Sequential activation of individual PKC isozymes in integrin-mediated muscle cell spreading: a role for MARCKS in an integrin signaling pathway

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Summary

To understand how muscle cell spreading and survival are mediated by integrins, we studied the signaling events initiated by the attachment of muscle cells to fibronectin (FN). We have previously demonstrated that muscle cell spreading on FN is mediated by α5β1 integrin, is associated with rapid phosphorylation of focal adhesion kinase and is dependent on activation of protein kinase C (PKC). Here we investigated the role of individual PKC isozymes in these cellular processes. We show that α, δ and εPKC are expressed in muscle cells and are activated upon integrin engagement with different kinetics – εPKC was activated early, whereas α and δPKC were activated later. Using isozyme-specific inhibitors, we found that the activation of εPKC was necessary for cell attachment to FN. However, using isozyme-specific activators, we found that activation of each of three isozymes was sufficient to promote the spreading of α5-integrin-deficient cells on FN. To investigate further the mechanism by which integrin signaling and PKC activation mediate cell spreading, we studied the effects of these processes on MARCKS, a substrate of PKC and a protein known to regulate actin dynamics. We found that MARCKS was localized to focal adhesion sites soon after cell adhesion and that MARCKS translocated from the membrane to the cytosol during the process of cell spreading. This translocation correlated with different phases of PKC activation and with reorganization of the actin cytoskeleton. Using MARCKS-antisense cDNA, we show that α5-expressing cells in which MARCKS expression is inhibited fail to spread on FN, providing evidence for the crucial role of MARCKS in muscle cell spreading. Together, the data suggest a model in which early activation of εPKC is necessary for cell attachment; the later activation of α or δPKC may be necessary for the progression from attachment to spreading. The mechanism of PKC-mediated cell spreading may be via the phosphorylation of signaling proteins, such as MARCKS, that are involved in the reorganization of the actin cytoskeleton.

Key words: Integrin, PKC, Muscle, FAK, MARCKS, Fibronectin

Introduction

The binding of integrins to extracellular matrix proteins generates intracellular signaling events that mediate such processes as cell survival, cell growth and differentiation (Hynes, 1992; Bates et al., 1995). Focal adhesion kinase (FAK), a non-receptor tyrosine kinase, has been identified as a primary mediator of integrin signaling (Kornberg et al., 1992; Schaller et al., 1992; Hanks et al., 1992; Rozengurt and Rodriguez-Fernandez, 1997). Localized at focal adhesion sites, FAK is rapidly phosphorylated in response to integrin binding, which results in the activation of signaling cascades that promote cell attachment and spreading (Kornberg et al., 1992; Schaller et al., 1992; Hanks et al., 1992; Richardson and Parsons, 1996; Rozengurt and Rodriguez-Fernandez, 1997). On the basis of in vivo and in vitro studies (Schaller et al., 1995; Chen et al., 2000), FAK not only interacts physically with integrins but also plays a role as an adaptor protein for different cytoskeleton proteins such as talin, tensin, paxillin and vinculin (Clark and Brugge, 1995). The attachment of the skeletal muscle cells to fibronectin (FN) through α5β1 integrin mediates an 'outside-in' signaling pathway that is initiated by the activation of integrin by FN. We found that PKC activation as well as FAK phosphorylation are both critical events in integrin-mediated cell spreading (Disatnik and Rando, 1999). We demonstrated that PKC is also involved in 'inside-out' signaling that mediates the activation of α4β1 integrin and leads to cell spreading on FN even in the absence of α5β1 integrin. Both 'outside-in' and 'inside-out' pathways are necessary for muscle cell spreading and survival (Disatnik and Rando, 1999).

The importance of integrin signaling in the survival of skeletal muscle cells is demonstrated by the recent reports of muscular degenerative disorders in mice with specific integrin deficiencies (Mayer et al., 1997; Taverna et al., 1998). These reports show that a deficiency in either α5 or α7 integrins causes muscular dystrophies, indicating that the expression of each integrin is necessary for long-term survival of myofibers. These results indicate that integrins are true signaling molecules, transmitting information from the extracellular milieu into the cell. However, the integrin-signaling cascade that regulates this survival pathway in muscle cells remains to be elucidated.

Several studies using different systems have highlighted the importance of protein kinase C (PKC) in integrin-mediated cell adhesion and spreading (Vuori and Ruoslahti, 1993; Schlaepfer et al., 1994; Disatnik and Rando, 1999), as well as in cell migration, FAK phosphorylation and focal adhesion formation (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993; Haimovich et al., 1996). Different approaches have been used to study the specific role of PKC in integrin signaling. Pharmacological activators of PKC have been reported to enhance the adhesion and spreading of cells (Mercurio and Shaw, 1988; Disatnik and Rando, 1999). Pharmacological inhibitors of PKC prevent not only focal adhesion formation but also stress fiber formation in fibroblasts plated on FN (Woods and Couchman, 1992). PKC also appears to be a key intermediate between integrins and FAK signaling in muscle cells and other cell types (Vuori and Ruoslahti, 1993; Disatnik and Rando, 1999; Miranti et al., 1999). Several studies have indicated that PKC activation is required for FAK phosphorylation in cells plated on FN (Vuori and Ruoslahti, 1993; Haimovich et al., 1996; Disatnik and Rando, 1999). Although PKC and FAK colocalize at focal adhesion sites (Schaller et al., 1992; Liao and Jaken, 1993), the precise functional relationship between these two kinases is not known.

The PKC family of serine-threonine kinases can be classified into three major families (Ron and Kazanietz, 1999). The classical PKCs consist of α, βI, βII and γPKC, which are Ca^{2+}/diacylglycerol-dependent kinases. The novel PKCs, δ, ε, η and θPKC, are Ca^{2+} independent but require diacylglycerol for activation. The third family, atypical PKCs, consists of ζ and ι/λPKC, which are neither Ca^{2+}- nor diacylglycerol-dependent. The PKC isozymes responsible for mediating cell attachment and spreading are unknown and may be tissue and stimulus specific. The lack of isozyme-selective modulators has limited the information available regarding the role of specific PKC isozymes in integrin signaling. Studies in different cell types have demonstrated α, δ and εPKC localization to focal adhesions (Liao and Jaken, 1993; Haimovich et al., 1996; Haller et al., 1998), suggesting that all these isozymes may be linked to the integrin-signaling pathway.

One of the most predominant intracellular substrates for PKC that may play a role in cell spreading is the myristoylated alanine-rich C kinase substrate protein (MARCKS) (Aderem, 1992b). MARCKS contains three highly conserved domains: an N-terminal myristoylation domain, a region of conserved sequence at the single intron splicing and an internal phosphorylation site domain (PSD), containing serines phosphorylated by PKC. This domain also serves as the site of high affinity calmodulin binding. Moreover, this region has also been shown to crosslink actin filaments in vitro (Hartwig et al., 1992; Swierczynski and Blackshear, 1995). PKC-mediated phosphorylation of the PSD domain decreases MARCKS affinity for the plasma membrane, calmodulin and actin, followed by its translocation from the cell membrane to the cytosolic fraction. Several reports have highlighted the potential role of MARCKS in cell attachment and spreading, but the mechanism of action is still unknown (Li et al., 1996; Manenti et al., 1997; Myat et al., 1997; Spizz and Blackshear, 2001).

In this report, we present evidence for the activation of distinct PKC isozymes leading to the phosphorylation of FAK and mediating spreading of skeletal muscle cells. In response to integrin engagement, there was a rapid but transient activation of α, δ and εPKC. Peptide modulators of individual PKC isozymes have been recently developed that inhibit or promote binding of individual PKC isozymes to their anchoring proteins, RACKs (Receptors for Activated C Kinase) (Mochly-Rosen, 1995; Souroujon and Mochly-Rosen, 1998; Dorn et al., 1999; Hu et al., 2000; Mochly-Rosen et al., 2000). The function of these short peptides conjugated to a cell-permeable peptide derived from the Antennapedia protein has been previously described in a variety of cells, including cardiac myocytes (Mochly-Rosen, 1995; Souroujon and Mochly-Rosen, 1998; Dorn et al., 1999; Hu et al., 2000; Mochly-Rosen et al., 2000; Chen et al., 2001). The 6-10 amino acid peptides derived from individual PKC isozymes were shown to act selectively on their corresponding isozymes by inducing (for the activator peptides) or inhibiting (for the inhibitor peptides) PKC translocation and cellular activity (for a review, see Souroujon and Mochly-Rosen, 1998). To assess further the role of individual PKC isozymes, we used α, δ and εPKC-selective activator and inhibitor peptides and examined their effects on cell spreading and FAK phosphorylation. The results of these studies suggest that εPKC activation is necessary to promote muscle cell attachment with a concomitant activation of α and δPKC that mediate cell spreading. Our results further demonstrate that MARCKS might be downstream of PKC in the integrin-signaling pathway that mediates muscle cell spreading. MARCKS may be the intermediate signaling molecule that lead to cell attachment and spreading. Taken together, these results support the link between specific PKC isozymes, MARCKS and the integrin-signaling pathway in muscle cell attachment and spreading.

