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Protein kinase C isoforms in muscle cells and their regulation by phorbol ester and calpain

Dong-hyun Hong [a,b,1,2], Jianya Huan [a,1], Bor-rung Ou [a,3], Jan-ying Yeh [a,3], Takaomi C. Saido [c], P.R. Cheeke [a], Neil E. Forsberg [a,*,4]

[a] *Department of Animal Sciences, Oregon State University, Corvallis, OR 97331-6702, USA*

[b] *Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331-6702, USA*

[c] *Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Tokyo 113, Japan*

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Abstract

Objectives were to identify the PKC isoforms in cultured muscle cells, to examine roles of Ca^{2+}-dependent proteinases (calpains) in processing of various muscle PKC isozymes and to obtain a mechanistic description of the processing of PKCs by examining the temporal relationships between phorbol ester-dependent translocation of muscle PKCs and calpains between cytosolic and membrane compartments. Using six isoform (α, β, γ, δ, ϵ, ζ)-specific polyclonal antibodies, PKC α, δ and ζ were detected in rat skeletal muscle and in L8 myoblasts and myotubes. PKC α and ζ were primarily localized in the cytosolic fraction of L8 myotubes whereas PKC δ was more abundant in the membrane fraction. Phorbol ester (TPA) caused rapid depletion of myotube PKC α and PKC δ isoforms from the cytosolic compartment and rapid appearance of these isoforms in the membrane fraction. However, long-term exposure of myotubes to TPA eventually caused down-regulation of PKCs in the membrane compartment. Down-regulation of PKCs in the membrane fraction was partially blocked by calpain inhibitor II. However, the rapid TPA-dependent cytosolic depletion of PKCs was unaffected by calpain inhibitor. This suggests that calpains may be responsible for membrane-associated down-regulation of PKCs but not for cytosolic depletion. In the final study we assessed the effects of phorbol ester on compartmentation of m-calpain with PKCs in muscle cells. Like the PKCs, TPA caused rapid association of m-calpain with the membrane fraction followed by down-regulation. This demonstrates that phorbol esters cause translocation of both PKCs and calpains to membranes where processing of PKCs may occur via the limited proteolysis exerted by calpains.

Keywords: Protein kinase C; Calpain; Phorbol ester; Muscle cell

1. Introduction

Protein kinase C (PKC), a serine/threonine kinase, is thought to play important roles in the control of a wide range of cellular processes, which include secretion, contraction, membrane receptor function, cell differentiation and tumor promotion [1,2]. Protein kinase C is activated by Ca^{2+} and diacylglycerol, which are generated from the receptor-mediated hydrolysis of membrane phospholipids (PL). Molecular cloning studies revealed that the PKC gene family contains at least ten different isozymes that can be divided into two or three major groups [3–6]. The first group consists of four conventional PKCs (cPKC): α, βI, βII and γ. The cPKC isoforms are activated by Ca^{2+}, phosphatidylserine and diacylglycerol or phorbol ester (PE). The second group consists of four novel PKCs (nPKC): δ, ϵ, η(L), and θ which are activated by phosphatidylserine and diacylglycerol or PE but not by Ca^{2+}. A third group consists of two atypical PKCs (aPKC): ζ and λ. aPKCs require only phosphatidylserine for activation. Because the individual isozymes have different cofactor requirements, subcellular distribution and substrate specificity, the profile of PKC isoforms expressed in a particular tissue is likely an important determinant of that tissue's

* Corresponding author. Fax: +1 (503) 7374174.

[1] These two authors (D.H.H. and J.H.) contributed equally to the project.

[2] Present address: Department of Pediatrics, University of Toronoto Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8.

[3] Current address: Department of Animal Science, Tunghai University, Taichung, Taiwan, R.O.C.

[4] Reprint requests

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response to an endocrine challenge. Differential tissue distribution of PKC isoforms have been reported by many authors [7–10].

Calpains are Ca^{2+}-activated neutral proteinases expressed as house-keeping proteinases. Two isoforms exist in most tissues (μ- and m-calpain) and house-keeping functions include cytoskeletal protein turnover, receptor processing and processing and/or activation of protein kinase C isoforms [3,11–13]. A membrane-activation model for the calpains was proposed by Mellgren [14] and Suzuki et al. [15] in which calpains exist normally as inactive cytosolic pro-enzymes but, in response to an increase in cell Ca^{2+}, calpains translocate to the cell membrane where they undergo autolysis. Hence, membrane-bound proteins are believed to be ideal calpain substrates. Once at the membrane, calpain activities are modulated by phospholipids [15].

In skeletal muscle, PKCs and calpains play key roles in differentiation and growth processes. PKC has been implicated in hypertrophy [16], contraction-induced muscle metabolism [17], glucose transport [18], myoblast differentiation [19,20] and proliferation [21]. We have determined that TPA increased muscle protein degradation [5] in myotubes. However, very little is known about the PKC isoforms in muscle, their regulation and individual functions. Although calpains may play a key role in control of myofibrillar protein degradation [11], their roles in the processing and activation of muscle PKCs have not been studied. In this study, we characterized the isoenzymes of PKC, their subcellular distribution in skeletal muscle and their differential down-regulation by phorbol ester (TPA). Furthermore, we investigated the role that calpains play in the TPA-dependent down-regulation of muscle PKCs. These studies demonstrate that phorbol esters not only induce translocation of the PKCs to membranes but also coordinate translocation of the protease responsible for their processing to the same compartment.

