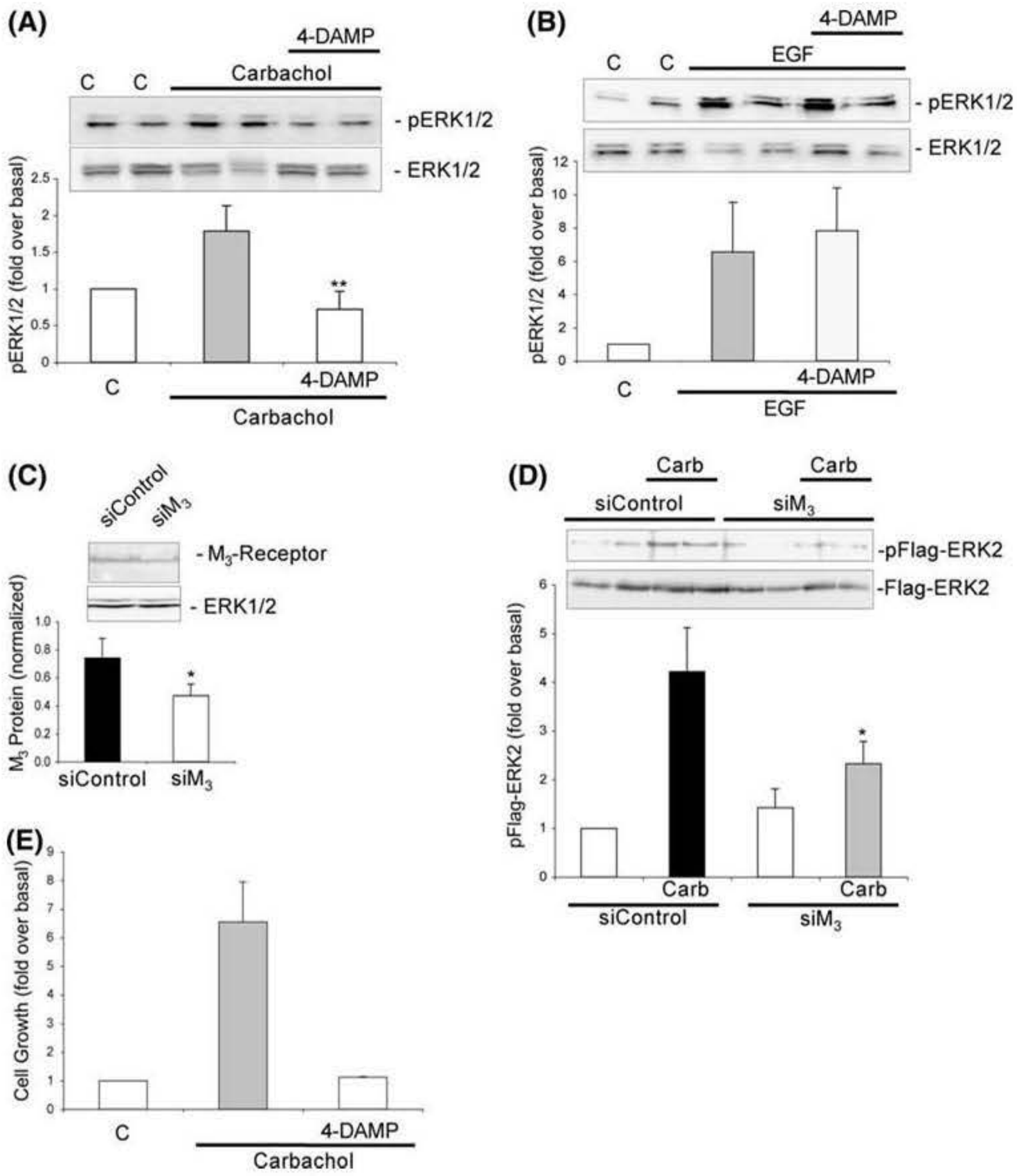

Fig. 2 Carbachol stimulation of ERK and MCF-7 cell growth requires the M_3-subtype of GPCRs. **a** Carbachol activation of ERK is blocked by the M_3-subtype receptor inhibitor, 4-DAMP. MCF-7 cells received no pretreatment or were pretreated with 4-DAMP (2 μM) and then stimulated with carbachol (10 μM) for 5 min. Endogenous ERK activation was measured by western blotting for phosphorylated-ERK1/ERK2 (pERK1/2). Phospho-ERK bands were densitized, normalized to ERK1/2, and quantitated. Data are presented as fold ERK1/2 phosphorylation over control ($N = 6 \pm$ SE, ** $P \leq 0.01$). **b** 4-DAMP does not effect EGF activation of ERK1/2. MCF-7 cells received no pretreatment or were pretreated with 4-DAMP (2 μM) and then stimulated with EGF for 5 min. Endogenous ERK activation was measured by western blotting and analyzed as in panel "A" ($N = 6 \pm$ SE). **c** siRNA to the M_3-subtype of GPCRs reduces its expression. MCF-7 cells were transfected with either scrambled siRNA or siRNA against the M_3 receptor as described in "Experimental procedures" section. Endogenous M_3 expression was measured by western blotting and the bands were densitized, normalized to ERK1/2, and quantitated. Data are presented as fold M_3 expression over ERK1/2 ($N = 6 \pm$ SE). **d** The M_3 receptor mediates carbachol activation of ERK. MCF-7 cells were co-transfected with Flag-ERK2 along with the control siRNA or the siRNA against the M_3 receptor as indicated in "Experimental procedures" section. MCF-7 cells were then stimulated with carbachol (10 μM) for 5 min and Flag-ERK2 phosphorylation was analyzed as indicated in "Experimental procedures" section ($N = 6 \pm$ SE). **e** MCF-7 cell growth utilizes the M_3 receptor. MCF-7 cells received no pretreatment or were pretreated with 4-DAMP (2 μM) and then stimulated with carbachol (10 μM) for 96 h. Data are presented as cell growth over control ($N = 4$)

effect EGF's activation of ERK (Fig. 4c) suggesting that STO-609 had no effect on a classical mechanism of ERK activation or ERK itself. CaM KK has several direct substrates including the phosphorylation of CaM KI at threonine 177. In order to confirm that the effect of STO-609 on ERK was due to block of CaM KK activity, we examined the ability of STO-609 to block CaM KK phosphorylation and activation of CaM KI. Carbachol and ionomycin both activated CaM KK and its phosphorylation of CaM KI in our system and STO-609 strongly blocked activation of