Materials and Methods

Cell culture

Throughout these studies, two populations of cells were used – α5-integrin-expressing cells and α5-integrin-deficient cells. Unless indicated otherwise, the cells expressed α5. Cells deficient in α5 integrin were derived from limb muscle of neonatal mice that were chimeric for α5 integrin expression, as described previously (Taverna et al., 1998; Disatnik and Rando, 1999). Cells expressing α5 integrin were generated by retrovirus-mediated transfer of a human α5 cDNA into α5-deficient cells and were indistinguishable from wild-type cells, which also express α5 integrin (Disatnik and Rando, 1999). To control for the retroviral infection, α5-deficient cells were infected with a control retrovirus that expressed only the selectable marker (Taverna et al., 1998; Disatnik and Rando, 1999). For growth, all cells were plated on dishes coated with 5 μg/ml laminin (Gibco BRL, Gaithersburg, MD) and maintained in growth medium consisting of Ham's F-10 (Mediatech, Inc., Herndon, VA) supplemented with 20% fetal bovine serum (Mediatech, Inc.).

PKC peptides

Peptide activators are pseudo-RACK1 [amino acids 241-246 of αPKC (SVEIWD) (Ron and Mochly-Rosen, 1995; Souroujon and Mochly-Rosen, 1998)], pseudo-δRACK [amino acids 74-81 of δPKC

(MRAAEDPM) (Chen et al., 2001)], and pseudo-εRACK [amino acids 85-92 of εPKC (HDAPIGYD) (Dorn et al., 1999)]. Peptide inhibitors are αC2-4 [amino acids 218-226 of αPKC (SLNPQWNET) (Souroujon and Mochly-Rosen, 1998)], δV1-1 [amino acids 8-17 of δPKC (SFNSYELGSL) (Chen et al., 2001)] and εV1-2 [amino acids 14-21 of εPKC (EAVSLKPT) (Dorn et al., 1999)]. The peptides were synthesized and purified (>95%) at the Stanford Protein and Nucleic Acid Facility. The peptides were crosslinked via an N-terminal Cys-Cys bond to the *Drosophila* Antennapedia homeodomain-derived carrier peptide (CRQIKIWFQNRRMK-WKK) or carrier-carrier dimer as a control (Derossi et al., 1994; Theodore et al., 1995).

Adhesion and spreading

For assessment of cell adhesion and spreading, 60 mm dishes were coated with FN (5 μg/ml; Gibco BRL) for 24 hours at room temperature. 1 hour before plating, all dishes were coated with 1% bovine serum albumin (Sigma, St Louis, MO). Cells were trypsinized and treated as indicated. In PKC activation or inhibition experiments, the cells were treated in suspension with respective peptides at 1 μM and then plated on FN for 30 minutes in the presence of the peptides as indicated. The cultures were assessed and photographed using a 40× phase contrast immersion objective on a Zeiss Axioskop microscope.

Western blot analysis

After trypsinization, cells were plated on FN for 30 minutes. For PKC activation, phorbol 12-myristate 13-acetate (PMA; Alexis Biochemicals, San Diego, CA) or specific PKC peptides were added to the cells in suspension for 10 minutes at the indicated concentration. For PKC inhibition, respective peptides were added to the cells in suspension for 20 minutes prior to plating. After 30 minutes of plating, attached and unattached cells were collected, spun and washed with cold PBS. The cells from both fractions were pooled and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40) containing aprotinin (20 μg/ml), leupeptin (20 μg/ml), phenylmethylsulfonyl fluoride (10 μg/ml), sodium orthovanadate (1 mM), sodium pyrophosphate (10 mM) and sodium fluoride (100 mM). Proteins from total extracts were electrophoresed by 10% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose then incubated for 2 hours in PBS containing 0.05% Tween and 5% non fat dry milk. Phosphotyrosine-containing proteins were detected with a monoclonal anti-phosphotyrosine antibody, PY-99 (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA), as described previously (Disatnik and Rando, 1999) followed by a horseradish-peroxidase-coupled anti-mouse secondary antibody (Amersham Corp., Arlington Heights, IL). Duplicate blots were also probed (or blots were reprobed after stripping) with anti-FAK polyclonal antibodies (1:1000; Santa Cruz Biotechnology) followed by a horseradish-peroxidase-coupled anti-rabbit secondary antibody. Specific antibody binding was detected by an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). Where indicated, the bands were quantitated using a Bio-Rad Fluor-S MultiImager (Bio-Rad, Hercules, CA).

Fractionation analysis

α5-expressing or α5-deficient cells were trypsinized, and 3×10^6 cells were replated on FN-coated dishes. At different time points after plating, non-adherent cells from duplicate dishes were pooled and collected by centrifugation. Adherent cells were scraped from the dish in homogenization buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA) containing protease inhibitors and phosphatase inhibitors as indicated above. The adherent and non-adherent cells were pooled, and the extract was passed through a 26-gauge needle and then spun at 100,000 ***g*** for 40 minutes at 4°C. The cytosolic fraction was collected, and the membrane fraction was solubilized in RIPA buffer. 80 μg protein was loaded on a 7.5% SDS-polyacrylamide gel. The level of MARCKS in each fraction was detected by western blot analysis, as above, using a goat polyclonal MARCKS antibody at 1:100 dilution (Santa-Cruz Biotechnology) followed by a horseradish-peroxidase-coupled anti-goat secondary antibody (1:15000; Pierce Endogen, Rockford, IL). For a loading control, actin protein was analyzed using a rabbit polyclonal antibody at 1:5000 dilution (Sigma).

Kinase assay and immunoprecipitation

α5-expressing or α5-deficient cells were trypsinized, and 2×10^6 cells were replated on FN-coated dishes. At different time points after plating, non-adherent cells from duplicate dishes were pooled and collected by centrifugation. Adherent cells were lysed in 100 μl RIPA buffer and combined with spun cells. The lysates were incubated on ice for 1 hour, and insoluble proteins were then pelleted by centrifugation. Protein estimation was done on the soluble fraction, and equal amounts of protein were used for immunoprecipitation of αPKC, δPKC and εPKC using isozyme-specific antibodies (1:100; Santa Cruz Biotechnology). PKC isozymes were immunoprecipitated for 3 hours at 4°C. After the addition of protein G-agarose beads, the reaction was incubated for 1 hour. Immunocomplexes were washed three times with RIPA buffer and once with binding buffer (20 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, and 25 μM ATP). For inhibition experiments, chelerythrine (2 μM, Alexis Biochemicals) was added 10 minutes before the kinase assay. The PKC activity of immunoprecipitated fractions was assayed by adding 40 μl of binding buffer containing 5 μCi [γ^{32}P]ATP (5000 Ci/mmole, Amersham) and 40 μg histone III-S (Sigma) or 10 μg myelin basic protein (MBP). After 15 minute incubations at 37°C, assays were terminated by adding sample buffer. The samples were loaded on a 10% or 12% SDS acrylamide gel, and the levels of phosphorylated histone or MBP were quantified either by cutting the band and counting ^{32}P incorporated into the substrates using scintillation fluid or by exposing the gel to autoradiographic film and quantifying the bands using a Bio-Rad Fluor-S MultiImager. After exposure, the blots were probed with specific PKC isozyme antibodies (1:500) for normalization of the immunoprecipitated material. The results from eight separate experiments were analyzed.

MARCKS cDNA cloning and transfection

Poly(A)$^+$ RNA was extracted from the C2C12 cell line using MicroFastrack purification kit (Invitrogen). We generated the full-length mouse MARCKS cDNA (GenBank accession number M60474) with Titanium one-step RT-PCR Kit (Clontech) using primers 5′-CGTCGTTACACCAACCGAAGGCTCT-3′ and 5′-GAATTGCGTGAGGGCTCTGGAGCTT-3′ and following the protocol outlined by the manufacturer. The product of 1 kb was then cloned into pGEM-T-Easy vector (Promega) and fully sequenced to confirm its sequence. MARCKS cDNA was then subcloned in the forward (sense) and reverse (antisense) orientation in pcDNA3.1/hygro vector (Invitrogen). The MARCKS-sense, the MARCKS-antisense or the vector alone was transfected into α5-expressing cells with Lipofectamine 2000 (Invitrogen). Hygromycin-resistant colonies were pooled, and clones were isolated by limiting dilution. Antisense and control transfected cells were maintained in the presence of 200 μg/ml hygromycin.

