Depolarization of Skeletal Muscle Cells induces Phosphorylation of cAMP Response Element Binding Protein via Calcium and Protein Kinase Cα*

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Membrane depolarization of skeletal muscle cells induces slow inositol trisphosphate-mediated calcium signals that regulate the activity of transcription factors such as the cAMP-response element-binding protein (CREB), *jun*, and *fos*. Here we investigated whether such signals regulate CREB phosphorylation via protein kinase C (PKC)-dependent pathways. Western blot analysis revealed the presence of seven isoforms (PKCα, -βI, -βII, -δ, -ϵ, -θ, and -ζ) in rat primary myotubes. The PKC inhibitors bisindolymaleimide I and Gö6976, blocked CREB phosphorylation. Chronic exposure to phorbol ester triggered complete down-regulation of several isoforms, but reduced PKCα levels to only 40%, and did not prevent CREB phosphorylation upon myotube depolarization. Immunocytochemical analysis revealed selective and rapid PKCα translocation to the nucleus following depolarization, which was blocked by 2-amino-ethoxydiphenyl borate, an inositol trisphosphate receptor inhibitor, and by the phospholipase C inhibitor U73122. In C2C12 cells, which expressed PKCα, -ϵ, and -ζ, CREB phosphorylation also depended on PKCα. These results strongly implicate nuclear PKCα translocation in CREB phosphorylation induced by skeletal muscle membrane depolarization.

Ca^{2+} controls a huge variety of biological processes, including the cell cycle, gene expression, and cell death (1). In skeletal muscle, depolarization induces Ca^{2+} release and muscle contraction, in a process known as excitation-contraction coupling, which is mediated by dihydropyridine receptors in the T-tubule membrane and ryanodine receptors in the sarcoplasmic reticulum (2). Depolarization of muscle cells in culture also results in a slow nuclear Ca^{2+} transient, unrelated to contraction (3–6). Dihydropyridine receptors are considered the voltage sensors for this Ca^{2+} signal (7), whereas a transient increase in inositol trisphosphate (IP_3)[1] mass mediates, via IP_3 receptors (IP_3Rs), the calcium increase that is detectable in nuclei and the adjacent cytoplasm (4, 5). IP_3Rs have been implicated using different inhibitors, and these receptors have been detected both in the nuclear envelope and in the SR of cultured muscle cells (4, 8).

Slow Ca^{2+} transients in and around the nucleus have been proposed to link membrane depolarization to the regulation of gene transcription. On the one hand, myotube depolarization induces an increase in phosphorylation of both extracellular signal-regulated kinases 1 and 2 (ERK1/2), the transcription factor cAMP-response element-binding protein (CREB), as well as mRNA levels of early genes like c-*fos*, c-*jun*, and *egr-1*. All of these responses are significantly blocked by reducing or eliminating the slow, IP_3-mediated, Ca^{2+} transients (8, 9). On the other hand, in myotubes depolarized in the presence of ryanodine at concentrations that block fast but not the slow Ca^{2+} transients, increases in P-ERK, P-CREB, as well as mRNA levels of the early genes *jun* and *fos*, were still observed (7, 9).

CREB activation occurs in response to a wide variety of stimuli, and in neurons it plays a key role in Ca^{2+}-activated gene expression (10, 11). CREB activation by phosphorylation on serine 133 occurs in response to activation of many different kinases. Hence, CREB activation represents an important nuclear end point for multiple signal transduction cascades (11). In hippocampus neurons, for instance, depolarization-induced CREB phosphorylation is mediated by calcium/calmodulin kinase (CaMK) IV and by the ERK pathway (12–14), whereby only in the latter case persistent CREB phosphorylation and cAMP response element-dependent transcription are observed (10, 14). Consistent with this notion, a MAPK kinase inhibitor (UO126) blocked ERK activation and CREB phosphorylation in depolarized muscle cells far more efficiently than SB-203580, a specific inhibitor for the p38 MAPK (9). Therefore, ERKs appear to be important constituents of at least one of the pathways involved in CREB phosphorylation induced by depolarization in skeletal muscle cells.

In the present study, we have extended our analysis of upstream signaling pathways that lead to CREB phosphorylation, by focusing on protein kinase C (PKC). PKCs are a family of at least 11 serine/threonine kinases that are divided into three main subgroups based on their amino acid sequence and lipid-dependent activation requirements. Conventional (c) PKC isoforms (PKCα, -βI, -βII, and -γ) require Ca^{2+} and DAG for

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[1] The abbreviations used are: IP_3, inositol trisphosphate; IP_3R, IP_3 receptor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; CREB, cAMP response element-binding protein; CaMK, calcium/calmodulin kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; cPKC, conventional PKC; DAG, diacylglycerol; PKA, cAMP-dependent protein kinase; GFP, green fluorescent protein; PLC, phospholipase C.

activation, the novel PKC isoforms (-δ, -ϵ, -η, and -θ) are Ca^{2+}-independent but DAG-dependent, and atypical PKC isoforms (-ζ, -ι/λ, and -μ) are unresponsive to DAG and/or Ca^{2+} (15).

CREB is phosphorylated *in vitro* by kinases from this family (16), but most of the results in the literature suggest that CREB phosphorylation downstream of PKCs is indirect and occurs through the activation of the ERK pathway (16, 17). In our experimental model, depolarization induces a transient increase in IP_3 mass with kinetics comparable with those of the slow Ca^{2+} transient (4). PLC hydrolysis of phosphatidylinositol 4,5-bisphosphate generates IP_3 responsible for calcium release via IP_3R and DAG. Together these two messenger molecules activate cPKC isoforms (15). Hence, we were particularly interested in investigating the role of this group of PKCs in events leading to CREB phosphorylation/activation.

Here we provide evidence showing that cPKCα activation and translocation to the nucleus is essential for depolarization-induced CREB phosphorylation, both in primary skeletal muscle cells and in the C2C12 muscle cell line. Depolarization-induced CREB phosphorylation was blocked by PKC inhibitors but not by others specific for CaMKs or PKA. Alternatively, conditions that lead to selective down-regulation with phorbol ester of most sensitive isoforms except PKCα did not affect CREB phosphorylation. Depolarization resulted in selective PKCα translocation to the nucleus in a PLC/IP_3R-dependent manner that was required for CREB phosphorylation. Finally, cPKCα-dependent CREB phosphorylation did not appear to require an intermediate of the ERK pathway.

EXPERIMENTAL PROCEDURES

Materials—Antibodies directed against PKCα, -βI, -ϵ, and -ζ and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, were obtained from Sigma. Antibodies against PKCβII, -δ, and -θ; total CREB; and total ERK2 were obtained from Santa Cruz Biotechnology. Antibodies against dually phosphorylated forms of ERK1 and ERK2 and phosphorylated CREB were from Cell Signaling Technology. Secondary horseradish peroxidase-conjugated anti-rabbit antibody was from Pierce. ECL reagents were from Pierce or Amersham Biosciences. PKC inhibitor Gö6976 and PLC inhibitor U73122 were from BIOMOL Research Laboratories. Bisindoleylmaleimide I was from Calbiochem. All tissue culture medium were purchased from Invitrogen.

Cell Culture—Rat skeletal muscle cells in primary culture were prepared essentially as previously described (3). Briefly, hindlimb muscle from neonatal Sprague-Dawley rats were mechanically dispersed and treated with 0.2% (w/v) collagenase. The suspension was filtered through lens tissue paper, spun down at low speed, and preplated to remove contaminating fibroblasts. The cells were plated on 60-mm culture dishes or on coverslips in 35-mm culture dishes for immunostaining procedures. The plating medium was Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) supplemented with 10% bovine serum, 2.5% fetal bovine serum, antibiotics, and antimycotic. To eliminate remaining fibroblasts, 10 μM cytosine arabinoside was added at the beginning of myoblats alignment. To induce differentiation, the cells were cultured in serum-free medium. The experiments were performed in 6–7-day-old cultures.