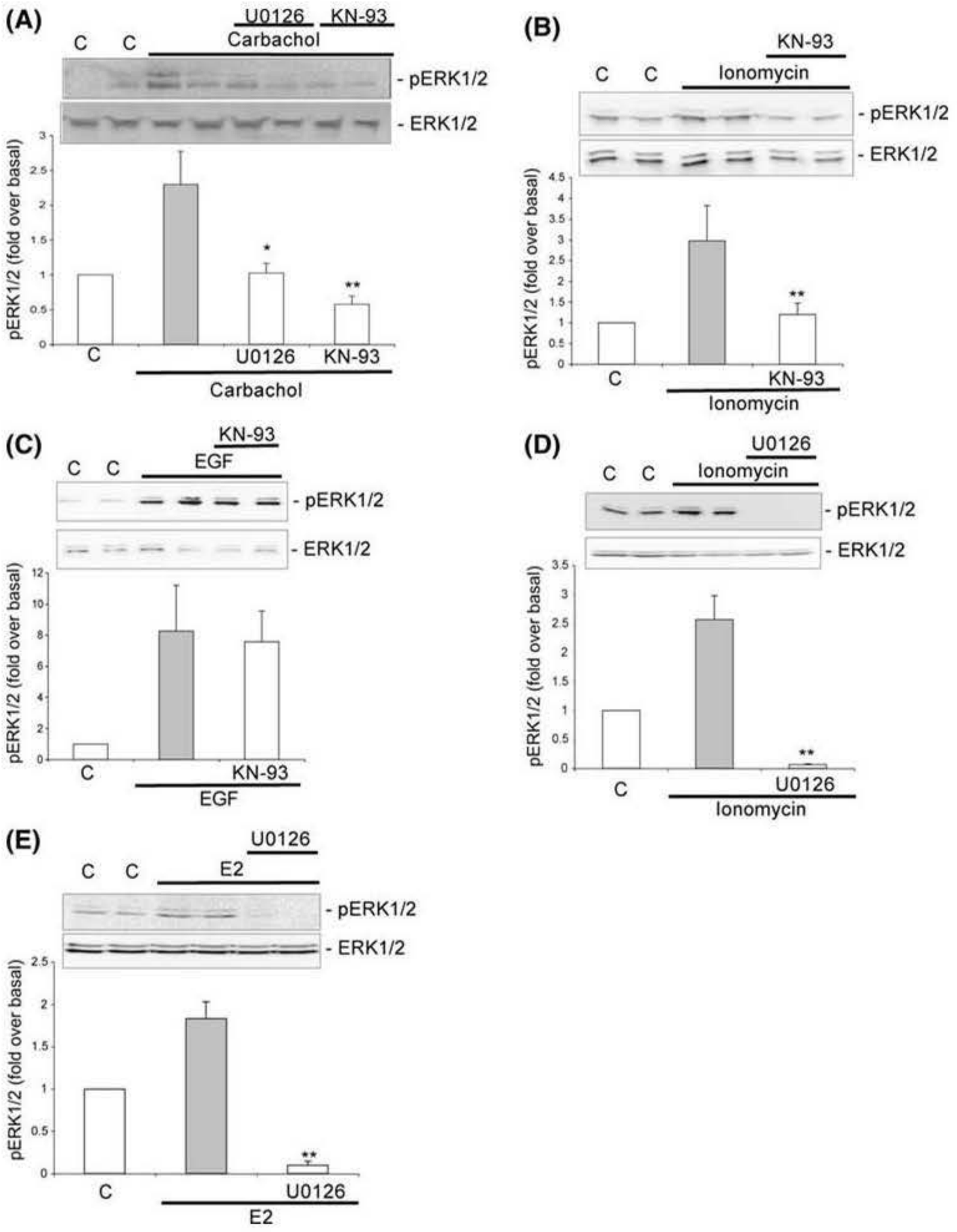

Fig. 3 Agonist activation of ERK depends on CaM kinases and MEK. **a** U0126 and KN-93 block carbachol activation of ERK. MCF-7 cells received no pretreatment or were pretreated with either U0126 (10 μM) or KN-93 (5 μM) and then stimulated with carbachol (10 μM) for 5 min. Endogenous ERK activation was measured by western blotting for phosphorylated ERK1/ERK2 (pERK1/2). Phospho-ERK bands were densitized, normalized to ERK1/2, and quantitated. Data are presented as fold ERK1/2 phosphorylation over control ($N = 4 \pm$ SE). **b** KN-93 blocks ionomycin activation of ERK. MCF-7 cells were pretreated identically to panel A and then stimulated with ionomycin (1 μM) for 5 min. Endogenous ERK activation was measured and analyzed as in panel A ($N = 4 \pm$ SE). **c** KN-93 does not block EGF-stimulated phosphorylation of ERK. MCF-7 cells were pretreated identically to panel A, then stimulated with EGF (300 ng/ml) for 5 min and ERK was analyzed as in panel A. Endogenous ERK activation was measured and analyzed as in panel A ($N = 4 \pm$ SE). **d** U0126 blocks ionomycin activation of ERK. MCF-7 cells were pretreated identically to panel A and then stimulated with ionomycin (1 μM) for 5 min. Endogenous ERK activation was measured and analyzed as in panel A ($N = 4 \pm$ SE). **e** Estrogen activation of ERK requires MEK. MCF-7 cells were pretreated identically to panel A and then stimulated with β-estradiol ("E2", 10 nM) for 5 min. Endogenous ERK activation was measured and analyzed as in panel A ($N = 6 \pm$ SE)

CaM KI by CaM KK (Fig. 4d, e). This data suggest that CaM KK mediates ERK activation downstream of carbachol and estrogen stimulation of MCF-7 cells.