Immunocytochemistry

Myoblasts were plated on FN-coated chamber slides for different lengths of time and then fixed with cold methanol followed by acetone or with 4% paraformaldehyde. Non-specific binding was blocked for 1 hour with 1% normal goat serum or normal rabbit serum in PBS containing 0.1% Triton X-100 (blocking solution) followed by overnight incubation with PKC isozyme-specific antibodies at 1:100, polyclonal FAK antibodies at 1:1000, polyclonal skeletal actin antibodies (Sigma) at 1:1000, polyclonal MARCKS antibodies (Santa-Cruz Biotechnology) at 1:100 or monoclonal paxillin antibody at 1:1000 in blocking solution containing 2 mg/ml bovine serum albumin. The cells were washed with the blocking solution followed by 2 hour incubations with a fluorescein-conjugated anti-rabbit IgG antibody (ICN Pharmaceuticals, Aurora, OH, diluted at 1:1500), a rabbit anti-goat Alexa fluor 488 antibody (Molecular Probes, Inc., Eugene, OR diluted at 1:500) or a goat anti-mouse Alexa fluor 546 (Molecular Probes at 1:500) in the presence, as indicated, of 1 μg/ml TRITC-Phalloidin (Sigma), which binds to F-actin. The specificity of the PKC staining was determined as described previously (Disatnik et al., 1994). After washing the cells three times with blocking solution, the slides were mounted with Vectashield (Vector, Burlingame, CA) and viewed with a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) using a 63× oil immersion objective. Images were recorded on Kodak T160 film.

Statistical analysis

The results presented are from three to eight separate experiments, as indicated. Data are presented as means ±s.d. Student's paired *t*-tests were used for comparisons. $P<0.05$ was considered statistically significant.

Results

To characterize myoblast attachment and spreading, cells were plated on FN-coated dishes and photographed to illustrate the distinct morphological changes over time. Within 5 minutes of plating, the cells made contact with the substrate, and we observed small bleb-like membrane protrusions around the periphery of most of the cells (Fig. 1A). This was followed by membrane ruffling within 10 minutes of plating, typical of an early stage of cell attachment (Myat et al., 1997). Further morphological changes were observed after 15 minutes on FN, when circumferential lamellae were observed surrounding the spread cells (Fig. 1A). By 30 minutes, the cells were all spread, revealing a flat and elongated morphology typical of myoblast cells in culture (Fig. 1A). These morphological changes were correlated with actin stress fiber formation (Fig. 1B). The phenomenon of cell spreading is initiated by the actin-driven protrusion of membrane ruffles that adhere to the substratum and expand to form lamellipodia (Stossel, 1993). Cell spreading proceeds along with distinct actin stress fiber formation until the cells have flattened on the substrate (Conrad et al., 1993; Myat et al., 1997). In myoblasts, actin stress fibers were formed only after the cells had been plated for 30 minutes on FN; however, they appeared to be located around the nucleus in a circular orientation as well as at the periphery of the cells (Fig. 1B).

Since FAK localizes to focal adhesion sites in cells plated on FN (Schaller et al., 1992; Hanks et al., 1992), we analyzed the cells for focal adhesion formation and FAK localization at these sites as a function of time after plating. Within 15 minutes of plating on FN, FAK was localized to the nucleus and diffusely in the cytosol of skeletal muscle cells with faint staining at the periphery of the cells (Fig. 1B). At 30 minutes, predominant punctate FAK staining was detected at cell edges, accentuating adhesion contacts with the substrate. Distinct focal adhesion sites were identified after 60 minutes on FN

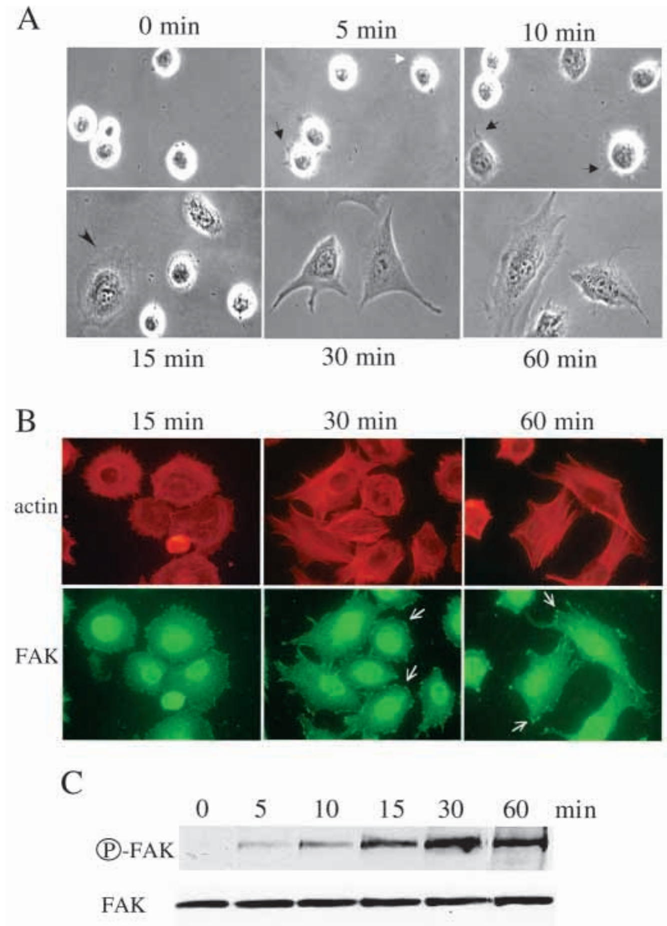

Fig. 1. Morphological and biochemical changes associated with cell attachment and spreading of skeletal muscle cells plated on FN. (A) Myoblasts were plated on FN and photographed at various times after plating. The time in minutes after plating is indicated. The panels show characteristic morphological changes of attachment and spreading, including bleb formation (5 minutes, white arrow), membrane ruffling (5 and 10 minutes, closed arrow) and circumferential lamella (15 minutes, arrowhead). (B) Actin stress fiber formation and FAK localization were determined in myoblasts at different time points after the cells were plated on FN. The upper panels show the development of stress-fiber formation by staining the cells with fluorescently labeled phalloidin. The lower panels show the change in FAK localization from a predominantly diffuse cytosolic localization at 15 minutes to a more focal adhesion site localization (arrows) at later time points. (C) FAK phosphorylation was determined as a function of time after plating on FN. At each time point, the cells were harvested in RIPA buffer. Phosphorylation of FAK was determined by immunoblot analysis using an anti-phosphotyrosine antibody. A duplicate blot was probed with an anti-FAK antibody (lower panel) to confirm equal loading of FAK protein in each lane.

(Fig. 1B). In addition to FAK translocation, we measured the phosphorylation of FAK by western blot analysis in cells plated on FN for different period of time (Disatnik and Rando, 1999). FAK phosphorylation is necessary for integrin-mediated cell attachment and spreading (Kornberg et al., 1992; Hanks et al., 1992; Richardson and Parsons, 1996). Our results show that there is phosphorylation of FAK within 5 minutes of plating (Fig. 1C), at a time when cells have just begun the process of