Myoblasts of the C2C12 cell line (ATCC) were cultivated in Dulbecco's modified Eagle's medium/Ham's F-12 medium (1:1) with 10% bovine serum and 2.5% fetal calf serum. For differentiation, the serum was replaced by 5% horse serum. The cells were studied 5–7 days after differentiation was initiated.

Depolarization Experiments—The cells were incubated with Krebs-Ringer under resting conditions for 30 min. This medium contains 4.7 mM KCl. Depolarization was induced by changing to a medium containing 84 mM KCl, whereas the osmolarity was maintained by decreasing the NaCl concentration. The times of incubation with the inhibitors or other conditions are specified under "Results. " The experiments were matched with vehicle-treated controls.

Down-regulation Assays—The myotubes were treated with 100 nM 4-αTPA or 100 nM 4-βTPA for 24 h. Phorbol ester was removed, and the cells were incubated in Krebs-Ringer solution for 1 h before depolarizing muscle cells as described above.

Western Blot Analysis—Stimulated cells were lysed in 60 μl of ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 5 mM Na_3VO_4, 20 mM NaF, 10 mM sodium pyrophosphate, and a protease inhibitor mixture (Calbiochem). The cell lysates were sonicated for 1 min, incubated on ice for 20 min, and centrifuged to remove debris. Protein concentration of the supernatants was determined with bovine serum albumin as standard. The lysate proteins were suspended in Laemmli buffer, separated in 10% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked at room temperature for 1 h in Tris-buffered saline containing 3% fat-free milk, with or without 0.5% Tween 20, and then incubated overnight with the appropriate primary antibody. After washing with Tris-buffered saline, the membranes were incubated with the secondary antibody at room temperature for 1.5 h. The immunoreactive proteins were detected using ECL reagents according to the manufacturer's instructions. The films were scanned, and the Scion Image program (National Institutes of Health) was employed for densitometric analysis of the bands. To correct for loading, the membranes were stripped in buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 50 mM β-mercaptoethanol, at 50 °C for 30 min and reprobed with the corresponding control antibodies.

Immunostaining and Confocal Imaging—Myotubes grown on coverslips were processed essentially as previously reported (18). Briefly, the myotubes were fixed in ice-cold methanol, blocked in phosphate-buffered saline containing 1% bovine serum albumin for 60 min, and incubated with primary antibodies at 4 °C overnight. The cells were washed five times with phosphate-buffered saline/bovine serum albumin and incubated with secondary antibody at room temperature for 90 min. The coverslips were mounted in Vectashield (Vector Laboratories, Inc.) to retard photobleaching. The samples were evaluated in a scanning confocal microscope (Carl Zeiss Axiovert 135 M-LSM Microsystem) and documented through computerized images.

Immunoprecipitation—The myotubes were solubilized in 200 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 0.1% Nonidet P-40, 1 mM EDTA, pH 8.0, EGTA, pH 7.8, 1 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 10% glycerol, 140 mM NaCl, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin). A 15,000 × *g* supernatant fraction was incubated with 10 μl of A/G-Agarose beads (Santa Cruz, Biotechnology) for 30 min. The beads were pelleted by centrifugation and washed three times with washing buffer (25 mM Hepes, pH 7.5, 0.2% Nonidet P-40, 140 mM NaCl, 0.1% bovine serum albumin, and 10% glycerol). After the precleaning procedure, the whole cell extract was incubated with 1 μl of anti-phosphorylated CREB for 1 h and 50 μl of protein A-Sepharose (Santa Cruz Biotechnology). Immunoprecipitates were resolved by SDS-PAGE, transferred to polyvinylidene difluoride filters, and blotted with the corresponding antibody.

Semi-quantitative Reverse Transcription-PCR—cDNA was amplified using c-*fos* primers, and the DNA concentration was normalized to glyceraldehyde-3-phosphate dehydrogenase expression. PCR amplification was maintained in the exponential phase for each product. The c-*fos* primers used were 5′-AGGCCGACTCCTTCTCCAGCAT-3′ (sense) and 5′-CAGATAGCTGCTCTACTTTGC-3′ (antisense), corresponding to bases 235–533 (9).

Data Analysis—All of the experiments were performed a minimum of three times. The results are expressed as the means ± S.E. The significance of differences among treatments was evaluated using *t* test for paired data or analysis of variance followed by Dunnett's post-test for multiple comparisons.

RESULTS

Depolarization-induced CREB Phosphorylation Required Calcium-dependent PKC Activity in Primary Skeletal Muscle Cells and in the Muscle Cell Line C2C12—Depolarization induces a transient increase in CREB phosphorylation in primary culture skeletal muscle cells (8, 9). This effect is mediated by an IP_3-dependent slow Ca^{2+} transient. In skeletal muscle cells, IP_3Rs are located to both the nuclei and the SR, and IP_3 mass increases following depolarization (4, 8). To determine whether PKCs may be implicated as downstream effectors of these events required for CREB activation, pharmacological studies were performed. BIM I (2.5 μM), a specific but broad spectrum PKC inhibitor (19) essentially abolished CREB phosphorylation (Fig. 1*A*). The calcium dependence of CREB phosphorylation prompted us to investigate whether calcium-sensitive PKC isoforms were involved. To this end, myotubes were depolarized in the presence of Gö6976, a cPKC inhibitor (20). Gö6976 (1 μM) essentially reduced P-CREB levels as efficiently

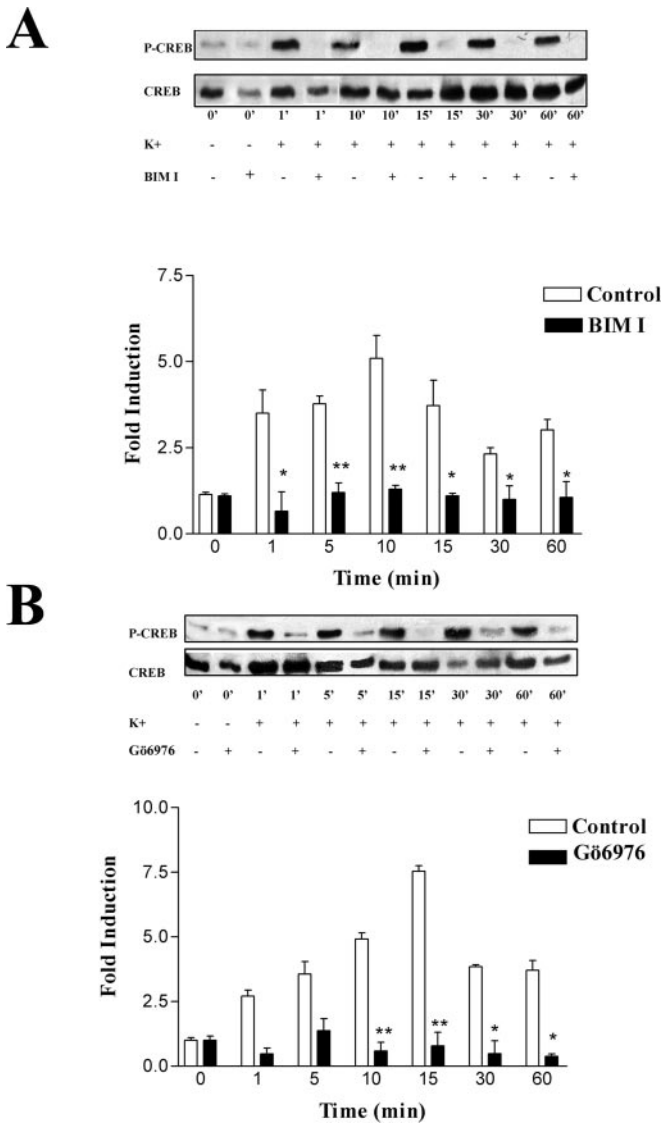

FIG. 1. **Potassium depolarization-induced CREB phosphorylation is blocked by PKC inhibitors.** *A*, *upper panel*. Western blot analysis of P-CREB or total CREB, as loading control, in myotubes preincubated with or without 2.5 μM bisindoleylmaleimide I, a general PKC inhibitor, and depolarized in the absence or presence of the inhibitor. *B*, *upper panel*, Western blotting for P-CREB or CREB in myotubes preincubated with or without 1 μM Gö6976, a specific inhibitor of calcium-dependent PKC isoforms, and depolarized in the absence or presence of the inhibitor reveals an almost complete inhibition of CREB phosphorylation. *Lower panels* in *A* and *B*, *bar graphs* represent P-CREB/total CREB levels expressed as the average fold increase (mean ± S.E.) over basal levels ($n = 5$). *, $p < 0.05$; **, $p < 0.001$, compared with the correspondent stimulus without inhibitor (*t* test for paired data).