Recent data suggest that the *alpha* isoform of CaM KK and the *gamma* isoform of CaM KI mediate ERK activation in neurons [19]. In addition to the pharmacological

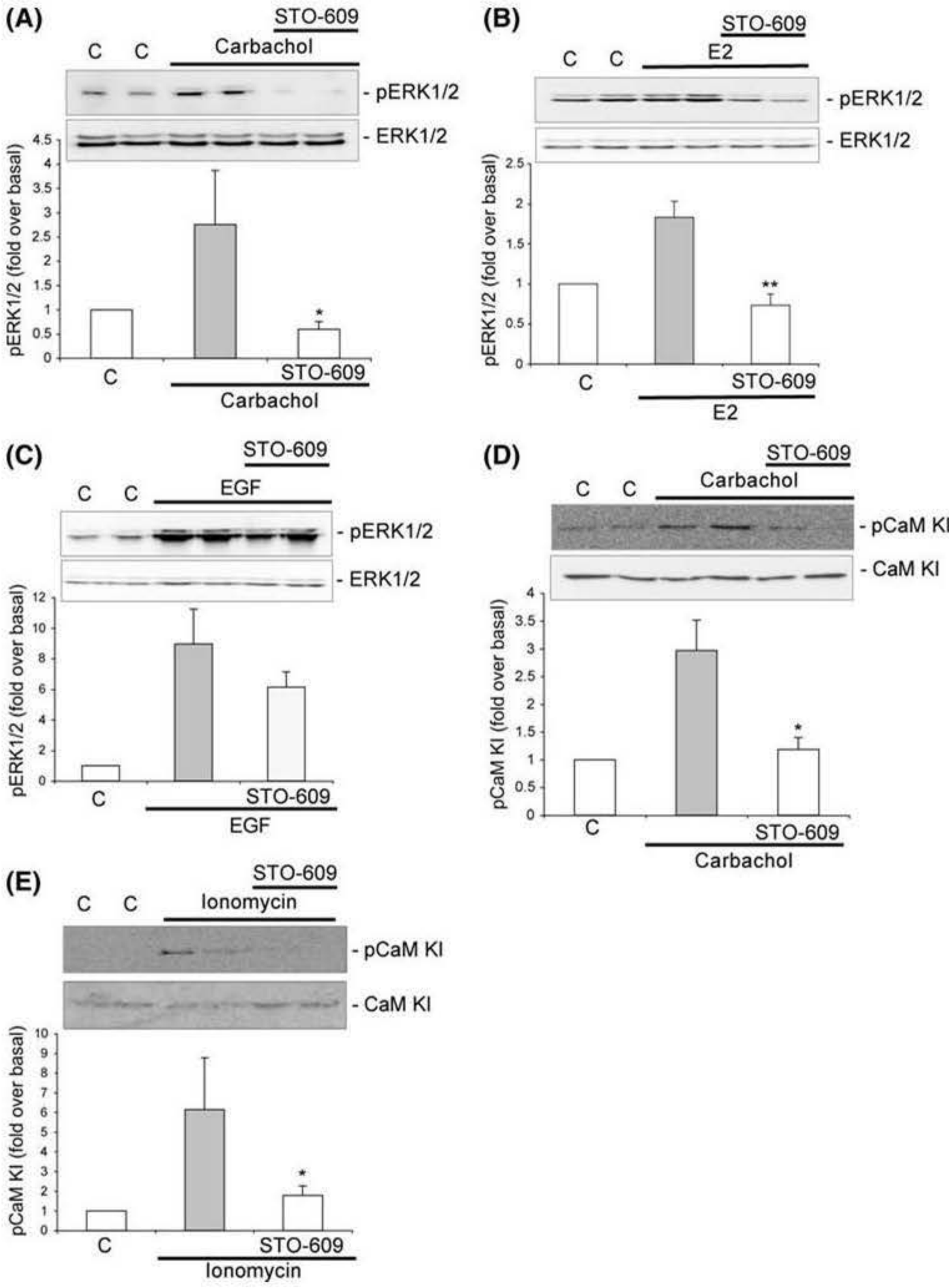

Fig. 4 ERK activation by carbachol, estrogen, and ionomycin is CaM KK dependent. A, STO-609 inhibits carbachol activation of ERK. MCF-7 cells received no pretreatment or were pretreated with STO-609 (5 μM) and then stimulated with carbachol (10 μM) for 5 min. Endogenous ERK activation was measured by western blotting for phosphorylated ERK1/ERK2 (pERK1/2). Phospho-ERK bands were densitized, normalized to ERK1/2, and quantitated. Data are presented as fold ERK1/2 phosphorylation over control ($N = 6 \pm$ SE). **b** Estrogen activation of ERK requires CaM KK. MCF-7 cells were pretreated identically to panel A and then stimulated with β-estradiol ("E2", 10 nM) for 5 min. Endogenous ERK activation was measured and analyzed as in panel A ($N = 6 \pm$ SE). **c** EGF activation of ERK does not require CaM KK. MCF-7 cells were pretreated identically to panel A, then stimulated with EGF (300 ng/ml) for 5 min and ERK was analyzed as in panel A ($N = 4 \pm$ SE). **d** Carbachol activates CaM KI via CaM KK. MCF-7 cells were pretreated identically to panel A and then stimulated with carbachol (10 μM) for 5 min. Endogenous CaM kinase I activation was measured by western blotting for phosphorylated CaM kinase I (pCaM KI). Phospho-CaM KI bands were densitized, normalized to CaM KI, and quantitated. Data are presented as fold CaM KI phosphorylation over control ($N = 8 \pm$ SE). **e** Ionomycin stimulates CaM KI phosphorylation. MCF-7 cells were pretreated identically to panel A and then stimulated with ionomycin (1 μM) for 5 min. Endogenous CaM KI activation was measured and analyzed as in panel D ($N = 4 \pm$ SE)

approach to inhibiting CaM KK and CaM KI in our cells, we also transfected short-hairpin RNA (shRNA) directed against either CaM KKα, CaM KIγ, or CaM KIV and examined ERK activation. Transfection of cells with the control pcDNA3 had no effect on co-transfected Flag-CaM KKα, Flag-CaM KIγ, or Flag-CaM KIV (Fig. 5a). In contrast, transfection of cells with shCaM KKα, shCaM KIγ, or shCaM KIV significantly blocked each of their expression, respectively. The shRNAs nor pcDNA3 did not alter the expression of β-tubulin, Flag-ERK2, or Flag-CaM KI these proteins also functioned as loading controls (Fig. 5a). In order to determine the role of CaM KKα on ERK activation, MCF-7 cells were co-transfected with either pcDNA3 or shCaM KKα along with Flag-ERK2 and then stimulated with carbachol. Carbachol treatment of control cells triggered a twofold increase in ERK activation an effect that was significantly blocked by expression of shCaM KKα (Fig. 5b). In order to evaluate the role of the CaM KIγ in calcium-mediated ERK activation, cells were co-transfected with either pcDNA3 or shCaM KIγ along with Flag-ERK2 and then stimulated with carbachol. As can be seen in Fig. 5c, carbachol increased ERK activation was completely inhibited by the presence of the shCaM KIγ. These data suggest that CaM KK and CaM KI are necessary for ERK activation. In order to confirm the specificity of the CaM kinases involved in ERK activation, we also examined whether shCaM KIV effected ERK activation. Inhibition of CaM KIV did not block ERK phosphorylation downstream of carbachol stimulation (Fig. 5d). In order to investigate the ability of CaM KK and CaM KI to stimulate ERK activation independent of agonist stimulation, cells were co-transfected with either pcDNA3 or constitutively active (ca) CaM KK, caCaM KI, or both caCaM KK/KI along with Flag-ERK2. Both caCaM KK and caCaM KI were sufficient to trigger ERK activation in our system independent of agonist stimulation (Fig. 5e). Taken together, the above data suggest that CaM KK and CaM KI are both necessary and sufficient for ERK activation in MCF-7 cells.