2. Materials and methods

2.1. Materials

Fetal bovine (FBS) and horse (HS) sera were from Hyclone (Logan, UT). Dulbecco's modified Eagle's medium, penicillin/streptomycin solution, trypsin solution, and affinity-purified anti-PKC isozyme-specific (α, β, γ, δ, ϵ, and ζ) antibodies were from GIBCO (Grand Island, NY). Calpain inhibitor II and leupeptin were from Boehringer Mannheim (Indianapolis, IN). Cytosine-β-D-arabinofuranoside (Ara-C), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), dimethyl sulfoxide and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Hybond-C Super and Hybond-ECL nitrocellulose membranes, horseradish peroxidase (HRP) conjugated donkey anti-rabbit IgG and ECL Western blotting analysis system were from Amersham (Arlington Heights, IL). Alkaline phosphatase (AP) conjugated goat anti-rabbit (GAR) IgG, alkaline phosphatase conjugate substrate kit, SDS-PAGE low molecular weight standard, and Bio-Rad protein assay dye reagent were from Bio-Rad (Richmond, CA). Culture dishes were from Corning (Corning, New York). Ca^{2+}-channel blockers, nifedipine and ryanodine, were obtained from Calbiochem Corporation (San Diego, CA).

2.2. Cell culture

L8 rat skeletal muscle cells were obtained from American Type Culture Collection (Rockville, MD). Cells, stored in liquid nitrogen, were thawed and maintained by repeated sub-culturing at low density on 10-cm culture dishes. Cells were grown in Dulbecco's-modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 44 mM $NaHCO_3$, 110 μg of sodium pyruvate/ml in a humidified atmosphere of 5% CO_2 and 95% air at 37° C. The medium was changed every 2 days. Cells (2.5×10^4 cells/cm^2) were grown in the presence of 10% FBS until they reached confluency. At this point the medium was replaced by DMEM containing 2% HS to induce differentiation. Approximately 3 days later, when myotube formation was observed, the cells were treated with 10 μM cytosine arabinoside for 48 h to remove any remaining dividing myoblasts. Microscopic examination was used to monitor differentiation. All studies were conducted at times during which 90–95% of all nuclei were myotubular. After the appropriate incubation time, cells were harvested for Western blotting analysis.

2.3. Preparation of m-calpain antibody

An antipeptidic antibody against the amino-terminal 18-mer segment of rat m-calpain was developed using a synthetic peptide, AGIAAKLAKDREAAEGLGC, conjugated to keyhole limpet hemocyanin and affinity-purified as previously described [22]. This antibody was designed to specifically recognize native (pre-autolysis 80 kDa isoform) m-calpain [22]. The antibody binds only to 80 kDa m-calpain and not to other calpain (μ-) isoforms and is sensitive enough to detect its antigen in crude cell lysates [22]. This antibody has also been used in previous studies to monitor the autolysis of m-calpain in intact cells with concomitant proteolysis of calpain substrate [22].

2.4. Preparation of tissue and cell extracts for Western blotting

The following method was used for extraction of total PKC from tissues and cultured cells for preliminary studies

[5] Hong and Forsberg, unpublished observations

on PKC isoforms in muscle tissues and brain. Male rats were killed by cervical dislocation and the brain and muscle were immediately removed and homogenized in ice-cold Buffer A (20 mM Tris-HCl [pH 7.5], 0.25 M sucrose, 50 mM β-mercaptoethanol, 1 mM PMSF, 200 μg/ml of leupeptin, 5 mM EDTA, 2 mM EGTA, 10 mM benzamidine, 0.2% Triton X-100) using a Polytron. L8 myotubes and myoblasts were prepared by washing cells with ice-cold PBS, after which they were scraped from their plates in Buffer A and homogenized in this buffer by sonication. Homogenates were kept at 4° C for 1 h then centrifuged at 100 000 × g for 30 min at 4° C. The supernatant was either used immediately or frozen at −80° C for SDS-PAGE. Protein concentration was determined by the method of Bradford [23].

The following method was used for isolation of membrane and cytosolic fractions from cultured L8 myotubes [24]. Myotubes were recovered from culture plates (by washing with ice-cold PBS followed by scraping) following various treatments with either phorbol ester or calpain inhibitor and homogenized in Buffer B (Buffer A lacking Triton X-100) using a Dounce homogenizer. Cells were centrifuged at 100 000 × g for 20 min at 4° C. Supernatants were used as the cytosolic fraction. To obtain membrane fractions, L8 cell pellets were washed and extracted with Buffer B containing 1% Triton X-100. After 30 min incubation at 4° C, the samples were centrifuged at 12 000 × g for 20 min yielding the solubilized membrane fraction. Protein concentrations of individual samples were determined by the method of Bradford [23]. Details of individual studies are presented in text and legends to figures.

2.5. Western blot analysis

Protein samples were subjected to 10% SDS-PAGE according to the method of Laemmli [25]. The separated proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-C Super or Hybond-ECL) at 4° C overnight using a Bio-Rad Transfer Blot apparatus (30 mAmp) by the method of Towbin et al. [26]. Nonspecific sites were blocked by incubation of nitrocellulose membranes with 2% BSA in TBST (50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween-20) for 1 h at room temperature. Anti-PKC antibodies or m-calpain pAb were incubated with the membranes for 2 h at room temperature. The antibody dilutions were as follows: α, 1:333; β, 1:333; γ, 1:333; δ, 1:200; ϵ, 1:333; ζ, 1:200; and m-calpain 1:500. After three washes (15 min) with TBST, secondary antibody (AP-conjugated goat anti-rabbit IgG, at 1:3000 dilution; or HRP-conjugated donkey anti-rabbit IgG, at 1:2000) was added and incubated for 1 h at room