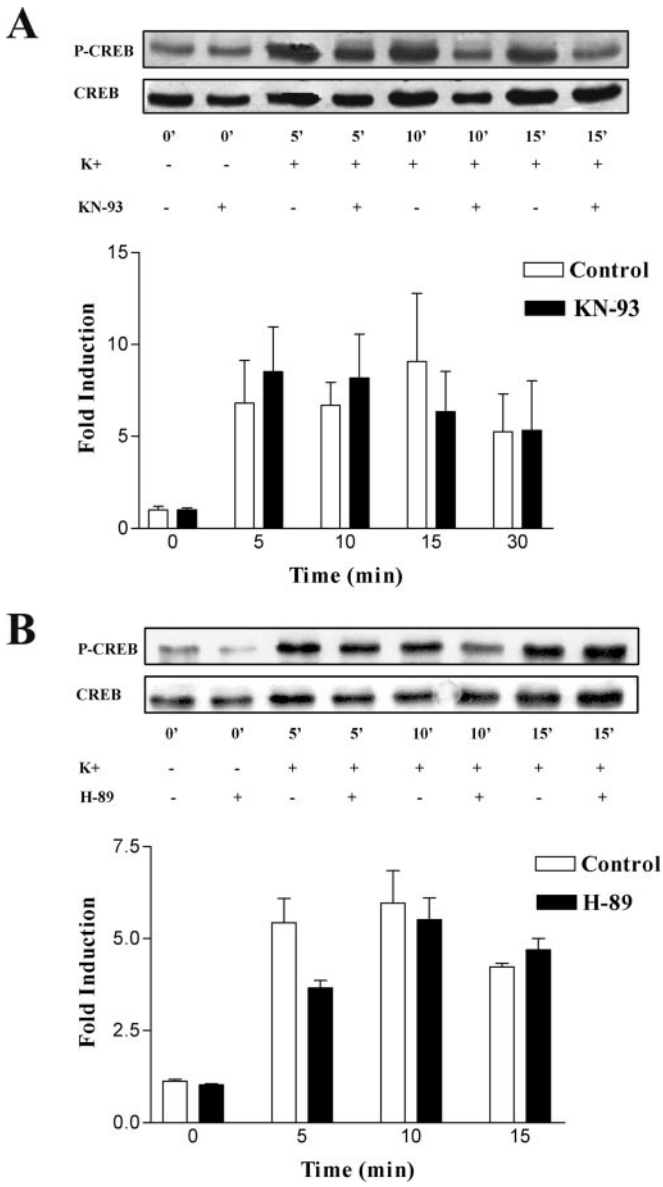

FIG. 2. **Depolarization-induced CREB phosphorylation is unaffected by CaMK or PKA inhibitors.** Western immunoblotting data showing that P-CREB levels were not changed by 10 μM KN-93, a CaMK inhibitor (*A*), or by 4 μM H89, a PKA inhibitor (*B*). The *bar graphs* represent P-CREB/total CREB levels expressed as the average fold increase (mean ± S.E.) over basal levels ($n = 3$).

as BIM I (Fig. 1*B*). Alternatively, rottlerin (10 μM), a novel PKC inhibitor (21), had no effect on CREB phosphorylation (data not shown; $n = 3$ experiments).

To determine whether CaMK or PKA participate in depolarization-induced CREB phosphorylation in muscle cells, additional non-PKC inhibitors were employed. CREB phosphorylation was neither decreased by 10 μM KN-93 (Fig. 2*A*) or by 4 μM H-89 (Fig. 2*B*), inhibitors of CaMK and PKA, respectively. Taken together, the results obtained with inhibitors implicated cPKCs rather than CaMK or PKA in CREB phosphorylation and activation.

To further investigate the role of PKCs, primary myotubes were exposed to the active (4β) and inactive (4α) forms of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Phorbol esters initially activate cPKCs and novel PKC but not the atypical PKCs isoforms (15). Prolonged treatment, however, tends to trigger PKC down-regulation of responsive PKC isoforms by promoting proteolytic degradation. In myotubes incubated short term with the biologically active 4β-TPA (1 μM), CREB phosphorylation increased transiently 4-fold over basal levels, reaching a maximum between 10 and 15 min (Fig. 3). By contrast, the inactive 4α-TPA (1 μM) did not induce CREB phosphorylation (Fig. 3). Moreover, the presence of BIM I (2.5 μM) abolished CREB phosphorylation induced by 4β-TPA (data not shown).

Additional experiments were carried out using the mouse muscle C2C12 cell line. K^+-induced depolarization triggers both fast and slow Ca^{2+} transients in this cell line, essentially by the same mechanisms as in primary myotubes (5). Depolarization of C2C12 cells also induced transient CREB phosphorylation similar to that observed for primary myotubes (Fig. 4*A*). P-CREB production in response to depolarization was

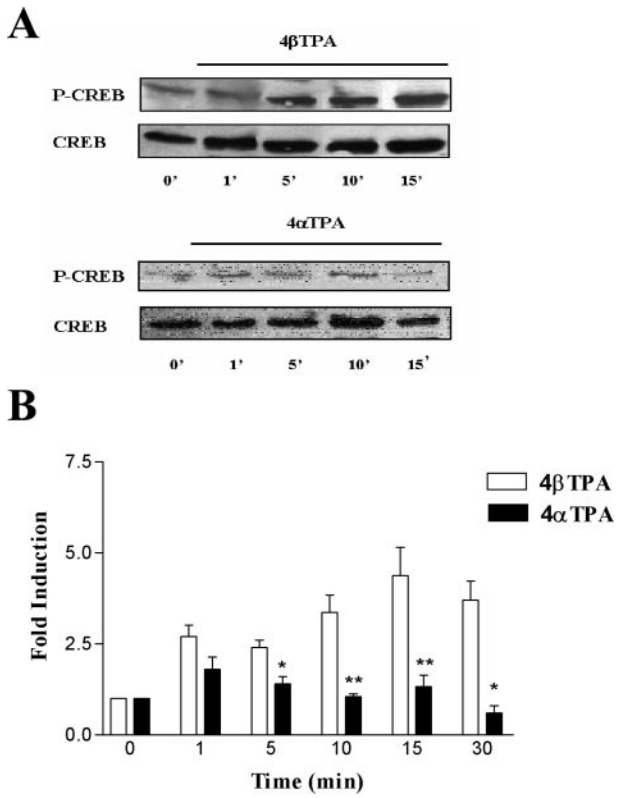

FIG. 3. **Phorbol ester-induced CREB phosphorylation in skeletal muscle cells.** *A*, Western immunoblot showing increased P-CREB levels induced by 1 μM 4β-TPA, the active form of the phorbol ester, whereas 1 μM 4α-TPA, a biologically inactive TPA-derivative, had no effect on CREB phosphorylation. *B*, *bar graphs* represent P-CREB/total CREB levels expressed as the average fold increase (mean ± S.E.) over basal levels ($n = 3$). *, $p < 0.05$; **, $p < 0.001$, with respect to 4α-TPA stimulation (*t* test for paired data).