In order to determine if carbachol-mediated ERK activation controlled downstream substrates and physiological targets we evaluated the phosphorylation state of the transcription factor Elk-1 on serine 383. Elk-1 has been suggested to activate transcription as a downstream substrate of ERK and has been implicated in cell growth and proliferation [29, 47–49]. We initially used a pharmacologic approach to evaluate the role of CaM kinases and ERK in Elk-1 phosphorylation. Similar to ERK regulation, Elk-1 was phosphorylated in response to carbachol treatment of MCF-7 cells in a MEK/ERK- and CaM KK-dependent manner as its phosphorylation was inhibited by U0126, KN-93, and STO-609, respectively (Fig. 6a, b). In order to assess the ability of carbachol to stimulate Elk1-mediated gene transcription, cells were transfected with Gal4-Elk-1 and the Gal4 luciferase reporter gene plasmids in the presence or absence of U0126 and STO-609. Carbachol-stimulated Elk-1 luciferase activity was significantly blocked by inhibiting either ERK or CaM KK (Fig. 6c). In order to further evaluate whether CaM KK or CaM KI were upstream of Elk-1 in our system, cells were transfected as in panel c along with either pcDNA, shCaM KKα, or shCaM KIγ and then stimulated with carbachol. Carbachol-stimulated Elk-1 luciferase activity was blocked by either shCaM KKα or shCaM KIγ (Fig. 6d). The relationship between ERK phosphorylation of Elk-1 was further investigated in cells transfected with either control siRNA or siERK2. Transfection of MCF-7 cells with siERK2 significantly knocked down its expression relative to control-transfected cells (Fig. 6e). As a control for specificity, neither the control siRNA nor siRNA to ERK2 had an effect on ATP-citrate lyase expression (Fig. 6e). Furthermore, carbachol stimulation of control siRNA transfected cells triggered phosphorylation of Elk-1, an effect that was completely blocked by expression of siERK2 transfected cells (Fig. 6f). This data suggest that in MCF-7 cells CaM KK, CaM KI, and ERK2 are required for phosphorylation and activation of Elk-1 following carbachol stimulation.

Previous research has suggested that calcium and CaM kinases are capable of regulating the expression of cyclin D1 in MCF-7 cells [24]. Cyclin D1 is a critical molecule that promotes progression of cells through the cell cycle leading to their growth and proliferation. Based on this information, we investigated the ability of carbachol to induce cyclin D1 expression through CaM KK and ERK in our system. Carbachol was capable of increasing cyclin D1 expression over twofold within 4 h of cell stimulation, an effect that was sustained for at least 24 h (Fig. 7a). In contrast, pretreatment of cells with U0126 (U0) to inhibit MEK and ERK attenuated the ability of carbachol to increase cyclin D1 expression at all time points examined (Fig. 7a, middle panels and graph). Similarly, inhibition of CaM KK with STO-609 (S) also blocked carbachol's induction of cyclin D1 (Fig. 7a, lower panels and graph). We also sought to examine whether CaM KK and ERK may be participating in estrogen's induction of cyclin D1 expression. Consistent with the previous work [50], estrogen induced cyclin D1 expression by 2 h and was maintained for at least 6 h (Fig. 7b). Inhibition of either ERK with U0126 or CaM KK with STO-609 substantially reduced the ability of estrogen to induce cyclin D1 expression (Fig. 7b). Consistent with these results, blockade of either CaM KK or ERK activities resulted in the inhibition of carbachol-stimulated cell growth (Fig. 7c). In order to control for the specificity of STO-609 in our cell growth assay, MCF-7 cells were also stimulated with EGF