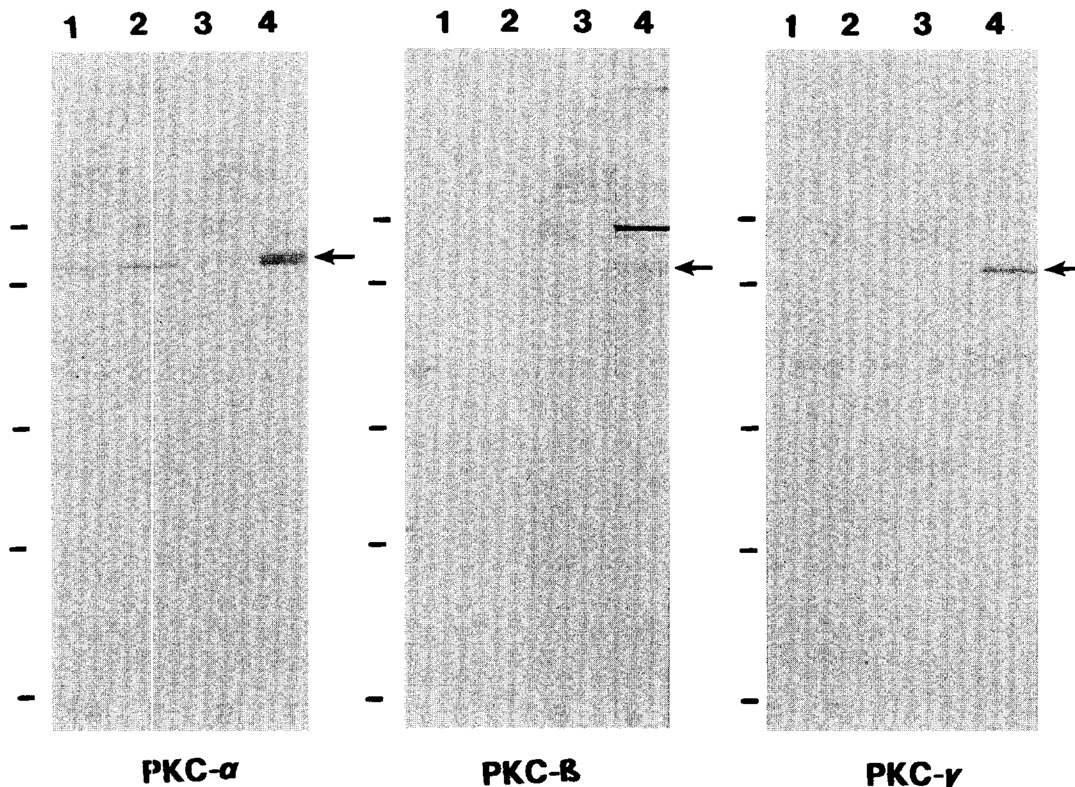

Fig. 1. Western blot analysis of protein kinase C isozymes (α, β, and γ) in muscle cytosol. Protein samples (100 μg) from rat L8 myoblasts (lane 1), L8 myotubes (lane 2), rat skeletal muscle (lane 3) and rat brain (100 μg for α and δ, 50 μg for β; lane 4) were subjected to Western blot analysis as described in Section 2. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, 31.0 and 21.5 kDa. Arrows indicate positions of PKC isozymes.

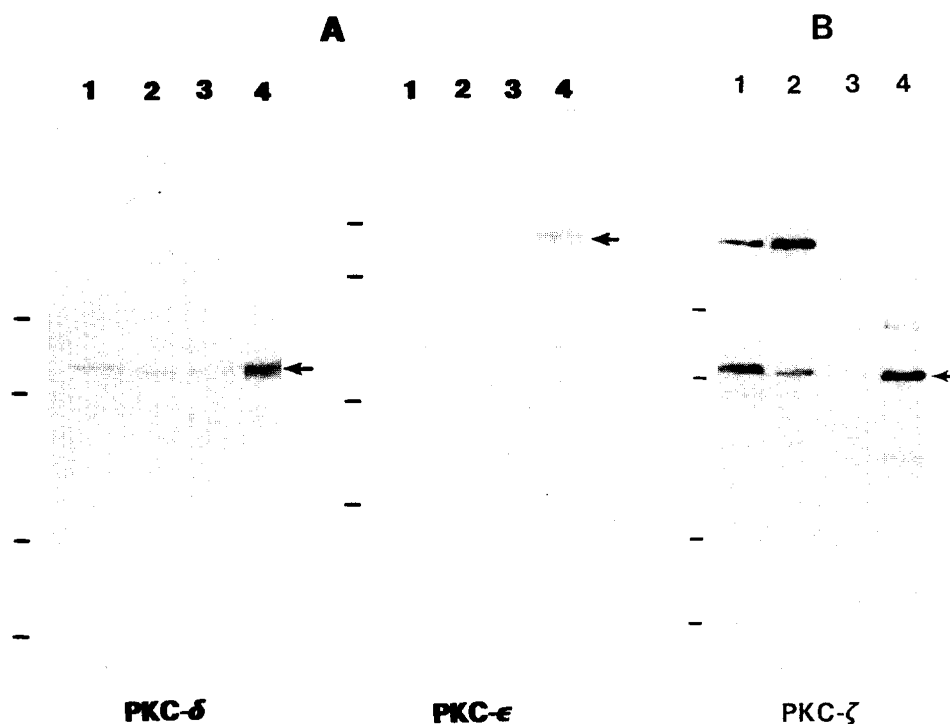

Fig. 2. Western blot analysis of protein kinase C isozymes (δ and ε; panel A, and ζ; panel B) in muscle cytosol. Protein samples from rat L8 myoblasts (lane 1), L8 myotubes (lane 2), rat skeletal muscle (lane 3) and rat brain (lane 4) were subjected to Western blot analysis as described in Section 2. For PKC δ, ε and ζ, 150, 100 and 150 μg of protein were electrophoresed, respectively. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7 and 31.0 kDa. Blots on panel A were processed using the alkaline phosphatase detection system. Panel B was developed using the more sensitive ECL detection system. Arrows indicate positions of PKC isozymes.