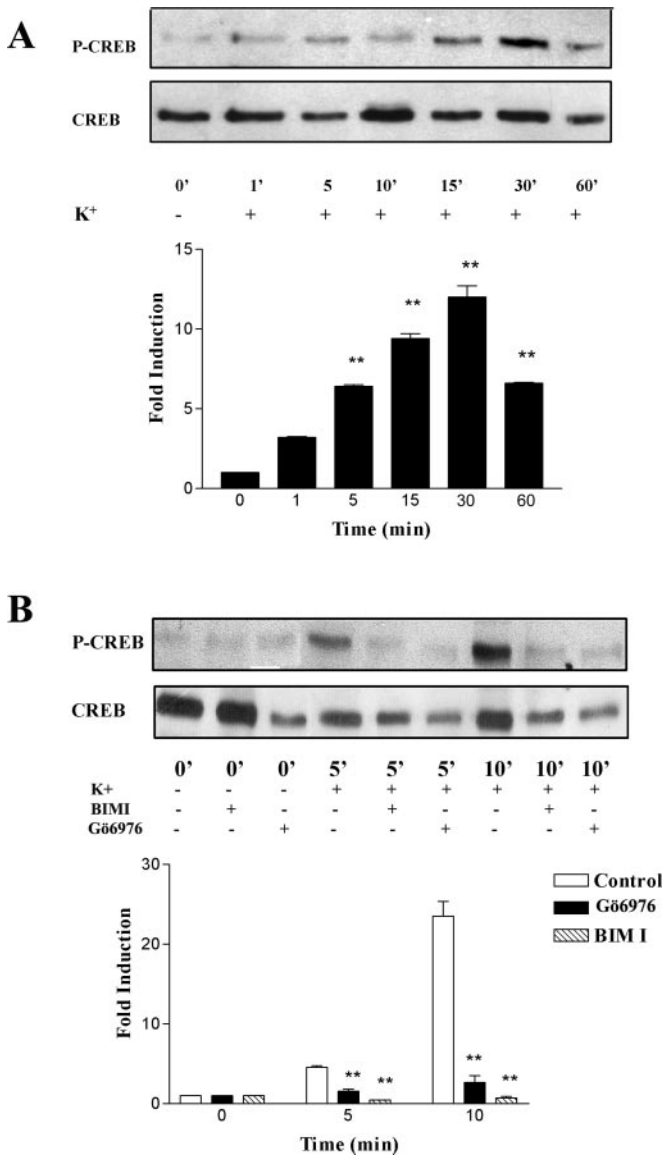

FIG. 4. **Depolarization-induced CREB phosphorylation in the C2C12 cell line is also blocked by PKC inhibitors.** *A*, *upper panel*, Western immunoblot showing activation of CREB in extracts of myotubes from the C2C12 cell line. P-CREB levels were significantly increased 5 min after depolarization and remained elevated until 60 min. *Lower panel*, *bar graphs* represent P-CREB/total CREB levels expressed as the average fold increase (mean ± S.E.) over basal levels ($n = 3$). **, $p < 0.001$, with respect to control condition without the inhibitor (one-way analysis of variance followed by Dunnett's multiple comparison post-test). *B*, *upper panel*, Western immunoblot showing complete inhibition of depolarization-induced CREB activation by PKC inhibitors. C2C12 myotubes were preincubated with or without 2.5 μM BIM I or 1 μM Gö6976 and depolarized in the presence or absence of the inhibitors for the times indicated. *Lower panel*, *bar graphs* represent P-CREB/total CREB levels expressed as the average fold increase (mean ± S.E.) over basal levels ($n = 5$). **, $p < 0.001$, with respect to control condition without the inhibitor (*t* test for paired data).

completely suppressed in the presence of either BIM I (2.5 μM), or Gö6976 (1 μM) (Fig. 4*B*). Overall, these data implicated cPKCs in depolarization-induced CREB phosphorylation both in primary culture skeletal muscle and C2C12 cells.

Characterization of PKC Isoforms in Skeletal Muscle Cells—The expression of different PKC isoforms was investigated by Western blotting using isoform-specific (α, βI, βII, ϵ, δ, θ, and ζ) anti-PKC antibodies in both primary culture skeletal muscle cells and in the C2C12 cells. All seven PKC isoforms (α, βI, βII, ϵ, δ, θ, and ζ) were detected in primary cultures of rat skeletal muscle (Fig. 5, *left column*). In the C2C12 cell line, however, only three isoforms were detected, namely PKCα, -ϵ, and -ζ (Fig. 5, *right column*). Because CREB phosphorylation was suppressed in C2C12 cells by the PKC inhibitors BIM I and Gö6976, these observations suggest that PKCα was the cPKC isoform involved in CREB phosphorylation in this cell line.

CREB Phosphorylation Following TPA-induced PKC Down-regulation—To identify in primary myotubes the specific isoform(s) that may be involved in depolarization-induced CREB phosphorylation, the effect of long term treatment with TPA was investigated. Myotubes were incubated with 100 nM TPA for 24 h, and down-regulation of the PKCs was assessed by Western blotting analysis. Under these conditions, the PKC isoforms βI, βII, δ, and θ were essentially eliminated. A small amount of PKCϵ was still apparent, whereas PKCζ remained unaffected. Unexpectedly, despite the complete loss of PKCβI and PKCβII protein, PKCα levels decreased only to 40% of controls. (Fig. 6*A*). Following chronic TPA (100 nM) treatment for 24 h, CREB phosphorylation in response to depolarization was assessed (Fig. 6*B*). Phosphorylation levels were similar to those attained in untreated cells. Myotubes treated in this way with either 4α- or 4β-TPA did not display significant differences in their ability to induce CREB phosphorylation in response to K^+-dependent depolarization. These results corroborate findings from C2C12 cells suggesting that the calcium-dependent PKCβI and PKCβII isoforms were not involved in CREB phosphorylation. Instead, residual cPKCα appeared sufficient to stimulate the activation of this transcription factor in primary skeletal muscle cells.

Depolarization-induced Nuclear Translocation of PKCα—In-

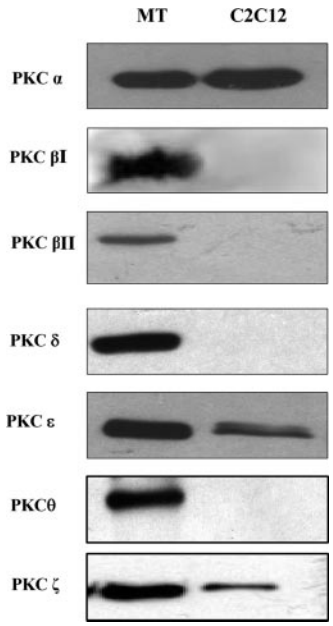

FIG. 5. **Expression of PKC isoforms in primary skeletal muscle cells and in the C2C12 cell line.** Western immunoblot data revealed the presence of seven different PKC isoforms in rat primary skeletal muscle cells (*left column*), whereas in C2C12 cell line only three PKC isoforms were detected (*right column*). The figure illustrates representative results from three independent determinations. As a control for loading, the membranes were stripped and reprobed with anti-PKCα or anti-PKCζ antibodies.

active forms of PKCs are often diffusely distributed throughout the cytosol or sometimes localized to specific regions or structures in the cell. A characteristic trait of PKCs is that activation often correlates with translocation between different intracellular compartments (22).