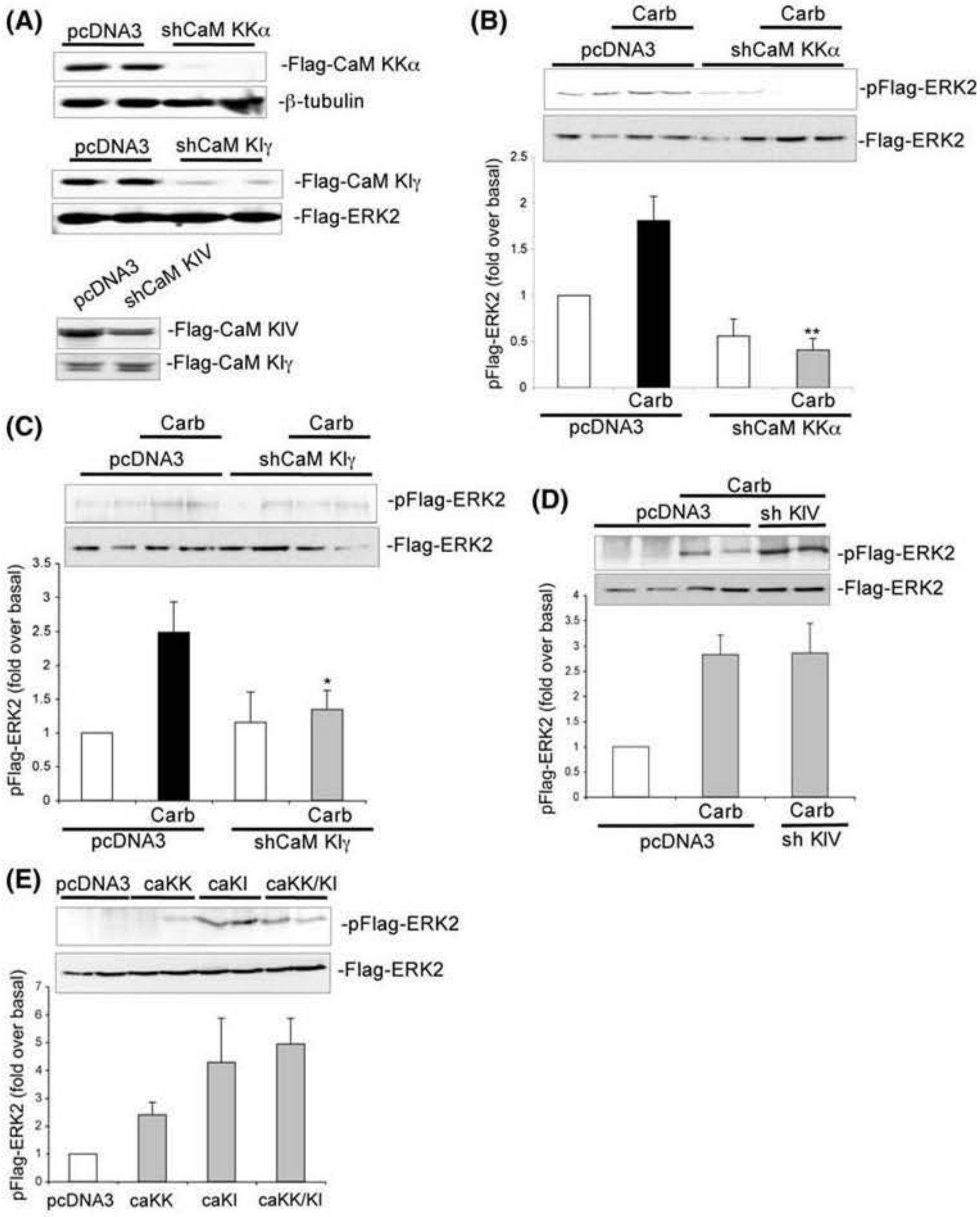

Fig. 5 Carbachol activation of ERK requires CaM KK and CaM KI. **a** shRNA to CaM KKα, CaM KIγ, or CaM KIV inhibit their own expression. Cos-7 cells were transfected with either pcDNA3 or shRNA plasmids directed against either CaM KKα, CaM KIγ, or CaM KIV and Flag-CaM KKα, Flag-CaM KIγ, or Flag-CaM KIV as described in "Experimental procedures" section. Flag expression was measured by western blotting, and the bottom panels are western blots of β-tubulin, Flag-ERK2, or Flag-CaM KIγ, demonstrating equal loading of proteins used for western blotting assays. **b** ERK activation requires CaM KKα. MCF-7 cells were co-transfected with Flag-ERK2 along with the control vector pcDNA3 or shRNA to CaM KKα. MCF-7 cells were stimulated with carbachol (10 μM) for 5 min and Flag-ERK2 phosphorylation was densitized, normalized to Flag-ERK2, quantitated and analyzed as indicated in "Experimental procedures" section ($N = 6 \pm$ SE). **c** Carbachol activation of ERK requires CaM KIγ. MCF-7 cells were co-transfected with Flag-ERK2 along with the control vector pcDNA3 or shRNA to CaM KIγ. MCF-7 cells were stimulated with carbachol (10 μM) for 5 min and Flag-ERK2 phosphorylation was analyzed as in panel B ($N = 6 \pm$ SE). **d** Carbachol activation of ERK is CaM KIV independent. MCF-7 cells were co-transfected with Flag-ERK2 along with the control vector pcDNA3 or shRNA to CaM KIV (sh KIV). MCF-7 cells were stimulated with carbachol (10 μM) for 5 min and Flag-ERK2 phosphorylation was analyzed as in panel B ($N = 4 \pm$ SE). **e** CaM KK and CaM KI are sufficient to activate ERK. MCF-7 cells were co-transfected with Flag-ERK2 along with the control vector pcDNA3, caCaM KK, caCaM KI, or CaM KK and CaM KI. Flag-ERK2 phosphorylation was analyzed as in panel B ($N = 4 \pm$ SE)

in the presence or absence of the CaM KK inhibitor. As can be seen in Fig. 7d, STO-609 did not block EGF-stimulated cell growth. Taken together, our results suggest that both carbachol and estrogen treatment of MCF-7 cells induce the expression of cyclin D1 and promotes cell growth in a CaM KK and ERK-dependent manner.