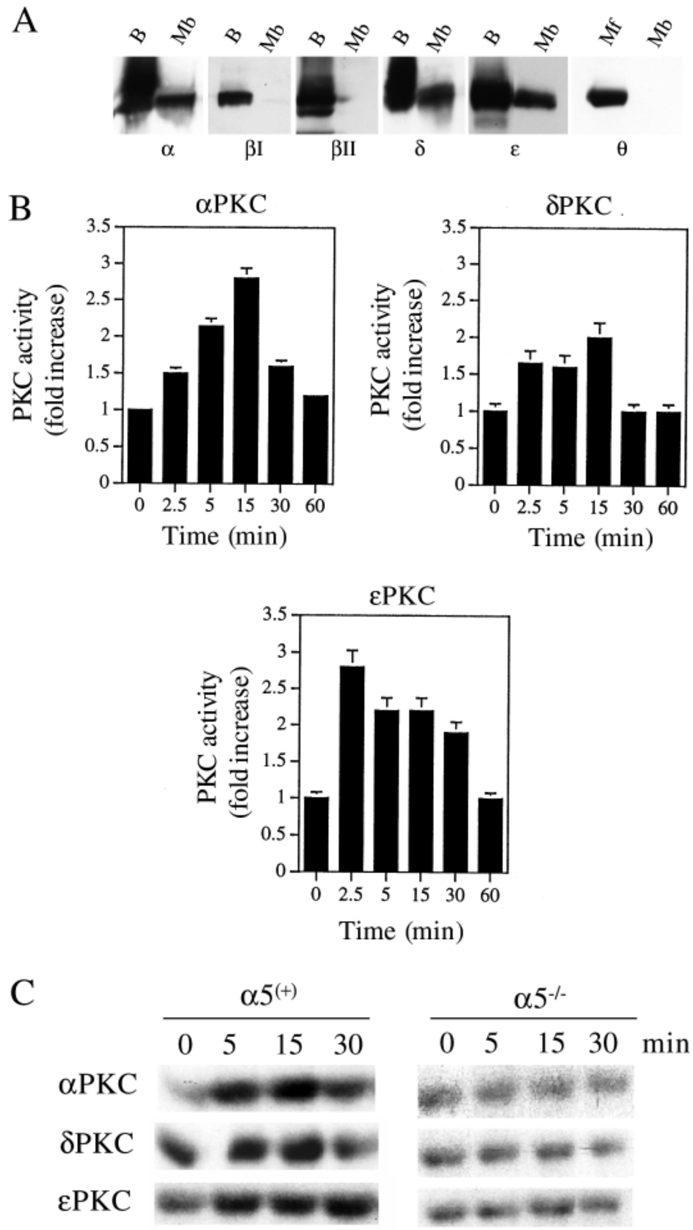

Fig. 2. PKC isozyme expression and activation in muscle cells. (A) PKC isozyme expression was determined by western blot analysis in mouse myoblasts. Myoblasts were grown for 2 days then lysed in RIPA buffer. 80 μg of proteins were chromatographed on a 7.5% SDS gel then probed with antibodies specific for individual PKC isozymes. The lanes containing protein from myoblast cultures are labeled 'Mb'. Adjacent lanes include positive controls for each isozyme. 'B' refers to mouse brain extract (20 μg/lane), which is known to highly express all the isozymes except for θPKC and was therefore used as a positive control. For θPKC, extract of skeletal muscle tissue ['Mf' (myofibers)] was used (80 μg/lane). (B) PKC isozyme activity increases upon integrin binding and activation. PKC isozymes were immunoprecipitated from total cell extracts of myoblasts plated on FN for different lengths of time, and the activity of each isozyme was determined by the level of ^{32}P incorporation into histone III-S. These results are normalized to the activity at time zero and to the amount of PKC isozymes immunoprecipitated from each sample determined by western blot analysis. These results represent the mean±s.d. from eight separate experiments. (C) α5-expressing and α5-deficient myoblasts were plated on FN for various times. PKC isozymes were immunoprecipitated as described in A, and phosphorylated histone was analyzed on 10% SDS-polyacrylamide gel. A representative autoradiogram is shown. As in A, PKC isozyme activity increases transiently in α5-expressing myoblasts ($\alpha5^{(+)}$) plated on FN. By contrast, there was no increase in PKC isozyme activity in the α5-deficient myoblasts ($\alpha5^{-/-}$) on FN. The protein levels of each isozyme in the two cell populations were indistinguishable by western blot analysis.

attachment and spreading. FAK phosphorylation increases with time after plating, as cell spreading, stress fiber formation and focal adhesion formation proceed. The phosphorylation of FAK reaches a maximum at 60 minutes when cell spreading is complete (Fig. 1C), and it does not change thereafter.

Our previous results demonstrated that PKC activation promotes muscle cell spreading on FN and that this activation is necessary for the interaction of α5 integrin with FN to promote cell spreading and FAK phosphorylation (Disatnik and Rando, 1999). To determine which of the PKC isozymes are involved in this integrin-signaling pathway in muscle cells, we first determined which isozymes are expressed in skeletal muscle cells by western blot analysis using isozyme-specific antibodies (Fig. 2A). We found that only α, δ and εPKC were expressed at detectable levels. Surprisingly, θPKC, which is known as a muscle specific isozyme (Osada et al., 1992), was not expressed in cultured myoblasts nor in myotubes. The same pattern of PKC isozyme expression was found in differentiated myotubes in culture (data not shown).

Previously, we found that inhibition of PKC with either calphostin C or bisindolylmaleimide I completely prevented integrin-mediated cell spreading and FAK phosphorylation (Disatnik and Rando, 1999). To determine which of the PKC isozymes were activated upon myoblast attachment to FN, cells were plated on FN-coated dishes for various lengths of time, and individual PKC isozymes were immunoprecipitated from total cell lysates. The activation of the PKC isozymes was determined by measuring the kinase activity in immunoprecipitates using histone III-S as a substrate. The incorporation of phosphate into histone was quantified, and the results for α, δ and εPKC activity are shown in Fig. 2B. The temporal pattern of activation differed among these three isozymes, suggesting a different role for each isozyme in cell attachment and spreading. αPKC was activated upon cell attachment to FN and reached a maximum activation after 15 minutes, followed by a rapid decline. We found that δPKC was activated during the first 15 minutes of plating, although the magnitude of the increase was less than that of α and εPKC. δPKC activation declined back to baseline levels over the next 15-30 minutes. By contrast, εPKC was highly activated as early as 2.5 minutes after plating and remained activated for at least 30 minutes. To determine the importance of the α5 integrin signaling pathway in PKC isozyme activation, we repeated this experiment using α5-deficient cells, which were described previously to fail to spread on FN (Disatnik and Rando, 1999). There was no activation of any of these isozymes when α5-deficient cells were plated on FN (Fig. 2C), providing further evidence that PKC activation is a downstream effector pathway for integrin signaling.

Increasingly, immunoprecipitation-based kinase assays are being used to evaluate the activity of individual PKC isozymes

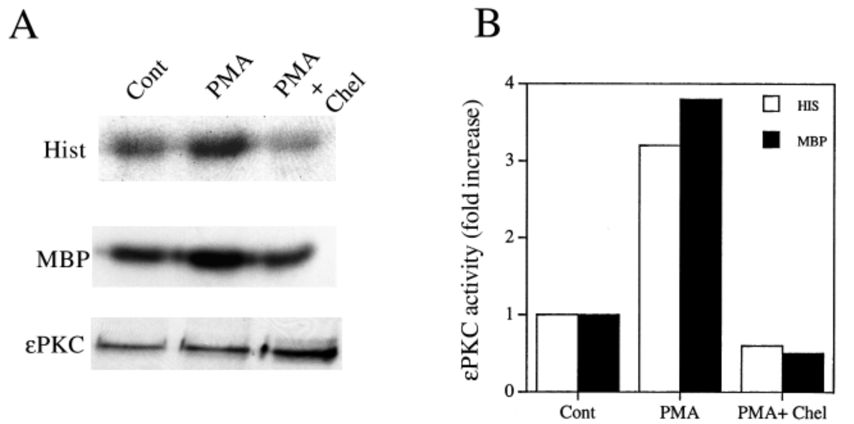

Fig. 3. Inhibition of immunoprecipitated kinase activity by a PKC-specific inhibitor. (A) εPKC was immunoprecipitated from myoblasts with or without PMA treatment. After immunoprecipitation with an antibody specific for εPKC, in vitro kinase assays were carried out in the presence and absence of the PKC inhibitor, chelerythrine (2 μM), using either histone or MBP as a substrate. The phospho-proteins were loaded on a 10% or 12% SDS-polyacrylamide gel then transferred to nitrocellulose followed by autoradiography to assess histone phosphorylation (upper row) or MBP phosphorylation (middle row). The blots were probed with an anti-εPKC antibody to confirm equal amounts of εPKC protein in each sample (lower row). (B) The incorporation of ^{32}P into histone or MBP from experiments such as that shown in A was quantified. These results presented are averaged from two separate experiments and demonstrate the marked induction of εPKC activity by PMA that is maintained after immunoprecipitation and inhibited by chelerythrine.

(Zang et al., 1997; Reyland et al., 1999; Bahr et al., 2000). However, to exclude the possibility that an unknown kinase that coimmunoprecipitates with PKC was responsible for histone phosphorylation, and also to show that immunoprecipitated PKC remained active, we performed a similar assay when εPKC was immunoprecipitated from untreated myoblasts and from myoblasts treated with 100 nM PMA for 5 minutes. We compared the ability of the immunoprecipitated material to phosphorylate histone and MBP in the presence and absence of chelerythrine, a specific inhibitor of PKC (Herbert et al., 1990). Fig. 3 shows that chelerythrine blocked the phosphorylation of these substrates, demonstrating that the kinase remains active following immunoprecipitation and that the activity is indeed caused by immunoprecipitated PKC.