Because CREB is a predominantly nuclear protein, we investigated by immunofluorescence labeling and confocal microscopy whether the subcellular distribution of the cPKCs α, βI, and βII was altered and in particular whether translocation to the nucleus occurred upon depolarization. Interestingly, PKCα translocated from the cytosol to the nuclear region (Fig. 7*A*) with kinetics comparable with those observed for CREB phosphorylation (Fig. 1). In the absence of stimulation PKCα was essentially absent from the nucleus. Following depolarization, PKCα was already detectable in the nucleus within 1 min (Fig. 7*A*, *inset*). PKCα levels increased until 5 min and were maintained until 15 min after stimulation. After 30 min the PKCα presence declined and then became undetectable at 60 min (Fig. 7*A*). PKCβI, in contrast, was only detected in the nucleus 60 min after stimulation (Fig. 7*B*), whereas PKCβII remained cytosolic at all times studied (Fig. 7*C*). In C2C12 cells PKCα translocation to the nuclear region was also observed during the first minute after depolarization but essentially disappeared by 60 min (Fig. 7*D*).

CREB and PKCα Co-immunoprecipitation—Because the interaction between kinases and their substrates *in situ* often involves multiple interaction sites, we speculated that PKC may be present in a protein complex together with CREB after depolarization. To test this possibility, P-CREB was immunoprecipitated from cellular extracts of primary culture myotubes that had been previously depolarized in high potassium solution. As expected, P-CREB and PKCα co-immunoprecipitated in extracts obtained 5 min post-depolarization (Fig. 7*E*). Moreover, no co-immunoprecipitation of PKCβI or βII with P-CREB was detected under the same conditions. These results support the notion that CREB forms a complex with PKCα (but not other cPKCs) following depolarization.

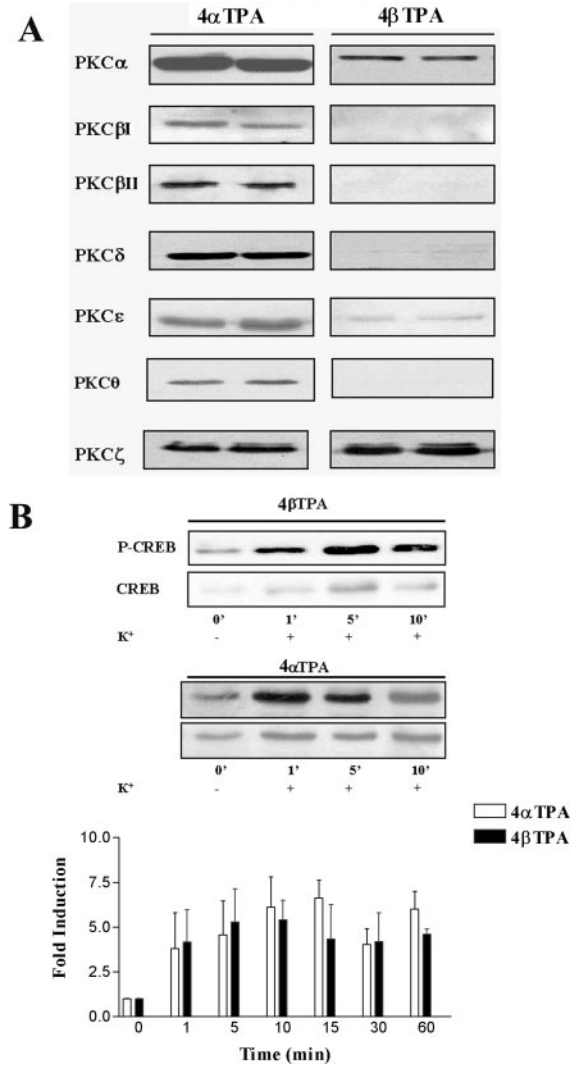

FIG. 6. **Residual PKCα levels following 4β-TPA treatment appeared sufficient to induce CREB activation in depolarized myotubes.** *A*, Western blot analysis showing complete down-regulation of several PKC isoforms but only a partial effect on the PKCα isoform. The cells were treated with either 4β-TPA or with 4α-TPA (1 μM) for 24 h. All of the lysates were resolved by SDS-PAGE followed by immunoblotting with different rabbit anti-PKC antibodies. Loading was controlled by stripping the membranes and reprobing with anti-PKCζ antibody. *B*, *upper panel*, Western blot analysis of P-CREB or total CREB from depolarized rat primary myotubes pretreated with either 4β-TPA or 4α-TPA (1 μM) for 24 h reveals no difference between both conditions. *Lower panel*, *bar graphs* represent P-CREB/total CREB levels expressed as the average fold increase (mean ± S.E.) over basal levels (n = 3).

Calcium Dependence of PKCα Translocation—Because CREB activation is highly dependent on the slow calcium transient induced by depolarization, PKCα translocation was also assessed under conditions that abolish this calcium signal. In the presence of either 50 μM 2-aminoethoxydiphenyl borate (IP_3 system inhibitor), 100 μM 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (intracellular calcium chelator), or 10 μM U73122 (PLC inhibitor), treatments that block the slow calcium transient (5, 8), no PKCα translocation to the nucleus was observed in myotubes depolarized for 5 or 10 min (Fig. 8). Similar results were also obtained using C2C12 cells (data not shown).

CREB Phosphorylation by PKC Did Not Involve ERK1/2 as Intermediates—Most published data suggest that PKC-dependent CREB phosphorylation is an ERK-mediated event, although ERK-independent mechanisms have also been reported