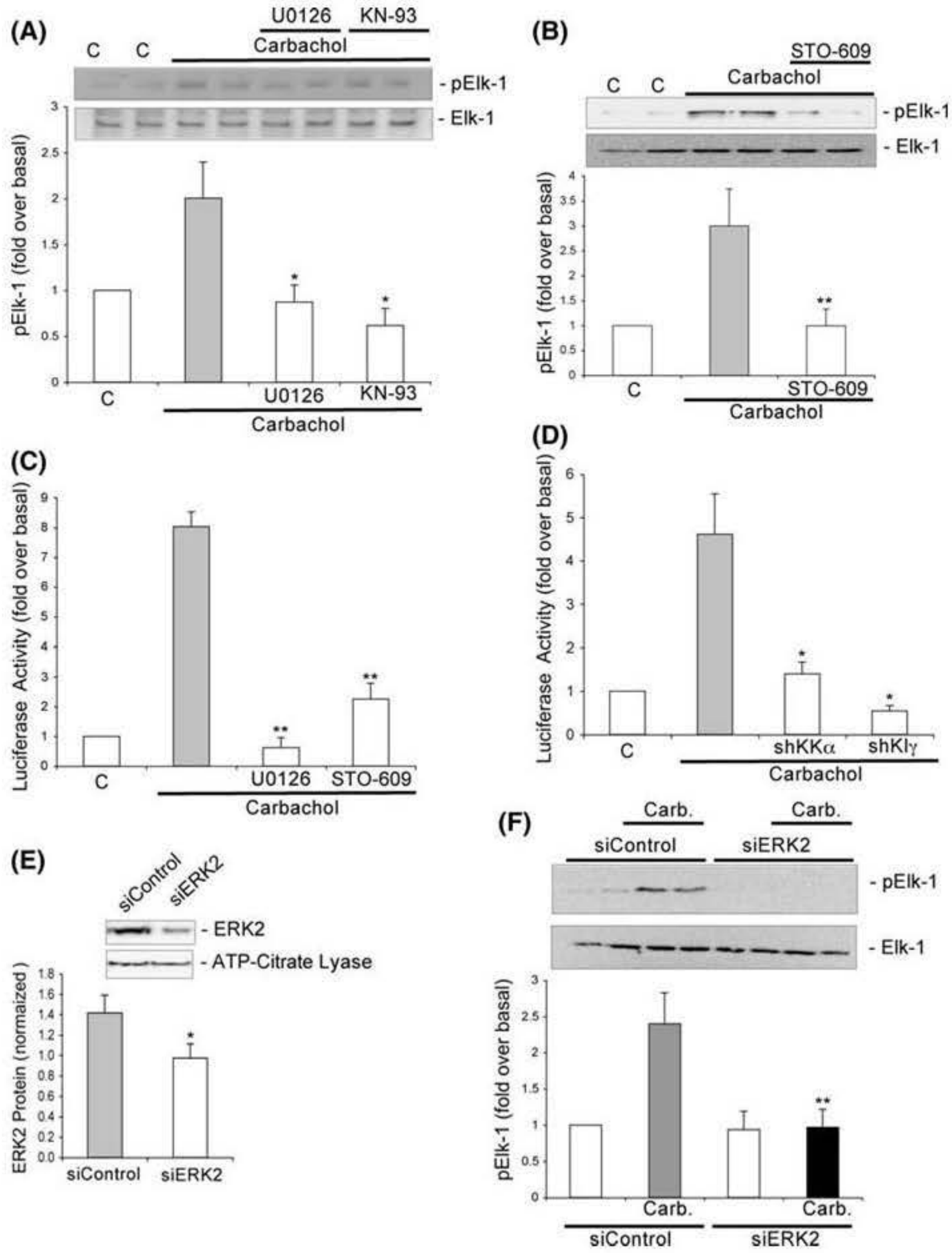

Fig. 6 Carbachol phosphorylation of the transcription factor Elk-1 is ERK-, CaM KK-, and CaM KI-dependent. **a** Carbachol-stimulated phosphorylation of Elk-1 is blocked by U0126 and KN-93. MCF-7 cells received no pretreatment or were pretreated with either U0126 (10 μM) or KN-93 (5 μM) and then stimulated with carbachol (10 μM) for 5 min. Endogenous Elk-1 phosphorylation (serine 383) was measured by western blotting (pElk-1). Phospho-Elk-1 bands were densitized, normalized to total Elk-1, and quantitated. Data are presented as fold Elk-1 phosphorylation over control ($N = 4 \pm$ SE). **b** Carbachol-stimulated phosphorylation of Elk-1 is inhibited by STO-609. MCF-7 cells received no pretreatment or were pretreated with STO-609 (5 μM) and then stimulated with carbachol (10 μM) for 5 min. Endogenous Elk-1 phosphorylation (serine 383) was measured and analyzed as in panel A. Data are presented as fold Elk-1 phosphorylation over control ($N = 6 \pm$ SE). **c** Carbachol stimulates Elk transcriptional activity via ERK and CaM KK. MCF-7 cells were transfected with a Gal4-luciferase reporter and a Gal4-Elk1 fusion protein and luciferase activity was measured as described in "Experimental procedures" section. MCF-7 cells received no pretreatment or were pretreated with U0126 (10 μM) or STO-609 (5 μM) and then stimulated with carbachol (10 μM) for 5 min. Luciferase activity was quantitated and normalized to untreated controls ($N = 6 \pm$ SE). **d** Elk transcriptional activity requires CaM KKα and CaM KIγ. MCF-7 cells were transfected with a Gal4-luciferase reporter, Gal4-Elk1 fusion protein and pcDNA or shRNA to either CaM KKα or CaM KIγ as indicated. MCF-7 cells were then stimulated with carbachol (10 μM) for 5 min and luciferase activity was measured as in panel C ($N = 5 \pm$ SE). **e** siRNA against ERK2 knocks down its expression. MCF-7 cells were transfected with either scrambled siRNA or siRNA complementary to ERK2 as described in "Experimental procedures" section. Endogenous ERK2 expression was measured by western blotting, and the bands were densitized, normalized to ATP-Citrate Lyase, and quantitated. Data are presented as fold ERK2 expression over ATP-Citrate Lyase ($N = 9 \pm$ SE). **f** Carbachol-stimulated Elk-1 phosphorylation requires ERK. MCF-7 cells were transfected with control siRNA or the siRNA against ERK2 as in panel E. MCF-7 cells were then stimulated with carbachol (10 μM) for 5 min and Elk-1 phosphorylation was measured by western blotting (pElk-1). Phospho-Elk-1 bands were densitized, normalized to total Elk-1, and quantitated. Data are presented as fold Elk-1 phosphorylation over control ($N = 6 \pm$ SE)