To complement the biochemical studies of PKC isozyme activation (Fig. 2), we compared the subcellular localization of α, δ and εPKC by immunocytochemistry in myoblasts plated on FN over time (Fig. 4). After the cells had been plated on FN for 15 minutes, we were able to assess the localization of PKC isozymes. Prior to this time point, the rounded morphology of most cells prevented any reliable assessment of isozyme localization by microscopic examination. Since, on the basis of the data from the kinase assay, we knew that PKC activation returns to basal levels by 1 hour after plating, we assessed cells for PKC isozyme localization between 15 minutes and 1 hour of plating on FN to correlate cellular localization with biochemical activation. Fig. 4 shows the differential localization of α, δ and εPKC at 15 minutes and 1 hour. There was little difference in the localization of any of the isozymes between 15 and 30 minutes after plating, suggesting that most of the cellular translocation occurred between 30 and 60 minutes after plating. We found that αPKC was localized at focal adhesion sites 15 minutes after plating cells on FN whereas, at later time points, αPKC was distributed more diffusely in the cytosol (Fig. 4). δPKC was found predominantly in a perinuclear, Golgi-like distribution after 15

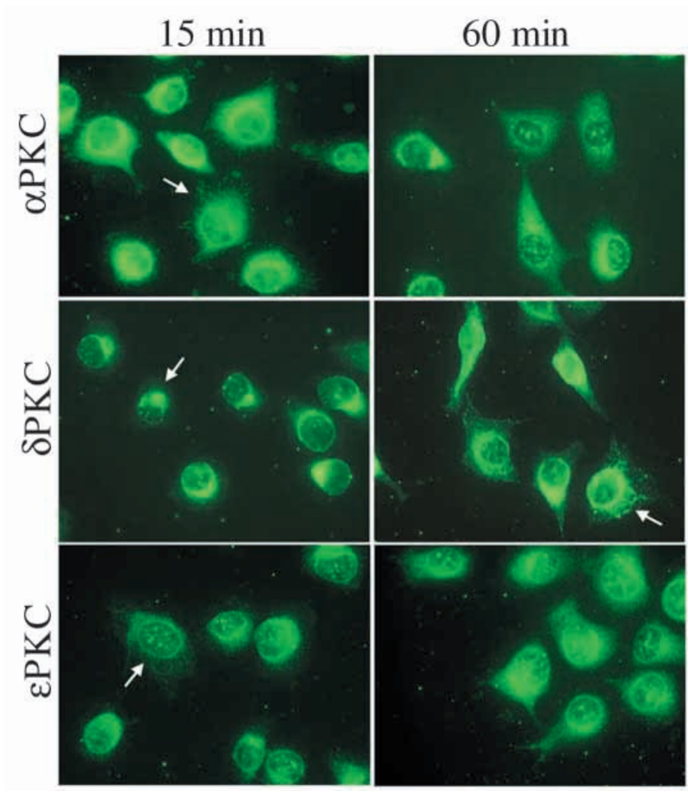

Fig. 4. PKC isozyme localization in myoblasts plated on FN. Myoblasts were plated on FN for various times, methanol/acetone fixed and stained for individual PKC isozymes. This figure shows characteristic patterns of localization of α, δ and εPKC as the cells attach and spread. These results were observed in more than 90% of the cells. αPKC is localized at focal adhesion sites (arrow) after 15 minutes on FN. After 15 minutes on FN, δPKC revealed a Golgi-like staining (arrow), whereas after 1 hour, δPKC showed a punctate staining pattern at the cell periphery (arrow). After 15 minutes, εPKC was detected in the nucleus and in perinuclear regions (arrow), and became localized diffusely in the cytosol as well as in the nucleus after 1 hour on FN.

minutes. After 1 hour on FN, δPKC was found in the cytosol in a punctate staining pattern (Fig. 4). εPKC was initially localized to the nucleus and to perinuclear regions. At 1 hour, εPKC was found diffusely in the cytosol as well as in the nucleus (Fig. 4). Therefore, these three different isozymes translocate to distinct locations in the cell after integrin activation, which may further indicate distinct roles for these PKC isozymes in cell attachment and spreading.

We previously demonstrated that activation of PKC was necessary for integrin-mediated cell spreading and FAK phosphorylation in muscle cells plated on FN and that PKC activation promoted α5-deficient cell spreading on FN, indicating that PKC signaling is a downstream effector pathway for integrin signaling (Disatnik and Rando, 1999). To test which individual PKC isozyme, when activated, is sufficient to promote spreading and FAK phosphorylation in α5-deficient cells, we used peptide activators (see Materials and Methods) that have been shown to activate individual PKC isozymes (Ron and Mochly-Rosen, 1995; Souroujon and Mochly-Rosen, 1998; Dorn et al., 1999; Chen et al., 2001). These small peptides were conjugated to a cell-permeable peptide derived from the Antennapedia protein (Dorn et al., 1999). It was previously shown that about 10% of the applied peptide enters into greater than 95% of the cells (Souroujon and Mochly-Rosen, 1998). We observed no toxic effects on the cells with peptide concentrations up to 1 μM. To test the propensity of individual PKC isozymes to promote muscle cell attachment and spreading, α5-deficient cells were plated on FN in the presence or absence of the individual activator peptides. Activation of αPKC with pseudo-RACK1 peptide led to α5-deficient cell spreading on FN nearly as well as that seen in cells treated with 3 nM PMA, a general PKC activator (Fig. 5A). Although pseudo-RACK1 activates all classical PKC isozymes (Ron and Mochly-Rosen, 1995), we could use it as a selective αPKC activator in the cells, since none of the other classical isozymes are expressed (Fig. 2A). In response to the activation of αPKC, approximately 80% of the cells were spread 30 minutes after plating. δPKC activation with pseudo-δRACK (Chen et al., 2001) promoted cell attachment and the formation of distinct lamellipodia in 90% of the cells within 30 minutes of plating, indicating the beginning of cell spreading. The activation of εPKC with the εPKC-selective agonist pseudo-εRACK (Dorn et al., 1999) promoted cell attachment very effectively (Fig. 5). Within 30 minutes of plating, 100% of the cells were attached but revealed a rounded morphology. The subsequent phases of cell spreading were not as effectively promoted by the activation of εPKC, suggesting perhaps that activation of αPKC and δPKC may be important for the progression from attachment to spreading. In the presence of all the activators together, the process of cell