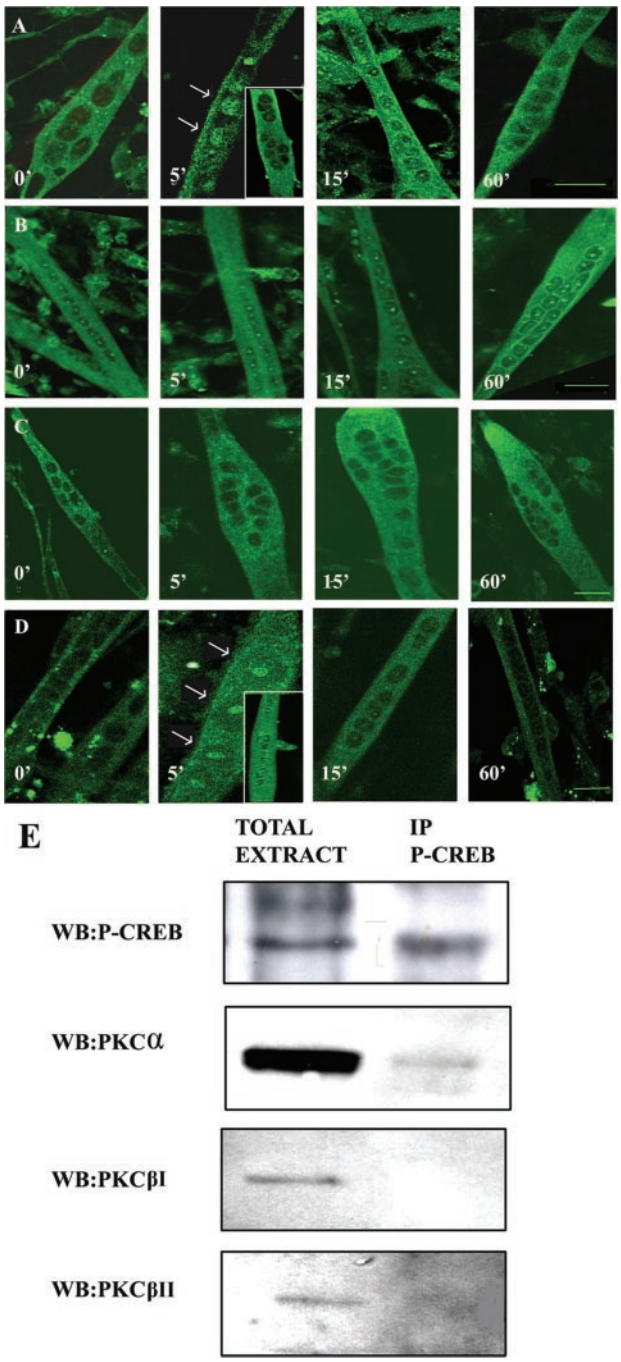

FIG. 7. **Potassium depolarization induced PKCα translocation to the nucleus in primary myotubes and in the C2C12 cell line.** *A*, immunofluorescence analysis of PKCα translocation in rat primary myotubes at different time points after depolarization. PKCα translocation to the nucleus was detected within 1–15 min and returned to the basal condition within 60 min. The *arrows* highlight nuclear PKCα translocation. Translocation at 1 min is shown as an inset in the 5-min panel. *Scale bar*, 20 μm. *B* and *C*, immunofluorescence analysis of PKCβI and PKCβII, respectively, showing that these isoforms did not translocate following depolarization. *Scale bar*, 20 μm. *D*, depolarization of C2C12 myotubes resulted in efficient PKCα translocation to the nucleus 1–15 min following stimulation (result after 1 min shown as *inset* in 5-min panel). The *arrows* indicate nuclear translocation. *Scale bar*, 20 μm. All of the results shown are representative of results obtained in five independent experiments. *E*, PKCα and phosphorylated-CREB co-immunoprecipitate after depolarization. Lysates from rat primary myotubes depolarized for 5 min were immunoprecipitated (*IP*) with anti-P-CREB antibody. The presence of PKCα, PKCβI, or βII in the immunoprecipitate was determined by Western blotting (*WB*) with specific antibodies. A representative Western blot shows the co-immunoprecipitation of P-CREB and PKCα. There was no evidence for co-immunoprecipitation of the PKCβI or PKCβII isoforms. The results shown are representative of three independent experiments.

(16, 17). We previously demonstrated that the ERK pathway plays a central role in CREB activation induced by depolarization in primary myotubes (9). Hence, we considered it important to determine whether PKC-dependent regulation of CREB phosphorylation actually involves an ERK intermediate. To this end, ERK1/2 phosphorylation was assessed in primary myotubes depolarized both in the absence and presence of the PKC inhibitors bisindoleylmaleimide I (Fig. 9*A*) or Gö6976 (Fig. 9*B*). No effect of these inhibitors on ERK phosphorylation was detectable at concentrations that blocked CREB phosphorylation (Fig. 1). Similar results were also obtained with C2C12 cells (Fig. 9*C*). These results and the observed co-immunoprecipitation of P-CREB with PKCα (Fig. 7*E*) strongly suggest that there is a direct effect of PKCα on CREB after depolarization.

PKC Inhibition Decreased c-fos mRNA Levels—Considering that PKC inhibition results in an important decrease in CREB phosphorylation, we tested whether PKC inhibitors affect the up-regulation of the early gene c-*fos* that follows depolarization of skeletal muscle cells in primary culture (9). Gö6976, a cPKC inhibitor that significantly decreased CREB phosphorylation both in primary culture skeletal muscle cells and in C2C12 cells (Figs. 1 and 4), also decreased c-*fos* mRNA levels detected by semi-quantitative reverse transcription-PCR, as previously described (9). The maximal stimulation with depolarization, obtained at 15 min of exposure to high K^+, was compared with that obtained in the presence of the inhibitor. The values (means ± S.E.), expressed as percentages of a normalized non-stimulated control cells (100%), were 211.7 ± 12.8% in control myotubes and 155.7 ± 20.7% in myotubes exposed to 1 μM Gö6976 ($n = 3$, $p < 0.05$).

DISCUSSION

Ca^{2+} release induced by depolarization of skeletal muscle cells activates many downstream effectors including transcription factors. In doing so, depolarization is linked to changes in gene expression (6, 8). In particular, a slow calcium transient, generated by IP_3R activation, has been shown to be required for both the activation of CREB and that of the early genes c-*fos*, c-*jun*, and *egr-1* (9). In these experiments, both CREB phosphorylation and up-regulation of early genes were found to be dependent on ERK1/2 activation (9). Here, we provide evidence showing that PKCα is also involved in CREB phosphorylation and in c-*fos* up-regulation triggered by depolarization and by the subsequently resulting slow calcium transient.

Neither CaMKs nor PKA were found to play a significant role in this process. Although we did not determine directly whether CaMK is activated in skeletal muscle cells by depolarization, previous results showed that KN-93 significantly reduced up-regulation of c-*fos* mRNA induced upon depolarization (9), suggesting that CaMK is likely to be activated under these experimental conditions.

CREB activation by depolarization in hippocampal neurons, on the other hand, is mediated by ERK1/2 and CaMKIV signaling pathways (10, 12, 14). The MAPK pathway, in particular, is thought to promote prolonged formation of P-CREB (14). In a different model, the neuroendocrine cell line PC12, depolarization-induced CREB phosphorylation is also mainly mediated by ERKs (10). PKA, a classical activator of CREB either directly or through the ERK pathway (23), does not participate in depolarization-induced CREB phosphorylation in the model of K^+-depolarized hippocampal neurons in culture (14). Recent observations indicate that synaptic activity in hippocampal neurons does not increase cAMP levels, even when large nuclear calcium signals and robust CREB-dependent transcription are observed (24).