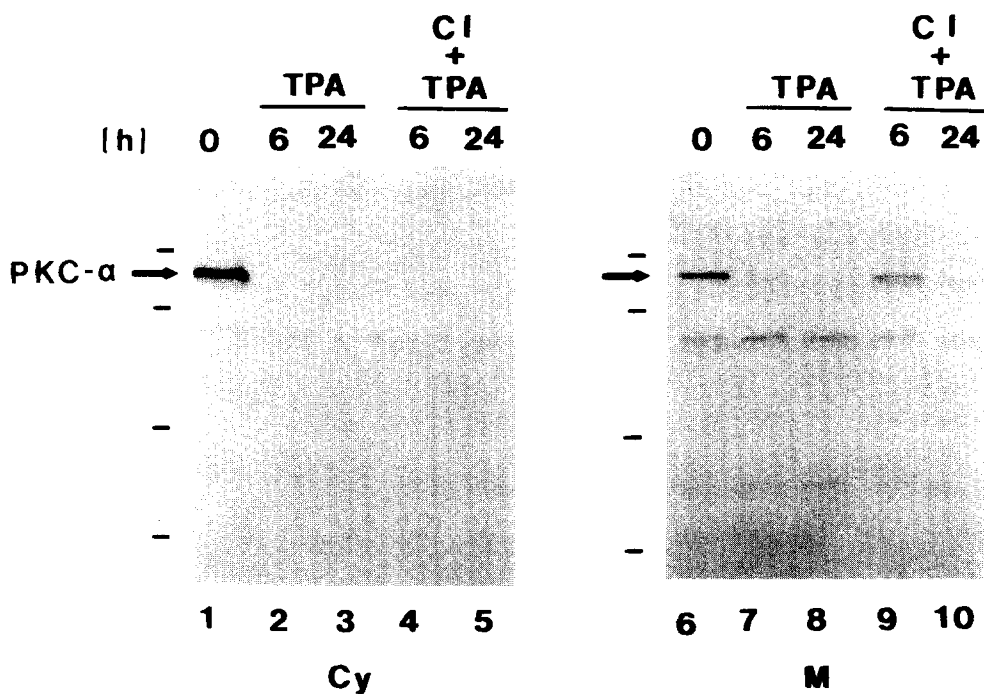

Fig. 3. Effects of calpain inhibitor II on TPA-induced down-regulation of PKC-α in L8 myotubes. L8 myotubes were treated with 300 ng/ml TPA (phorbol ester) in the absence or presence of 100 nM calpain inhibitor II (CI; *N*-acetyl-leucyl-leucyl-methioninal) for 0, 6 or 24 h. Cytosolic (Cy; lanes 1–5) and membrane (M; lanes 6–10) fractions were prepared for Western blot analysis. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, and 31.0 kDa. Arrows indicate positions of PKC isozymes. 250 μg of protein were loaded per lane.

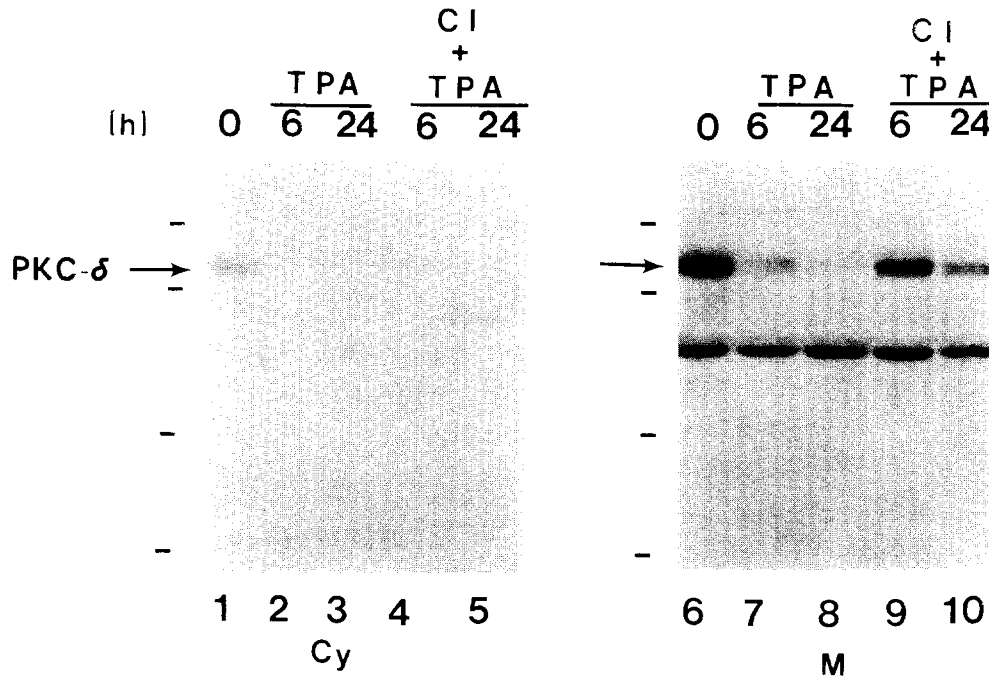

Fig. 4. Effects of calpain inhibitor II on TPA-induced down-regulation of PKC-δ in L8 myotubes. L8 myotubes were treated with 300 ng/ml TPA in the absence or presence of 100 nM calpain inhibitor II (CI; *N*-acetyl-leucyl-leucyl-methioninal) for 0, 6 or 24 h. Cytosolic (Cy; lanes 1–5) and membrane (M; lanes 6–10) fractions were prepared for Western blot analysis. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2, 42.7, and 31.0 kDa. Arrows indicate positions of PKC isozymes. 150 μg of protein were loaded per lane.

temperature. Following this incubation, the membrane was washed (15 min) three times with TBST. Specific binding of anti-PKC or anti-m-calpain antibodies was detected by either the alkaline phosphatase detection kit (Bio-Rad) or the ECL detection system (Amersham). Where necessary, blots were scanned using a Hewlett-Packard Scan-Jet II CX scanning densitometer using NIH Image Software (Version 1.54) and a Power MacIntosh (Model 7100/66).