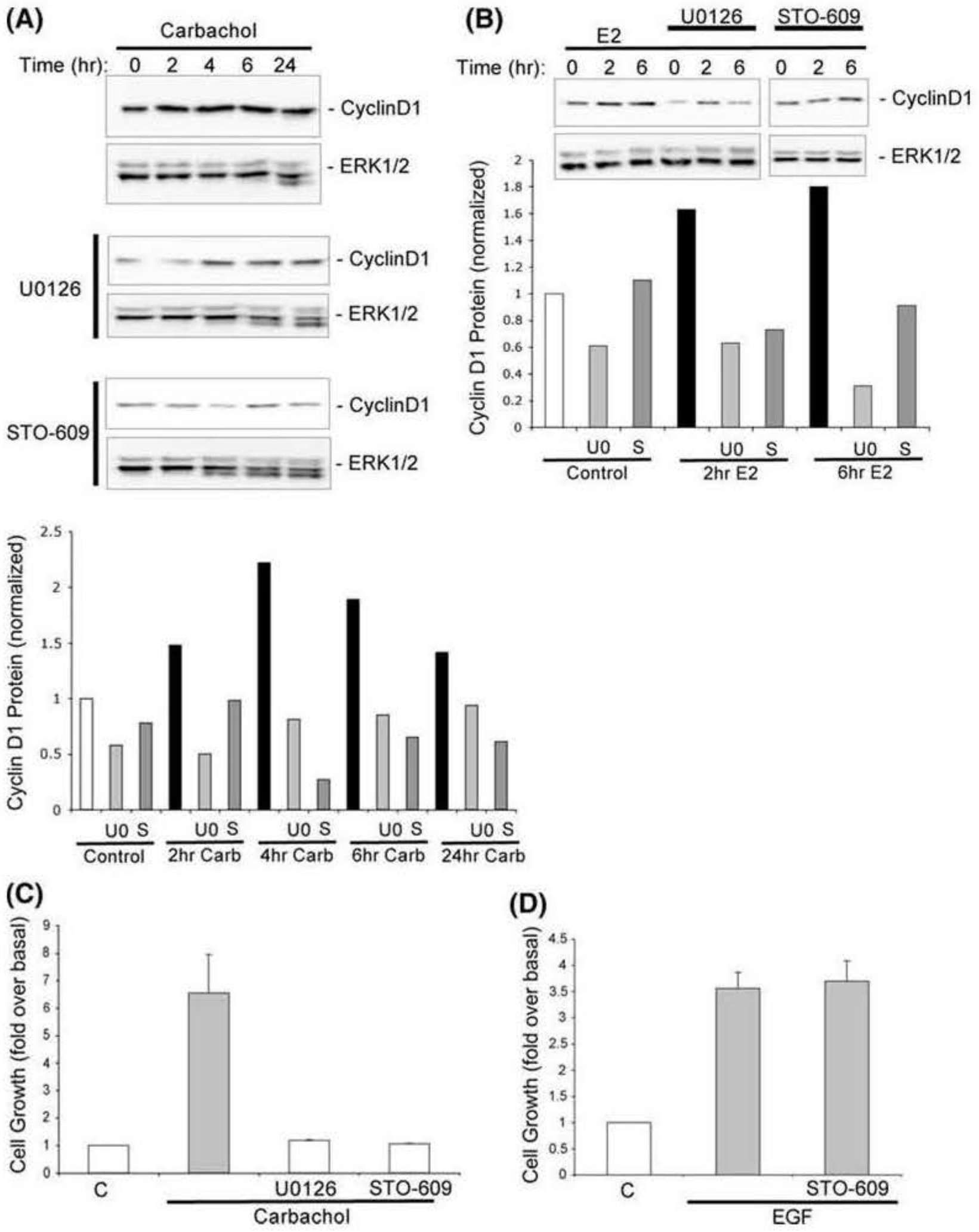

Fig. 7 Carbachol and estrogen-stimulated cyclin D1 expression and cell growth requires CaM KK and ERK. **a** U0126 and STO-609 block carbachol-stimulated cyclin D1 expression. MCF-7 cells received no pretreatment (*top panels*) or were pretreated with either U0126 (10 μM; *middle panels*) or STO-609 (5 μM; *bottom panels*) and then stimulated with carbachol (10 μM) for the indicated times and in the presence or absence of inhibitors. Endogenous cyclin D1 expression (cyclin D1) was measured by western blotting. Western blotting of corresponding cellular extracts for ERK1/2 serves as loading controls. Cyclin D1 bands were densitized, normalized to total ERK1/2, and quantitated. Data are presented as fold cyclin D1 expression over untreated control. **b** Estrogen triggers cyclin D1 expression through ERK and CaM KK. MCF-7 cells received no pretreatment or were pretreated with either U0126 (10 μM) or STO-609 (5 μM) and then stimulated with β-estradiol (E2; 10 nM) for the indicated times and in the presence or absence of inhibitors. Endogenous cyclin D1 expression (cyclin D1) was measured by western blotting. Western blotting of corresponding cellular extracts for ERK1/2 serves as loading controls. Cyclin D1 bands were densitized, normalized to total ERK1/2, and quantitated. Data are presented as fold Cyclin D1 expression over untreated control. **c** MCF-7 stimulated cell growth is blocked by U0126 and STO-609. MCF-7 cells received no pretreatment or were pretreated with U0126 (10 μM) or STO-609 (5 μM) and then stimulated with carbachol (10 μM) for 96 h. Data are presented as cell growth over control ($N = 4 \pm$ SE). **d** EGF-stimulated MCF-7 cell growth is STO-609 insensitive. MCF-7 cells received no pretreatment or were pretreated with STO-609 (5 μM) and then stimulated with EGF (300 ng/ml) for 96 h. Data are presented as cell growth over control ($N = 4 \pm$ SE)

Discussion

Understanding the basic biology and mechanisms that underlie cell growth and proliferation are important areas of current cancer biology research. Calcium is an established second messenger that is known to promote cell proliferation; however, the downstream targets responsible for these effects have not been well defined. We have utilized both pharmacological as well as molecular biochemical approaches to examine the mechanism of carbachol and estrogen signaling in MCF-7 breast cancer cells. Results from previous studies suggest that carbachol and estrogen are sufficient to trigger an increase in intracellular calcium concentration as well as ERK activation [4, 7, 13]. Here, we demonstrate that estrogen and carbachol treatment of MCF-7 cells activates ERK and cell growth. Carbachol activation of ERK and cell growth appears to be mediated through a M_3-subtype of muscarinic GPCR. Significantly, estrogen and carbachol utilize CaM KK and CaM KI to trigger calcium-dependent ERK activation in MCF-7 cells. CaM KK and CaM KI are also sufficient to trigger ERK activity as constitutively active versions of the kinases increase ERK phosphorylation. Inhibition of CaM KK and ERK blocked phosphorylation of the transcription factor Elk-1 and cyclin D1 expression downstream of carbachol signaling. CaM kinases, in particular CaM KK and CaM KI, have been proposed to regulate MCF-7 cell cycle progression perhaps through ERK [24]. Our results are the first to demonstrate that ERK, Elk-1, cyclin D1, and cell growth are regulated by CaM KK and CaM KI in MCF-7 cells.

Carbachol and estrogen have previously been shown to activate ERK via calcium in MCF-7 cells, consistent with the results presented here. A major receptor for intracellular calcium is calmodulin and its targets the CaM kinases. The function of the CaM kinases has been the focus of numerous recent breakthroughs in the field of cell signaling [51]. The best studied of the CaM kinases is CaM KII that has been established as a key participant in synaptic plasticity, neuronal development such as axon attraction, and mammalian behavior [15]. Recent studies have implicated CaM KII in regulating proliferation of muscle, myeloid leukemia, and osteosarcoma cells [52–54]. For example, work by Si et al. demonstrated that CaM KIIγ is found in numerous leukemia cell lines, activated by the *bcr-abl* oncogene, and mediates myeloid leukemia cell proliferation [52, 55]. CaM KII has also been suggested to crosstalk with Notch-1 signaling and Hes-1 gene expression to promote proliferation of PC3 and C4-2B prostate cancer cells [55]. Interestingly, the expression of siRNAs to either the *alpha*, *beta*, or *gamma* isoforms of CaM KII had no effect on Hes-1 gene expression, but C4-2B proliferation was completely blocked by KN-93 at 72 h. The proposed role for CaM KII in many cells including neurons is largely based upon the ubiquity of the protein and ability of the inhibitory compounds KN-93 and KN-62 to block CaM KII activity. However, it has been recently well established that the KN-62 and KN-93 compounds also inhibit CaM KI and CaM KIV thus complicating the originally proposed roles for CaM KII in cellular function [56].