To our knowledge, no reports describing a role for PKCs in

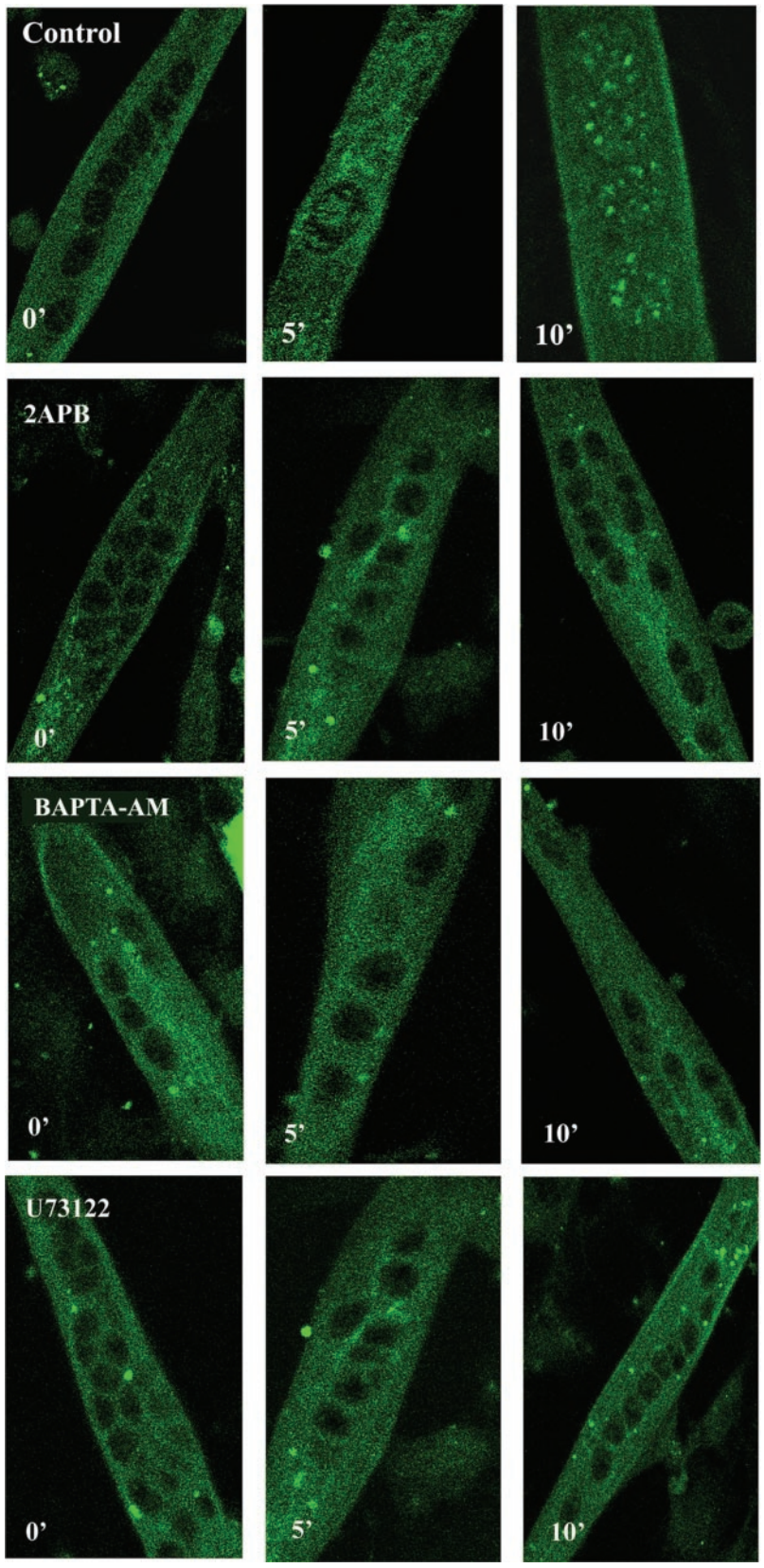

FIG. 8. **PKCα translocation induced by depolarization was inhibited by blocking the slow Ca^{2+} transient.** Immunofluorescence analysis of PKCα translocation in rat primary myotubes at different time points after depolarization, under control conditions, and in the presence of either 50 μM 2-aminoethoxydiphenyl borate, 100 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, or 10 μM U73122. PKCα translocation was not detectable 5–10 min after depolarization in the presence of any of the slow calcium transient blockers.

depolarization-induced CREB activation are available. However, PKC-dependent CREB phosphorylation in response to neurotransmitters has been described in hippocampal neurons (17), where CREB phosphorylation was observed upon stimulation via either muscarinic acetylcholine receptors or metabotropic glutamatergic receptors. The PKC effect in this case was predominantly mediated by the ERK pathway. In fact, several reports point to PKCs as key activators of the Raf/MAPK cascade in response to growth factors (25), and this mechanism was also shown to be present in chick skeletal muscle cells in response to 1α,25$(OH)_2$-vitamin D_3 (26). This is a relevant point in our cell system considering that ERKs are involved in

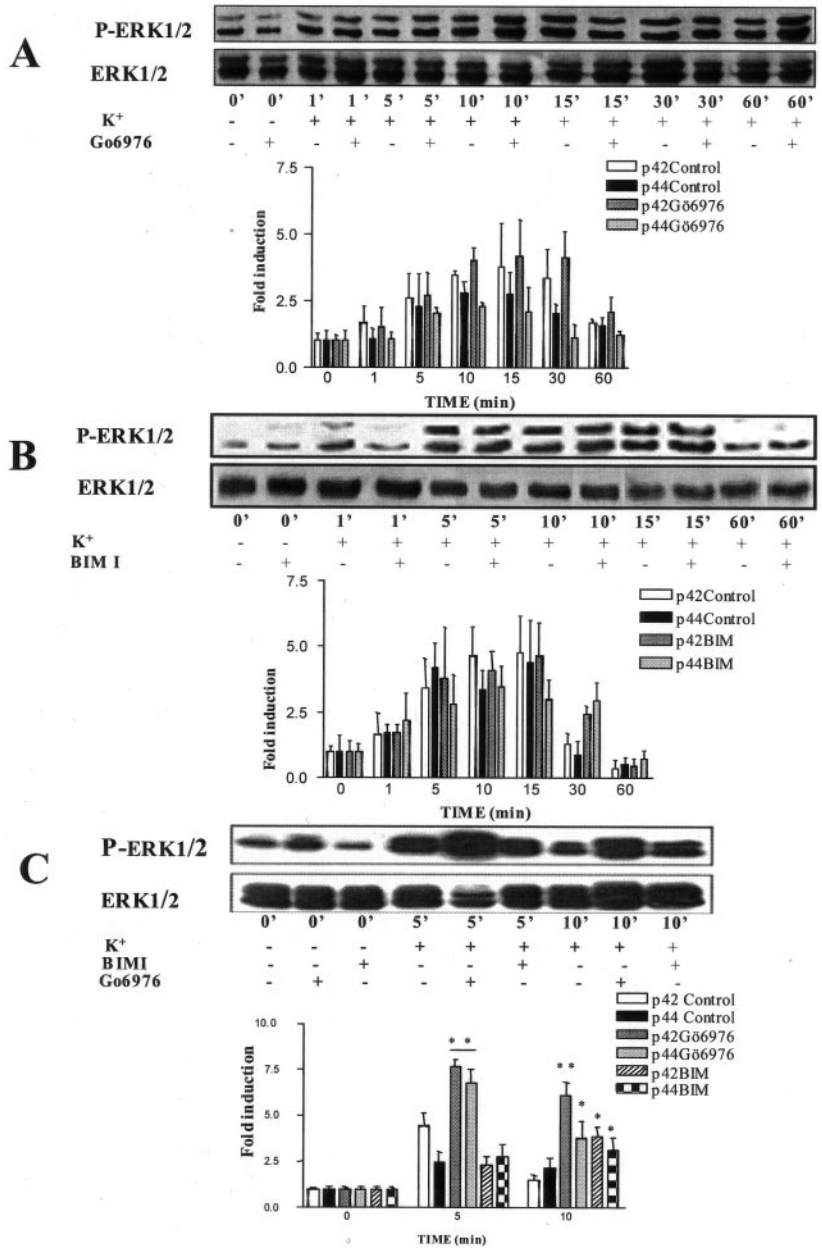

FIG. 9. **CREB phosphorylation induced by depolarization was independent of the MAPK/ERK1/ERK2 pathway.** *A*, *upper panel*, Western blot analysis of P-ERK1/2 or ERK2 as a loading control shows that the increased P-ERK1/2 levels observed following depolarization of rat primary myotubes were not affected by 2.5 μM bisindoleylmaleimide I. *B*, ERK1/2 activation was unaffected by Gö6976, a calcium-dependent PKC inhibitor. The cells were pretreated with or without 1 μM Gö6976 for 30 min and then depolarized in the absence or presence of the drug for the times indicated. *Upper panel*, representative Western blot is shown. *C*, C2C12 myotubes were pretreated with or without 2.5 μM BIM I or 1 μM Gö6976 for 30 min and then depolarized in the absence or presence of the inhibitors for the times indicated. *Upper panel*, representative immunoblotting data showing that phospho-ERK1/2 levels were not reduced by the PKC inhibitors. *Lower panels* in *A–C*, *bar graphs* represent P-ERK1 or P-ERK2/total ERK2 expressed as the average fold increase (mean ± S.E.) over basal levels (n = 3). No statistically significant differences were found between control and experimental condition (t test for paired data) in *A* and *B*. In *C*, bisindoleylmaleimide I induced a significant increase in ERK1/2 phosphorylation.

CREB phosphorylation (9). The results shown here provide evidence indicating that PKC-dependent CREB phosphorylation is not mediated by ERKs, suggesting that ERK- and PKC-dependent increases in CREB phosphorylation occur via independent pathways. First, PKC inhibition by either bisindoleylmaleimide I or Gö6976 did not decrease P-ERK levels in depolarized skeletal muscle cells; on the contrary, an increase was observed (Fig. 9). Second, both PKCα translocation to the nucleus and co-immunoprecipitation of P-CREB with PKCα further suggest that in depolarized skeletal muscle cells PKCα may directly interact with and phosphorylate CREB in the nucleus. In favor of this possibility, CREB has been shown to be phosphorylated by PKC *in vitro* (16). In a different but related model, activation of ERK1/2 by electrical stimulation of *ex vivo* rat skeletal muscle, was found not to be affected by PKC inhibition (27). It is also worth noting that participation of PKC in ERK activation has also not been detected in depolarized hippocampal neurons (28).