2.6. Studies with Ca^{2+}-channel blockers.

Effects of phorbol ester on translocation of calpains in the presence and absence of Ca^{2+} channel blockers was investigated. Blockers included nifedipine (1–10 μM) and ryanodine (5–50 μM). Nifedipine was dissolved in ethanol then added to culture media at either 5 or 10 min prior to additions of phorbol ester. Ryanodine was dissolved in

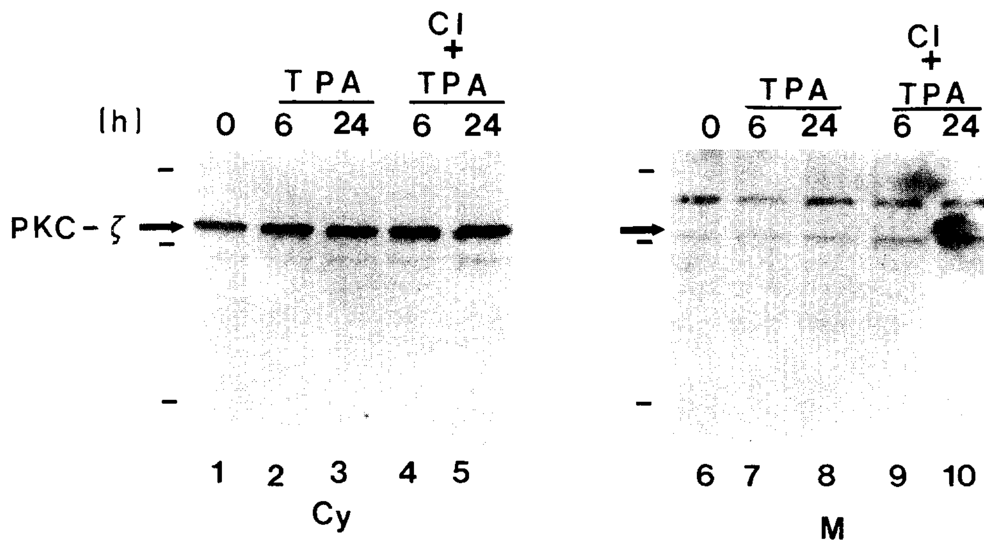

Fig. 5. Effects of calpain inhibitor II on TPA-induced down-regulation of PKC-ζ in L8 myotubes. L8 myotubes were treated with 300 ng/ml TPA in the absence or presence of 100 nM calpain inhibitor II (CI; *N*-acetyl-leucyl-leucyl-methioninal) for 0, 6 or 24 h. Cytosolic (Cy; lanes 1–5) and membrane (M; lanes 6–10) fractions were prepared for Western blot analysis. Positions of molecular weight markers are shown on the left: (from the top) 97.4, 66.2 and 42.7 kDa. Arrows indicate positions of PKC isozymes. 150 μg of protein were loaded per lane.