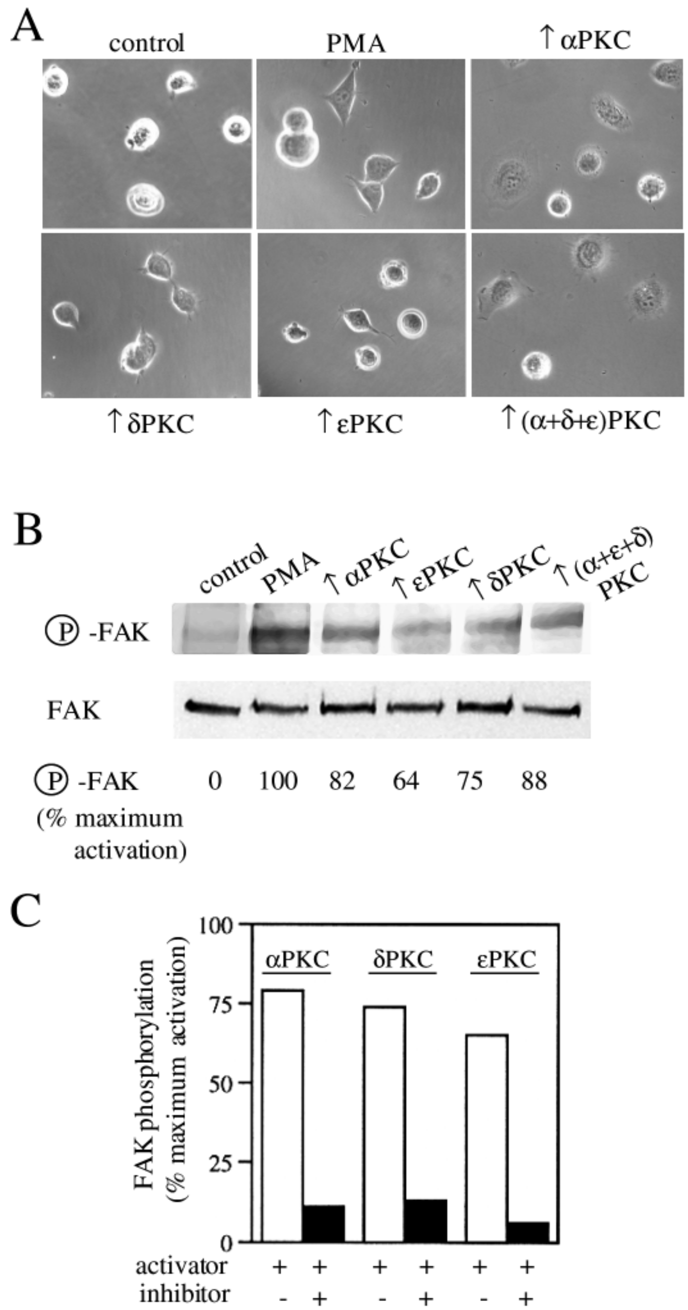

Fig. 5. α5-deficient cell spreading induced by activation of specific PKC isozyme. (A) α5-deficient cells were plated on FN for 30 minutes in the presence or absence of PMA (3 nM), α, δ or ε peptide activators (1 μM, labeled by the up arrow) or all three peptide activators together. In the absence of any activators, the cells do not attach. PMA treatment promotes rapid attachment and spreading. The activation of α, δ or εPKC all promote cell attachment, and α and δPKC activation promote cell spreading. All three activators together are nearly as effective as PMA. (B) α5-deficient cells treated as in A were assessed for FAK phosphorylation after 30 minutes on FN using an anti-phosphotyrosine antibody. FAK phosphorylation increased in the presence of the PKC activators in parallel with the effect on cell spreading shown in A. A duplicate blot was probed with an anti-FAK antibody to confirm equal loading (lower panel). FAK phosphorylation was quantified to calculate the percentage of activation (shown below each lane), with control levels being defined as no activation and PMA treatment defined as maximal activation. (C) α5-deficient cell spreading induced by α, δ and εPKC activators is inhibited by the corresponding specific inhibitors. α5-deficient cells were treated with individual PKC isozyme activators in the presence or absence of isozyme-specific inhibitors. FAK phosphorylation was determined by western blot analysis, and the level of phosphorylation was quantified. The experiment was repeated three times with similar results, and the results of a representative experiment are shown. The data were calculated as percentages of maximum activation obtained after 3 nM PMA treatment.

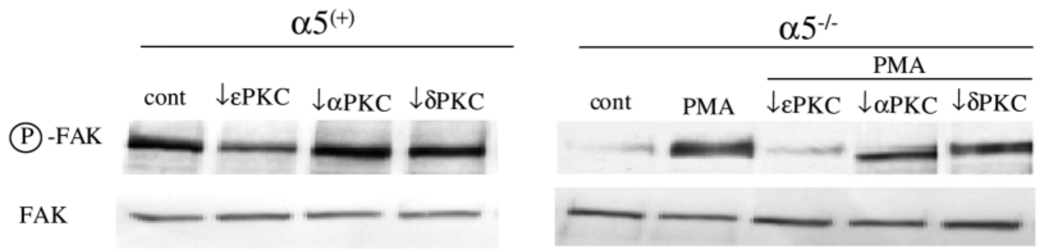

Fig. 6. εPKC is required for cell spreading and FAK phosphorylation in α5-expressing and α5-deficient myoblasts. α5-expressing (α5$^{(+)}$) and α5-deficient (α5$^{-/-}$) cells were plated on FN in the presence or absence of 3 nM PMA, α, δ or εPKC inhibitors (downward arrows), or PKC inhibitors in the presence of PMA (3 nM). FAK phosphorylation was determined after 30 minutes. The εPKC inhibitor blocks FAK phosphorylation both in α5-expressing cells and in α5-deficient cells activated by PMA, whereas inhibitors of αPKC and δPKC had little effect. For the loading control, the level of FAK protein was determined by probing duplicate blots with an anti-FAK antibody.

attachment and spreading was comparable to that seen in the presence of PMA (Fig. 5A). Fewer rounded cells (i.e. attached but not spread) were observed under this condition when compared with the cells plated in presence of individual isozyme activators alone.

Since FAK phosphorylation is such a critical determinant of cell attachment and spreading (Hanks et al., 1992; Burridge et al., 1992; Disatnik and Rando, 1999), we induced cell spreading using PKC activators as described above and assessed FAK phosphorylation by western blot analysis. Indeed, cell attachment and spreading induced by the PKC activators (Fig. 5A) correlated with increases in FAK phosphorylation (Fig. 5B). The increased progression from cell attachment to cell spreading promoted by activation of αPKC and δPKC as compared with εPKC is reflected in the somewhat greater FAK phosphorylation after 30 minutes of plating in cells treated with the respective activators.

To confirm the specificity of α, δ and εPKC activators, we tested whether these processes could be blocked by isozyme-specific PKC inhibitors. We tested the ability of isozyme-specific activators to promote α5-deficient cell spreading and FAK phosphorylation in the presence or absence of their respective isozyme-specific inhibitor peptides, αC2-4 for αPKC (Souroujon and Mochly-Rosen, 1998), δV1-1 for δPKC (Chen et al., 2001) and εV1-2 for εPKC (Dorn et al., 1999). The promotion of cell spreading and phosphorylation of FAK were nearly completely inhibited when cells were treated with the specific inhibitor of the isozyme that was being activated (Fig. 5C). None of the inhibitors had any effect on cells treated with activators of other isozymes.

The activation and translocation patterns of each of these isozymes suggest divergent roles in integrin-mediated muscle cell spreading. The results with the activators indicate that the activation of each isozyme is sufficient, at least partially, to promote cell attachment, spreading and FAK phosphorylation. To test for the necessity of individual isozyme activation in integrin-mediated muscle cell spreading, we plated α5-expressing cells on FN in the presence or absence of α, δ and εPKC inhibitors and measured the level of FAK phosphorylation. Similarly, we treated α5-deficient cells with PMA and then plated them on FN in the presence or absence of α, δ and εPKC inhibitors and measured the level of FAK phosphorylation (Fig. 6). εPKC inhibition by εV1-2 peptide reduced FAK phosphorylation in both cell populations. In contrast, selective inhibition of αPKC and δPKC did not affect the level of FAK phosphorylation or cell spreading (Fig. 6). However, treatment with either inhibitor did result in a delay in the progression from attachment to spreading (data not shown). Together with the results on isozyme activation and translocation, these data indicate that εPKC activation is sufficient to promote cell attachment and necessary to promote cell spreading and FAK phosphorylation in cultured skeletal muscle cells. Neither αPKC nor δPKC appears to be necessary, individually, for muscle cell spreading. However, each is capable of promoting attachment and spreading when activated. These data suggest that the early activation of εPKC is required and that the later activation of one additional isozyme may be necessary for the progression from

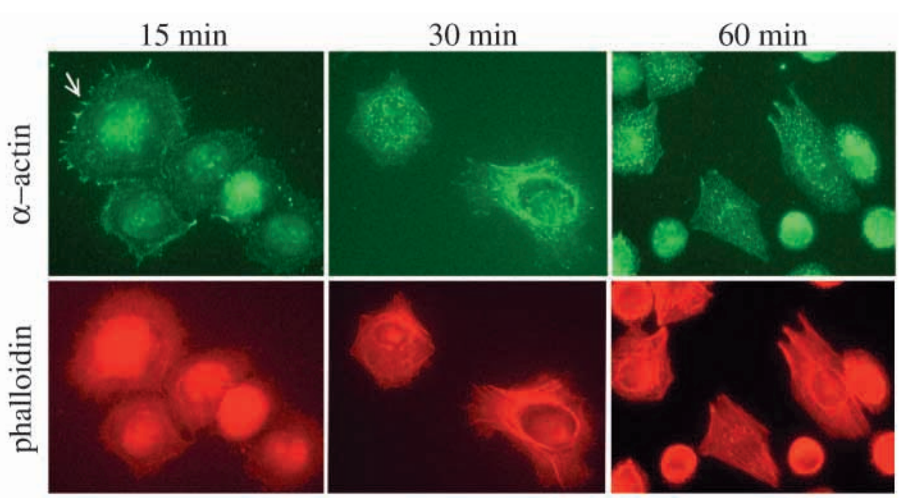

Fig. 7. Actin reorganization in α5-expressing cells plated on FN. Actin localization and stress-fiber formation were observed in α5-expressing cells plated on FN. Paraformaldehyde-fixed cells were stained for actin and stress fibers (F-actin) as indicated in the Materials and Methods. At early time points (15 minutes), actin was found at focal adhesion sites (arrow). With time, focal contacts were distributed uniformly across the cell surface, and fine stress fibers were found around the nucleus and at the periphery of the cell.

attachment to spreading, but that there may be redundancy in the effects of αPKC and δPKC activation for this process.

Some earlier studies had suggested that the promotion of cell spreading by PKC activation (Vuori and Ruoslahti, 1993) may be via its effects on the regulation of actin dynamics and stress fiber formation (Rosen et al., 1990). We first examined the reorganization of actin and formation of stress fibers in muscle cells plated on FN. Fig. 7 shows that actin is localized at focal contacts only at the periphery of the cells after 15 minutes on FN. After 1 hour, punctate focal contacts were visible, distributed more uniformly across the cell/substratum surface rather than just at the periphery (Fig. 7). This reorganization of actin is accompanied by the disassembly of stress fibers, which was also reported after PMA treatment (Rosen et al., 1990) and is consistent with reports of the disassembly, reorganization and reassembly of the actin network associated with cell attachment and spreading (Aderem, 1992a; Stossel, 1993; Defilippi et al., 1999). We also examined the dynamics of actin in stress-fiber formation associated with cell adhesion and spreading. Soon after plating (15 minutes), no stress fiber formation was evident (Fig. 7). By 30 minutes, and increasing up to 1 hour, stress fibers were found at the cell periphery and predominantly around the nucleus. This stress fiber reorganization parallels the changes in focal contact distribution regulated by actin cytoskeletal dynamics.