Indirect evidence on the independent PKC and ERK pathways converging to CREB comes from the results on inhibition of c-*fos* up-regulation induced by depolarization, obtained with cPKC inhibition. Although the decrease in P-CREB levels was comparable with both MAPK/ERK kinase (9) and PKC inhibitors (this work), the magnitude of the decrease in c-*fos* levels with both treatments was different. The results obtained in this work with Gö6976, represent a decrease of 50% in c-*fos* levels as compared with controls. When using UO126, a higher decrease (83%) was obtained (9).

Rat skeletal muscle cells in primary culture were found to express seven PKC isoforms belonging to three different subgroups. Similar results have already been described in rat, human, and chick skeletal muscle cells in primary culture (29–31). The highly significant decrease in CREB phosphorylation observed in the presence of PKC specific inhibitors (Fig. 1) but not with the PKA or CaMK inhibitors (Fig. 2) pointed toward PKCs as mediators of the response. In primary muscle cell cultures, complete down-regulation of PKCβI, PKCβII, PKCδ, and PKCθ was achieved upon prolonged TPA treatment. Surprisingly, PKCα levels were only reduced to 40%, and this residual PKCα level appeared sufficient to maintain CREB

phosphorylation in response to depolarization (Fig. 5). Marked differences in the responsiveness of phorbol ester-sensitive PKC isoforms to chronic stimulation has been reported in skeletal muscle cells (32). Identification of the PKC isoform involved in CREB phosphorylation was facilitated in C2C12 cells because only three isoforms were detected (α, ϵ, and ζ). PKCα was the only representative of the calcium-dependent subgroup expressed in these cells (Fig. 6). Taken together, these results strongly implicated cPKCα as the PKC-mediator involved in CREB phosphorylation in response to K^+-dependent muscle depolarization.

A large amount of data are available suggesting that PKC translocation to different subcellular locations occurs in response to physiological stimuli, including depolarization. In early experiments, stimulation of rat skeletal muscle gastrocnemius-plantaris-soleus via the sciatic nerve increased total PKC activity associated with the particulate fraction from 60 to 83% within 2 min of stimulation (33). More recently, confocal microscopy analysis of PKCα-GFP expressed in vascular smooth muscle cells revealed translocation of this protein from the cytosol primarily to the plasma membrane upon K^+-induced depolarization (34). Analysis of the response of this reporter to a number of stimuli indicated that local increases in calcium were responsible for targeting the protein to a variety of intracellular locations. Hence, localized changes in calcium levels determine PKCα-GFP distribution in these cells. Interestingly, PKCα targeting in this case depended both on the presence of the C2 as well as of a functional kinase domain (34). Likewise, analysis of PKCα-GFP translocation in A7r5 smooth muscle cells stimulated with a variety of agents, including phorbol esters, calcium mobilizing agents, and angiotensin II, indicated that the spatial and temporal characteristics of PKCα translocation may vary considerably (35). Furthermore, adenovirus-mediated overexpression of different PKC isoforms (α, βII, δ, ϵ, and ζ) in cardiomyocytes confirm the notion that individual isoforms translocate to distinct subcellular locations upon activation (36). Thus, localization of PKCs in muscle cells varies in both a stimulus- and isoform-dependent fashion, and such variations are likely to have important functional consequences.

In the experiments reported here we detected isoform-specific translocation of PKCα to the nucleus in response to skeletal muscle cell depolarization (Figs. 7 and 8). PKCα translocation to the nucleus has been described previously in several models (37). In muscle cells, perinuclear PKCα translocation occurs in cardiac myocytes after stimulation with norepinephrine or phorbol 12-myristate 13-acetate (38). Furthermore, PKCα activation and translocation to the nucleus is implicated in agonist-induced cardiomyocyte hypertrophy (36). In A7r5 smooth muscle cells phorbol ester stimulates nuclear PKCα localization (35), and translocation to such sites requires intact microtubules (39). However, depolarization-induced PKCα translocation to the nucleus in skeletal muscle cells has not been reported before.

Clearly, PKCα translocation is dependent on the previously reported slow calcium transient observed predominantly in the nuclear region (4), because both 2-aminoethoxydiphenyl borate and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid blocked this event. An increase in nuclear DAG also appears to be important for PKCα translocation. The mechanism potentially involved in DAG generation following depolarization of skeletal muscle cells remains to be determined. Preliminary experiments analyzing isolated nuclei from skeletal muscle cells in culture indicate that phosphatidylinositol 4,5-bisphosphate and PLCβI are present in the nucleus and, moreover, that there is an increase in IP_3 mass with cell stimulation.[2]

A phosphoinositide-PLC cycle that can generate IP_3 and DAG has been reported in several cell systems (40). In particular, a nuclear PLCβI has been demonstrated to form DAG in Swiss 3T3 cells in response to IGF-1, which does not affect PLC at the plasma membrane (41). Furthermore, a PLC inhibitor blocks both this response and nuclear translocation of PKCα. A potential role for activated nuclear PKCα could be to inhibit PLCβ activity in a negative feedback loop (42). Alternatively, nuclear PKCα may also promote DAG kinase ζ translocation from the nucleus to the cytosol and thereby prolong nuclear elevation of DAG levels as well as PKCα activation (43, 44).

PKC isoforms have been extensively studied in relation to their role in skeletal muscle proliferation and differentiation (30). A role for PKCs in gene expression regulated by skeletal muscle depolarization has been proposed to be involved in repression of nicotinic acetylcholine receptor expression in extrajunctional muscle by electrical activity. Recently, in chick skeletal muscle, PKCζ (atypical) was shown to participate in the pathway mediating such repression, in a complex mechanism also involving c-*jun* N-terminal kinase, and the transcription factor Sp1 (45). Sp1 phosphorylation, required to activate nicotinic acetylcholine receptor gene transcription, is thought to be prevented by upstream regulation of a kinase or phosphatase phosphorylated by c-*jun* N-terminal kinase or PKCζ. A different situation has been reported in mammalian muscle. In primary culture rat skeletal muscle, constitutively active PKCα or phorbol ester treatment suppressed nicotinic acetylcholine receptor gene expression, whereas the effect of electrical activity on gene expression was not mediated by PKCs (46). Therefore, PKCs would not be expected to play a physiological role in the activity-dependent regulation of this gene in mammalian muscle. Interestingly, in adult muscle fibers, activation of PKCs by muscarinic AChR is thought to be involved in regulation of the expression of slow myosin heavy chain isoform gene (47).

In this work we propose a role for PKCα in promoting transcription of c-*fos* through CREB phosphorylation. Transcription of this particular early response gene is regulated both by the CRE and by the SRE response elements (48). Interestingly, ERKs have been described to activate both cAMP response element- and serum response element-dependent transcription events (13). This observation may explain why in our current experiments, the increase in c-*fos* mRNA was only partially blocked (50%) by a PKC inhibitor (Gö6976) that almost completely blocked CREB phosphorylation (Figs. 1 and 4). Inhibition of ERKs, on the other hand, reduced c-*fos* expression by 83% (9). Taken together, these results suggest that increased c-*fos* transcription observed in response to muscle depolarization is mediated by at least two different pathways, one of which is PKCα-dependent.

Our results describing cPKCα involvement in depolarization-induced CREB phosphorylation can be summarized as follows. Skeletal muscle cell depolarization-induced Ca^{2+} increases at the nuclear level appeared to be mediated by the IP_3 system, as previously reported (6). Of the three cPKCs detected in the cytoplasm under resting conditions, only the α isoform was found in the nucleus at early time points after depolarization. CREB phosphorylation by cPKCα was not mediated by the ERK pathway but instead is more likely to be a direct effect of the cPKCα fraction that translocates to the nucleus upon stimulation. The mechanisms involved in the preferential activation of cPKCα rather than the isoforms βI or βII, in this particular cell model, require further investigation. Future

[2] C. Cárdenas, J. L. Liberona, and E. Jaimovich, unpublished data.

work will seek to unravel the precise role of cPKCα in depolarization-induced regulation of gene expression in muscle cells.

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