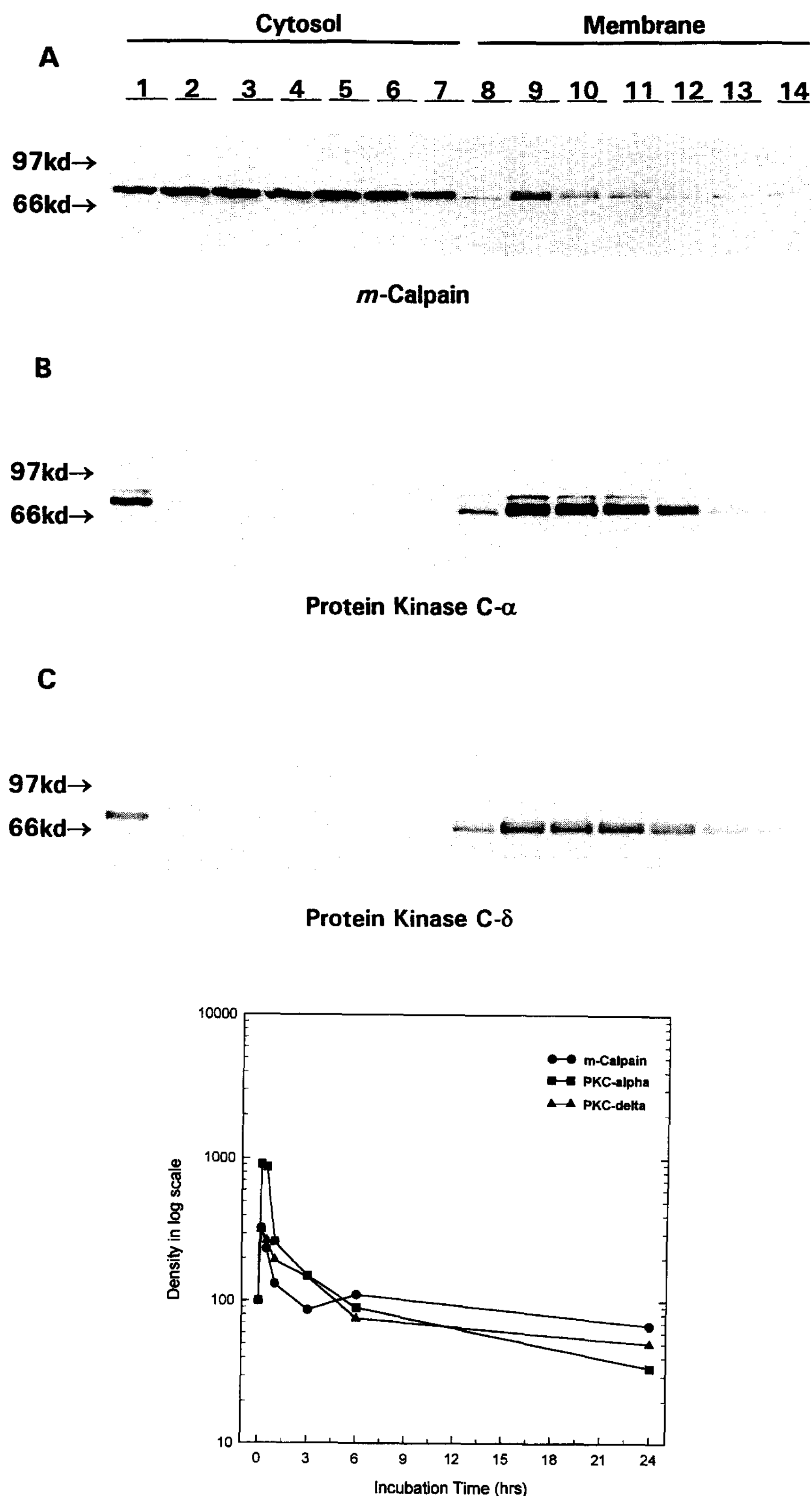

Fig. 6. Effects of TPA (300 ng/ml) on cytosolic and membrane-bound m-calpain (panel A), PKC α (panel B) and PKC δ (panel C). TPA was added to cultures of myotubes for 0 min (lanes 1 and 8), 10 min (lanes 2 and 9), 30 min (lanes 3 and 10), 1 h (lanes 4 and 11), 3 h (lanes 5 and 12), 6 h (lanes 6 and 13) or 24 h (lanes 7 and 14) after which concentrations of m-calpain and PKCs were determined by Western blot analysis. Lanes 1–7 correspond to the cytosolic fraction and lanes 8–14 correspond to the membrane fraction. Western blotting was completed as outlined in Section 2. 100 μg of protein were loaded per lane. Molecular weight markers of 66 kDa and 97 kDa are indicated. In this and following figures, myotubes were maintained in 2% HS and SDS-PAGE was performed on 7.5% SDS-PAGE gels. Scanning densitometry of data presented in the lower panel. For comparative purposes, values are expressed as a proportion of initial (unstimulated) antigens associated with the membrane fraction.

sterile water prior to being added to culture media at either 5 or 10 min prior to additions of phorbol ester. Effects of TPA on m-calpain compartmentation were examined following 2 min of exposure to TPA.

3. Results

3.1. PKC isoforms in muscle

We used affinity-purified isozyme-specific polyclonal antibodies to detect PKC α, β, γ, δ, ϵ and ζ isoforms in extracts from rat L8 myoblasts and L8 myotubes, skeletal muscle and brain. Extracts from rat brain were used as a positive control (Figs. 1 and 2). We detected PKC α (Fig. 1), PKC δ (Fig. 2A) and PKC ζ (Fig. 2B) in all muscle samples. However, PKC ζ concentration in adult muscle was very low. PKCs β, γ and ϵ were not detectable in the muscle samples. The apparent molecular weight of both PKC α and PKC δ was 80 kDa. The PKC ζ isozyme-specific antibody recognized a predominant 70 kDa protein in rat brain plus two uncharacterized proteins of 85 and 52 kDa. In myotubes, myoblasts and adult muscle, the PKC ζ antibody recognized a 70 kDa protein (PKC ζ) plus a larger protein (> 100 kDa). PKC ζ concentration in L8 myotubes and myoblasts and brain was much higher than that detected in adult skeletal muscle.

3.2. Subcellular distribution and down-regulation of PKC isoforms by TPA

Next we examined the subcellular distribution of three PKC isoforms in L8 myotubes and their response to TPA (Figs. 3, 4 and 5). TPA was administered either for 6 or 24 h. Phorbol ester reduced concentrations of myotube PKC α and PKC δ (Figs. 3 and 4) in both cytosolic and membrane fractions. PKC ζ, in both cytosolic and membrane fractions, was unaffected by TPA (Fig. 5). Although PKC α was depleted in the cytosolic fraction, trace amounts of PKC α were detected in the membrane fraction of L8 myotubes following both 6 and 24 h exposure to TPA (Fig. 3). Six and 24 h of TPA treatment eliminated PKC δ from the cytosolic compartment; however, trace quantities of this isozyme also remained associated with the membrane fraction following both 6 and 24 h exposure to TPA (Fig. 4). The TPA-dependent down-regulation of PKC α and PKC δ in the membrane fraction was partially blocked by calpain inhibitor II (Figs. 3 and 4). However, this inhibitor did not block the TPA-dependent translocation of the α and δ isozymes from the cytosolic fraction to the membrane fraction. PKC ζ, which does not bind TPA [3], was neither translocated nor down-regulated by TPA treatment and was unresponsive to calpain inhibitor II treatment (Fig. 5).

Because calpains may process and activate PKCs, we hypothesized that phorbol esters may also bring about translocation of calpains to membranes to facilitate this event. Hence, we investigated the temporal effects of TPA on the compartmentation of the 80 kDa m-calpain large subunit, PKC α and PKC δ in myotubes (Fig. 6A–C). Treatment of myotubes with TPA did not affect cytosolic m-calpain concentration but caused a several-fold accumulation of membrane-associated m-calpain (Fig. 6A). The effect of TPA on membrane m-calpain was maximal following 10 min of exposure after which membrane m-calpain concentration declined slowly with time. Ten min of TPA treatment were sufficient to deplete cytosolic PKC α and PKC δ (Fig. 6B) and this depletion was maintained for the 24-h duration of the study. Translocation of PKCs to the membrane fraction was complete within 10 min. Following 1 h of TPA treatment, membrane PKC concentrations began to decline. Of interest, m-calpain was cleared from the membrane fraction at a rate slightly faster than PKC α clearance (Fig. 6A). Phorbol ester-dependent down-regulation of membrane PKC δ, however, lagged behind the down-regulation of membrane PKC α and m-calpain.