Among the many known substrates of PKC, MARCKS is one that is known to play a critical role in the regulation of actin dynamics (Aderem, 1992a). MARCKS has been postulated to be involved in signaling initiated by interactions between cells and ECM, and MARCKS has been localized at focal contacts in macrophages (Rosen et al., 1990). To determine the localization of MARCKS in muscle cells, α5-expressing cells were plated on FN for 30 minutes then stained with anti-MARCKS antibody (Fig. 8A). After 30 minutes on FN, MARCKS was recruited to cellular structures resembling focal adhesions. To confirm that MARCKS is indeed at focal adhesion sites in muscle cells upon spreading, we co-stained cells with antibodies to MARCKS and to paxillin and found that they localized to the same sites after the cells were plated on FN for 30 minutes (Fig. 8A). Fig. 8B shows the localization of MARCKS in myoblasts plated for various times on FN. Soon after adhesion (15 minutes), MARCKS was found in punctate structures typical of focal adhesion throughout the cell. After 30 minutes and 1 hour on FN, MARCKS was observed more diffusely in the cytosol in most of the cells, although localization at focal contacts was still evident and very predominant at 30 minutes (Fig. 8A). At all time points MARCKS staining was also observed in perinuclear region.

To determine whether MARCKS activation is indeed downstream of integrin signaling, we examined MARCKS localization in α5-expressing and α5-deficient cells plated on FN. We observed MARCKS translocation from the membrane to the cytosol in α5-expressing cells plated on FN as early as 15 minutes (Fig. 8C). This translocation was as rapid and complete as that seen when the cells were treated in suspension with PMA, which is known to cause MARCKS translocation

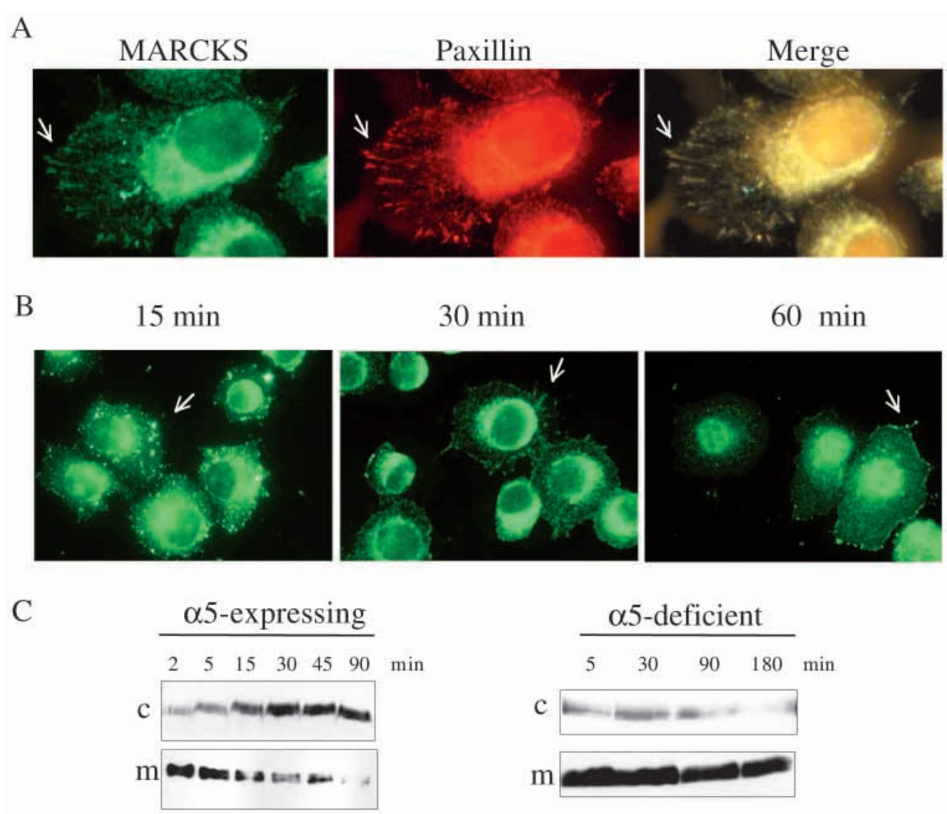

Fig. 8. MARCKS localization and translocation in α5-expressing cells plated on FN. (A) MARCKS is localized to focal adhesion sites during the initial phases of muscle cell attachment and spreading. α5-expressing cells were plated on FN, and cells were fixed and co-stained for MARCKS and the focal adhesion protein paxillin. The cells shown here were fixed 30 minutes after plating and show colocalization of MARCKS and paxillin, demonstrating the localization of MARCKS at focal adhesion sites (arrow). (B) MARCKS translocates from the membrane to the cytosol during muscle cell spreading. α5-expressing cells were plated on FN for various times prior to fixation and immunocytochemical assessment of MARCKS localization. MARCKS localization to focal adhesion sites (arrows in each panel) is most prominent at early time points, decreasing in intensity as the cells spread. With time, MARCKS becomes more diffusely distributed in the cytosol. (C) MARCKS translocation is mediated by integrin activation. To confirm the immunocytochemical translocation in B and to assess the role of integrin activation in the process, α5-expressing and α5-deficient cells were plated on FN, and MARCKS translocation was assessed by cellular fractionation. In α5-expressing cells, MARCKS is initially localized predominantly in the membrane compartment, consistent with the localization seen in B. With time, MARCKS translocates to the cytosolic fraction such that by 90 minutes, nearly all of the protein is in this compartment. By contrast, there is almost no translocation of MARCKS from the membrane to the cytosol in α5-deficient cells plated on FN.

in other cell types. In α5-deficient cells, which fail to spread on FN even 3 hours after plating, no translocation of MARCKS was observed over this time course (Fig. 8C). These data support the hypothesis that MARCKS translocation is mediated by integrin signaling.

To assess directly the importance of MARCKS in muscle cell spreading, we transfected α5-expressing cells with MARCKS cDNA in the antisense orientation or either with vector alone or MARCKS cDNA in the sense orientation as a control (both controls showed similar results). Transfected clones were selected and expanded, although clonal populations in which spreading was impaired (see below) were much more difficult to expand. Control transfected clones appeared to be normal in assays of cell spreading and expressed normal levels of MARCKS protein (Fig. 9). Clones transfected with the antisense vector, by contrast, displayed variable capacities to spread on FN. When these clones were analyzed by western blot analysis, there was a direct correlation between the reduction of MARCKS protein levels and the inhibition of cell spreading. Fig. 9A shows three clones with varying levels of MARCKS protein expression, showing the range of inhibition of protein expression by the antisense vector. The clones with the highest level of MARCKS expression (although still reduced compared to controls) showed mild impairment of spreading, whereas those with the lowest levels of MARCKS expression showed the most severe impairment of spreading (Fig. 9B). The clone in which MARCKS protein was undetectable by western blot analysis (clone 02) displayed almost no spreading on FN (Fig. 9B). To confirm that cell spreading is mediated by the activation of MARCKS by PKC, clone 02 was treated with PMA (100 nM) then plated on FN. PMA treatment did not activate clone 02 spreading on FN (Fig. 9C), demonstrating that cell spreading is mediated by MARCKS through PKC activation. These data suggest that MARCKS is essential for muscle cell spreading and, together with the data in previous figures, support the model that MARCKS is a key target of PKC phosphorylation in the regulation of the integrin-mediated process.