CaM KI and CaM KIV are both substrates for CaM KK and all three kinases have recently garnered significant attention downstream of calcium signaling [15, 46]. The development of the selective CaM KK inhibitor, STO-609, has enabled investigators to differentiate signaling pathways that may utilize CaM KK but not CaM KII [46]. For example, CaM KK signaling has been implicated (using STO-609) in macroautophagy, energy homeostasis, and hippocampal development [10, 17, 19, 20]. These studies are consistent with our results demonstrating the ability of STO-609 to inhibit CaM KK's activation of CaM KI, ERK, Elk-1, and MCF-7 cell proliferation. CaM KIV is a target of CaM KK and it is primarily a nuclear protein expressed in neurons, T cells, and testes [57–61]. CaM KIV has also been suggested to mediate activation of p53 gene expression by estrogen in MCF-7 cells. However, the prescribed role for CaM KIV was based on the use of KN-93 and over expression of constitutively active CaM KIV [62]. The ability of CaM KIV to participate in calcium signaling in MCF-7 cells remains controversial because it has been suggested that MCF-7 cells express little or no CaM KIV [24]. Our data suggest that CaM KIV does not mediate ERK activation downstream of carbachol signaling. In contrast, CaM KI appears to be a ubiquitously expressed cytosolic protein found in numerous cells including epithelial, neurons, fibroblasts, HeLa, MCF-7, and osteoblasts [24, 63–66].

CaM KI has recently been demonstrated to participate in the proliferation of mouse cholangiocytes and osteoblasts [67, 68]. Additionally, CaM KI mediates cyclin D1 expression, cell cycle progression, and proliferation of serum-stimulated WI-38 fibroblasts [65]. Consistent with these results, we propose that CaM KI mediates activation of ERK to regulate cyclin D1 expression and cell proliferation downstream of calcium signaling in MCF-7 cells. CaM KK and CaM KI are capable of regulating ERK activity and neurite development in neuroblastoma cells [19, 21, 23]. Furthermore, Wayman et al. demonstrated that CaM KK and CaM KIγ trigger Ras/MEK/ERK activation, CREB phosphorylation, and Wnt-2 transcription as a means to promote hippocampal dendritic arbor formation [19]. Previous studies have suggested that CaM KK and CaM KI participate in MCF-7 cell cycle progression, consistent with those observations, we now propose that they are capable of acting through ERK and the transcription factor, Elk-1. The exact mechanism for CaM KK/

KI coupling to the Ras/MEK/ERK pathway remains uncharacterized and is an area of active investigation.

CaM KI appears to have numerous substrates including the RacGEF, βPix, Numb protein, CREB, microtubule regulating kinase (MARK2), and p300 reviewed by Wayman et al. [15]. CaM KI also directly phosphorylates the dynamin-related protein 1 (Drp1) at serine 600 in neurons and HeLa cells [66]. Drp1 is a GTPase that associates with the mitochondrial membrane and facilitates mitochondrial fission downstream of calcium and CaM KI signaling. The function of CaM KI-provoked mitochondrial dynamics appears to be ERK independent but highlights the growing role for CaM KI in processes that may underlie cell growth. CaM KI has also been shown to be required for osteoblast proliferation and is highly expressed in endometrial carcinomas; however, the role of ERK or Elk-1 downstream of CaM KI in these systems was not evaluated [63, 68]. Interestingly, KN-93 blocked cyclin D1 and cyclin A expression in the Ishikawa endometrial cancer cell line consistent with our observed role of CaM kinases being upstream of cyclin D1 [63, 69].

Previous research has also shown that estrogen and carbachol are capable of increasing gene expression and promoting cell growth and proliferation. Carbachol has been shown to increase ERK activation, cyclin D1, cyclin E, and p21 expression in quiescent NIH3T3 cells although the pathway downstream of the M_3 receptor what not carefully examined [69]. Cyclin D1 is a key regulator of cell cycle progression through the G1 phase that, upon its induction, binds to cyclin-dependent kinases (CDKs) 4 or 6 [42]. The cyclin D1/CDK complexes are then capable of phosphorylating substrates essential to promote cell proliferation. Cyclin D1 is one of the most commonly overexpressed proteins in breast cancer and is found in nearly 50% of all breast cancer cases [41, 70]. Recent work has demonstrated that overexpression of cyclin D1 in MCF-7 cells increases their growth rates and may be a therapeutic target for anticancer drugs [71]. Our work here is the first to demonstrate that M_3 muscarinic receptor activation by carbachol activates Elk-1 transcription and increases cyclin D1 expression in MCF-7 cells via CaM KK and ERK. Cyclin D1 is important for MCF-7 cell cycle progression and has numerous transcriptional inducers including AP-1, CREB, JAK-STATs, Ets proteins (of which Elk-1 belongs), and Sp-1 [72–74]. Elk-1 is an established substrate and transcription factor target for ERK signaling in MCF-7 cells [29]. Expression of constitutively active CaM KK in PC-12 cells has previously been shown to trigger transcriptional activation through Elk-1 by phosphorylation of serine 383 [44]. The specific requirement for ERK in this activation was not examined. Consistent with these observations, we propose that carbachol operates through CaM KK, CaM KI, and ERK to stimulate Elk-1 phosphorylation and transcription in MCF-7 cells. Interestingly, carbachol and estrogen have also been suggested to increase Elk-1 phosphorylation and Egr-1 gene expression through ERK in SH-SY5Y neuroblastoma cells and MCF-7 cells, respectively [6, 29, 75]. Egr-1 is an early growth response gene that encodes for a zinc finger transcription factor and modulates transcription of several genes. We did not examine Egr-1 expression in MCF-7 cells; however, this is an intriguing area for future studies in our model system. CaM KI has also been proposed to regulate *c-fos* gene expression and proliferation of osteoblasts [68]. c-fos protein and genes are established targets of ERK in numerous cell types including fibroblasts and PC-12 cells [34, 76, 77]. It is interesting to speculate that perhaps ERK may also be operating downstream of CaM KI to regulate *c-fos* gene expression.

The various roles for CaM kinases in cellular function continue to generate exciting observations and the physiological properties of CaM KK and CaM KI is rapidly growing. Our observations expand our current understanding of CaM KK and CaM KI signaling to include their control of ERK, Elk-1, and cyclin D1 in breast cancer cells. We suggest a working model by which agonists that increase intracellular calcium concentration and CaM KK activation promote MCF-7 cell growth. Specifically, carbachol acting through the M_3 muscarinic receptor triggers CaM KK, CaM KI, and ERK activation leading to the phosphorylation and activation of Elk-1 and increases in cyclin D1 expression. The physiological ligand, estrogen, also appears to utilize a similar intracellular signaling pathway in MCF-7 cells. Dissecting the signaling pathways utilized by calcium in cancer cells may provide relevant targets for future studies in cancer biology.

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