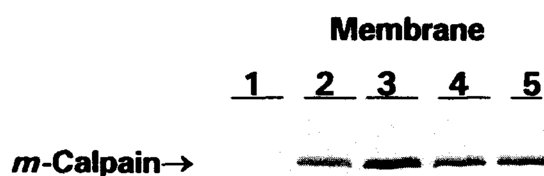

Fig. 7. Effects of calcium channel blockers on TPA-dependent translocation of m-calpain to the membrane fraction of L8 myotubes. The treatments included no pre-treatment (lane 1), TPA (300 ng/ml) for 2 min (lane 2), pre-treatment with 50 μM ryanodine for 10 min followed by addition of TPA for 2 min (lane 3), pre-treatment with nifedipine (10 μM) for 10 min followed by TPA (lane 4) and pre-treatment with both ryanodine and nifedipine followed by TPA (lane 5). Following TPA treatment, the membrane fraction was recovered and membrane-associated m-calpain concentration was determined by Western blotting.

To determine whether the TPA-dependent translocation of m-calpain to membranes was dependent upon sarcoplasmic- or voltage-dependent Ca^{2+}-channels, we tested ability of TPA to cause translocation of the 80 kDa m-calpain large subunit in the presence and absence of two Ca^{2+} channels blockers (nifedipine and ryanodine, respectively; Fig. 7). Again, phorbol ester caused translocation of m-calpain to the membrane fraction. However, nifedipine and ryanodine, added alone or in combination, did not interfere with TPA-dependent translocation of m-calpain.

4. Discussion

In this study, we demonstrated the presence of Ca^{2+}- and PL-dependent PKC α, Ca^{2+}-independent but PL-dependent PKC δ and Ca^{2+}- and PL-independent PKC ζ in

L8 myoblasts, L8 myotubes and rat adult muscle. PKCs α, δ and ζ were present in both undifferentiated and differentiated L8 muscle cells although the PKC ζ concentration, in adult muscle, was very low. These findings confirm other studies which reported PKC α [27] and PKC δ [28] in muscle. The presence of PKC β in muscle was reported by Hanaina et al. [29] and Nakano et al. [30]. However, we were unable to detect PKC β in myoblasts, myotubes or rat muscle even when loading much higher quantities of protein on gels. Perhaps only a small amount of PKC β is expressed in skeletal muscle. The quantitative immunoblotting by Yoshida et al. [31] indicated that PKC β in muscle was very low compared other tissues. We detected a 70 kDa single band with the PKC ζ antibody in muscle cells, whereas a 70- and 85 kDa doublet were detected in brain. These results are consistent with previous findings where multiple immunoreactive PKC ζs were detected in other tissues [18,32,33]. The lower molecular weight bands found in blots of PKC α and PKC δ (Figs. 3 and 4) may represent non-specific binding of the pAbs because phorbol ester had no effect on the cellular distributions or concentrations of these proteins. Alternatively, the low MW bands may be processed components of PKC which possess the epitope but are not responsive to phorbol ester.

A feature of the regulation of PL-dependent PKCs is their translocation to membranes following interaction with phorbol ester [3,10,34]. We studied this in detail in muscle cells using TPA: a diacylglycerol analog. The membrane fraction in this study consisted principally of sarcolemma but also included other membranous fractions including mitochondrial and sarcoplasmic reticulum. PKC α and PKC δ were translocated to membranes by TPA; however, the PKC ζ isozyme did not translocate or down-regulate in response to TPA. In contrast, Borner et al. [34] reported the translocation of rat fibroblast PKC ζ by phorbol ester. However, most studies indicate that PKC ζ is resistant to TPA-induced translocation or down-regulation [35–37].

Our results revealed two distinct TPA-dependent processes involved in control of muscle PKCs: one of which was calpain-independent, the second of which we suggest is calpain-dependent. Treatment of muscle cells with phorbol ester first caused the rapid translocation of PKC α and PKC δ from the cytosol to membranes. Two lines of evidence suggest that the disappearance of the α and δ isoforms from the cytosol was not the result of proteolysis. First, disappearance of PKCs from this pool was accompanied by concomitant appearance of immunoreactive proteins of the same molecular weights in the membrane fraction. Second, inhibition of calpains, the enzymes which appear to be responsible for processing of PKCs, by addition of calpain inhibitor II did not prevent the disappearance of PKCs from the cytosolic compartment.

The second effect of phorbol ester was to effect down-regulation of membrane PKCs. Two lines of evidence support the theory that calpains mediated this phenomenon. First, calpain inhibitor reduced the ability of TPA to deplete membrane PKCs. Second, native (80 kDa) m-calpain translocated to the membrane fraction in response to TPA and was eliminated from the membrane fraction in advance of the down-regulation of PKCs α and δ. Because our calpain antibody recognized only the intact 80 kDa pro-m-calpain, the loss of calpain immunoreactivity from the membrane prior to the loss of the PKCs may indicate the necessity for m-calpain to be proteolytically processed and activated prior to the processing and down-regulation of membrane-associated PKCs. The absence of immunoreactive PKC fragments in the cytosolic and membrane fractions following extended treatment with TPA suggests that the proteolyzed PKC is rapidly degraded.

The limitation of these studies is that calpain inhibitor II is not a fully selective inhibitor. It also inhibits the multicatalytic proteinase (MCP: proteasome) [38] and other cysteine proteinases including the lysosomal cysteine proteases. However, in this study the concentration of calpain inhibitor II used was less than that which is required to inhibit MCP [38]. Hence, it is very unlikely that calpain inhibitor II reduced the TPA-dependent down-regulation of PKC by inhibiting the MCP. Further, lysosomes typically do not degrade membrane-associated proteins, whereas calpains do. Our observations that a portion of m-calpain translocated to the membrane fraction and the considerable evidence that calpains degrade membrane-associated proteins including PKCs [3,39,40] causes us to suggest that calpain inhibitor II exerted its actions via the calpains.

The reason why cytosolic m-calpain concentration did not change despite the rapid appearance of immunoreactive m-calpain at the membrane is not clear. In other studies (data not shown) we verified that 6 and 24 h of TPA treatment did not change L8 myotube total m-calpain subunit concentration. Earlier studies [41] have determined that a TPA-responsive element (TRE) resides in the human m-calpain promoter and that TPA increases m-calpain transcription. Based on this it is possible that TPA rapidly increases synthesis of m-calpain in muscle cells in order to maintain fixed levels of this protease in the cytosol. Alternatively, there may simply exist an abundance of cytosolic m-calpain in unstimulated myotubes and the portion of m-calpain which is translocated to membranes in response to TPA represents only a small portion of this total. If true, this would leave the majority of m-calpain in an inactive cytosolic pool available for use for other housekeeping functions.

The mechanism by which phorbol esters induce down-regulation of membrane PKC is not fully understood. Savart et al. [13] suggested that TPA binding to the regulatory domains of PKCs (C1 region) may cause a conformational change in PKC, increasing its affinity for calpain and allowing its proteolysis at physiological concentrations of Ca^{2+}. A recent circular dichroism study showed that binding of TPA to PKC caused marked changes in secondary structure [42]. This may explain why

PKC ζ, which lacks TPA binding sites, is not down-regulated by TPA.

Earlier studies by Pontremoli et al. [43] have shown that phorbol esters induce translocation of calpain to neutrophil membranes. We found a similar phenomenon in cultured muscle cells and propose that a coordinated transfer of PKCs with their processing protease to membranes may represent a mechanism for PKC activation and/or degradation in muscle. However, the mechanism by which TPA caused translocation of m-calpain to the membrane fraction is not clear. The 'Mellgren/Suzuki model' proposes that an increase in cell Ca^{2+} concentration brings about calpain translocation to membranes [14,15]. Activation of PKCs can bring about changes in cell Ca^{2+} concentration [19,42,44]. Hence, the ability of TPA to cause calpain translocation could be direct (i.e., TPA may bind to calpain and thereby induce m-calpain translocation) or indirect (i.e., TPA may increase cytosolic Ca^{2+}). To test the second possibility, we examined the ability of TPA to induce m-calpain translocation following treatment of myotubes with nifedipine and ryanodine administered alone or in combination. Nifedipine is a selective blocker of cellular voltage-operated Ca^{2+}-channels. Ryanodine is an inhibitor of Ca^{2+} efflux from the sarcoplasmic reticulum. However, neither of these inhibitors interfered with the ability of TPA to cause translocation of m-calpain to membranes. Hence, the mechanism by which TPA induces translocation of m-calpain to membranes is unresolved.

Based on these and others' results, we present the model shown in Fig. 8 for PKC α and δ activation in muscle. This model builds upon an earlier model we proposed for corpus luteum [45]. Briefly stated, activation of phospholipase C by ligand releases two second messengers: IP_3 and diacylglycerol. While IP_3 releases intracellular Ca^{2+}, diacylglycerol acts directly upon PKCs α and δ and induces their translocation to the membrane fraction. Simultaneously, DAG, either directly or indirectly, causes the translocation of m-calpain to membranes. There, m-calpain, in the presence of sufficient Ca^{2+}, undergoes autolysis and may process PKC. This may either activate PKC (i.e., generating a constitutively-active PKM) or may represent the preliminary step in muscle PKC degradation. The unique elements of this model which were identified in this study include the identification of PKC isoforms in muscle, their translocation with m-calpain to membranes in response to phorbol ester and the processing of muscle PKC α and δ in membrane fractions by calpain.

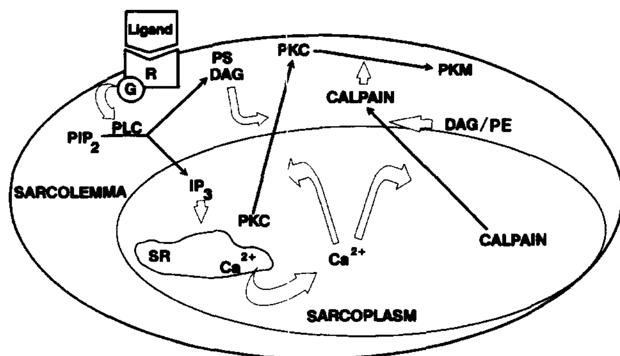

Fig. 8. Model for the relationship between PKC α and δ and calpain in muscle cells. Binding of ligand to its receptor (R) causes hydrolysis of phosphotidyl-inositol-4,5-bisphosphate (PIP_2) by membrane phospholipase C (PLC). This generates two second messengers: diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 causes release of Ca^{2+} from sarcoplasmic reticulum (SR). Phorbol ester (PE) or diacylglycerol (DAG) cause translocation of PKC α and δ and m-calpain to the membrane fraction. There, the newly-released Ca^{2+} regulates membrane-bound calpain activities which cause processing of PKC α and δ. This processing either activates PKC (by generating a PKC metabolite (PKM) which is constitutively active) or represents the first step in PKC degradation and inactivation. Phosphotidylserine (PS) and the newly-released Ca^{2+} also regulate PKC translocation and activity. This figure was adapted from our earlier work [45].

In conclusion, muscle cells contain at least three PKC isoforms (α, γ and ζ). Their presence is not dependent on differentiation. These isoforms permit three different signal transduction pathways to exist in muscle: Ca^{2+}- and PL-dependent (PKC α), Ca^{2+}-independent but PL-dependent (PKC δ) and Ca^{2+}- and PL-independent (PKC ζ). This diversity explains the wide range of functions in muscle that have been attributed to PKC. As in other cells, the α and δ isoforms, but not the ζ isoform, are translocated and down-regulated by TPA and are substrates for membrane-bound calpain. Of particular interest, translocation of PKCs with their putative processing proteinase is coordinated by phorbol ester. These observations point to a highly coordinately movement of signal transduction machinery to the membrane upon myotube stimulation. It is likely that these responses underlie many aspects of muscle cell physiology and it is now critical that we identify which hormones rely upon these transduction phenomena to coordinate their cellular events and to identify the cytosolic and nuclear events which follow PKC isoform activation and down-regulation.

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