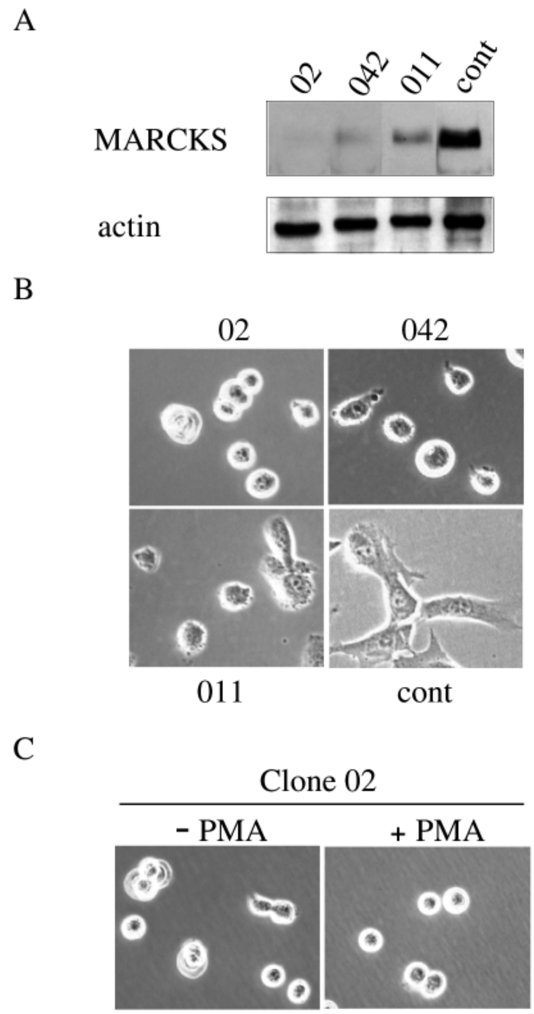

Fig. 9. MARCKS is essential for muscle cell spreading. α5-expressing cells were transfected with a MARCKS antisense vector or a vector control, and individual clones are selected for analysis of MARCKS expression and cell spreading. (A) MARCKS expression in transfected clones. Three clones (02, 042 and 011) transfected with the MARCKS-antisense cDNA showed reduced levels of MARCKS protein. A representative clone ('cont') transfected with control vector showed normal levels of MARCKS protein expression. Individual clones were photographed to illustrate the relationship between MARCKS protein expression and cell spreading. The clone transfected with the control vector (empty vector) showed normal cell spreading on FN. By contrast, each of the clones expressing the MARCKS antisense vector showed reduced cell spreading, and the inhibition of cell spreading correlated directly with the extent of reduction of MARCKS protein expression (A). MARCKS protein was undetectable by western blot analysis in clone 02, and this clone showed the most dramatic inhibition of cell spreading. Clone 02 was plated on FN with or without pretreatment with PMA (100 nM). Even activation of PKC by PMA was unable to promote spreading of this clone in which MARCKS protein was undetectable (A).

Discussion

We have reported that a deficiency in α5 integrin leads to apoptotic death of skeletal muscle cells (Taverna et al., 1998). Other investigators have likewise demonstrated apoptotic cell death when the interactions between integrins and matrix proteins have been disrupted (Meredith et al., 1993; Frisch and Francis, 1994; Bates et al., 1995; Zhang et al., 1995). These data suggest that integrin signaling induces cell survival pathways, whereas a deficiency of those signals may initiate cell death pathways. Muscle cells possess multiple integrins with different matrix binding capacities, and those integrins function to maintain the integrity of differentiated muscle fibers (Vachon et al., 1996). We previously presented a model that suggested a positive feedback loop of integrin engagement, signaling and activation in muscle cells in which PKC is involved (Disatnik and Rando, 1999). In this present study, we determined the respective roles of three different PKC isozymes in integrin-mediated muscle cell spreading. We used a new generation of PKC isozyme-specific inhibitors and activators (Mochly-Rosen, 1995; Souroujon and Mochly-Rosen, 1998; Dorn et al., 1999; Hu et al., 2000; Mochly-Rosen et al., 2000; Chen et al., 2001) to investigate the role of the individual PKCs in integrin signaling in muscle cells. We found that activation of α, δ or εPKC with respective peptides is sufficient for inducing attachment and/or spreading of α5-deficient cells on FN. Despite this commonality, the results of the kinase assay (Fig. 2) and translocation studies (Fig. 4) suggest that each PKC isozyme plays a distinct role in muscle cell spreading, as the temporal patterns of activation differ following integrin engagement.

We previously demonstrated using pharmacological and genetic approaches that PKC is involved in α5β1 integrin 'outside-in' and 'inside-out' signaling pathways, which lead to cell spreading and cell survival (Taverna et al., 1998; Disatnik and Rando, 1999). We showed that integrin engagement leads to FAK phosphorylation via a PKC signaling pathway and that PKC activation mediates a crosstalk between α5β1 and α4β1 integrin that induces muscle cell spreading on FN (Disatnik and Rando, 1999). PKC appears to be one of the key intermediates in integrin-mediated signaling in many cells (Juliano and Haskill, 1993; Clark and Brugge, 1995), and several reports have demonstrated that cell spreading is induced by PKC activation (Haller et al., 1998). Previous studies reported that the activation of PKC may result from the increase in phospholipase C activity induced following integrin engagement (Cybulsky et al., 1993; Plopper et al., 1995; Banno et al., 1996; Zhang et al., 1999). For example, in vascular smooth muscle cells, diacylglycerol content increases as early as 10 minutes after plating on FN (Haller et al., 1998). The activation of PKC by PMA also promotes cell attachment and spreading on extracellular matrix proteins (Mercurio and Shaw, 1988; Vuori and Ruoslahti, 1993; Disatnik and Rando, 1999; Miranti et al., 1999). PKC activation has been found to precede the morphological changes that are characteristic of cell spreading (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993), suggesting that a target of PKC activity may be important in regulating those morphological changes.

Our results have focused on the role of individual PKC isozymes in integrin-mediated muscle cell adhesion and spreading. The specific substrates of these isozymes are still not known. As focal adhesion formation is integrally linked to cell spreading (Kornberg et al., 1992; Hanks et al., 1992), the proteins that constitute these sites are obvious candidates as targets of PKC activity. Indeed, components of the cytoskeleton as well as focal adhesion proteins were reported to be regulated by PKC signaling (Woods and Couchman, 1992; De Nichilo and Yamada, 1996; Emkey and Kahn, 1997; Adams et al., 1999). The localization of αPKC at focal adhesion sites that we describe here and as has been reported by others (Liao and Jaken, 1993) may phosphorylate proteins at these sites. The focal adhesion protein, paxillin, is phosphorylated on serine and threonine and has been shown to shuttle from focal adhesions to a trans-Golgi-endosomal network, accompanied by vinculin (Brown et al., 1998; Norman et al., 1998). Our finding, in this report, of δ and εPKC at the Golgi apparatus and around the nucleus suggests that these isozymes may likewise be involved in the regulation of focal adhesion proteins.

MARCKS is a PKC substrate that cycles on and off membranes by a mechanism termed the myristoyl-electrostatic switch (Aderem, 1992b). It is a protein known to crosslink actin filaments regulated by PKC and therefore is important in the stabilization of the cytoskeletal structure (Hartwig et al., 1992). MARCKS has been reported to be involved in cell spreading in several systems (Rosen et al., 1990; Li et al., 1996; Manenti et al., 1997; Myat et al., 1997; Spizz and Blackshear, 2001). Myat et al. (Myat et al., 1997) demonstrated that MARCKS regulates membrane ruffling and fibroblast cell spreading. They reported that fibroblast spreading is inhibited in cells expressing a MARCKS mutant that fails to translocate upon PKC activation. To support these data, a recent report by Spizz and Blackshear (Spizz and Blackshear, 2001) demonstrated that the localization of MARCKS at the membrane may inhibit cellular adhesion. Myat et al. (Myat et al., 1997) reported direct evidence that PKC-dependent MARCKS activation regulates actin-dependent membrane ruffling and fibroblast adhesion. Actin crosslinking increases the viscosity and stiffness of the actin filament network, stabilizing the actin-rich cell cortex. The spreading mechanism of the cells requires that stress fibers are rapidly disassembled and filopodia and lamellipodia are extended at the leading edge of moving cells to make contact with the matrix (Haimovich et al., 1996). The rigidity caused by actin polymerization is probably a negative regulator of cell spreading (Aderem, 1992a). Indeed, it is known that PKC increases cell spreading and concomitantly inhibits stress-fiber formation and causes reorganization of actin filaments. We found that, in skeletal muscle cells, MARCKS is initially localized to focal adhesion sites and quickly translocates to the cytosol upon integrin activation, suggesting that MARCKS activation is an early event in cell attachment and spreading. Poussard et al. (Poussard et al., 2001) provided evidence of a MARCKS-PKCα complex in skeletal muscle by chromatography, consistent with our data showing MARCKS and PKCα at focal adhesion sites. In this report, we demonstrate that MARCKS is essential for muscle cell spreading. Muscle cells that do not express MARCKS protein failed to spread on FN. Together, these results indicate that MARCKS is a component of the integrin pathway, downstream of PKC, that mediates skeletal muscle cell spreading.

We have previously demonstrated that inhibition of PKC blocks FAK phosphorylation and muscle cell spreading on FN (Disatnik and Rando, 1999), which is comparable to similar responses in other cells (Woods and Couchman, 1992; Haimovich et al., 1996). However, the roles of respective PKC isozymes have not been widely studied. Haller et al. found that inhibition of specific PKC isozymes with pharmacological agents or antisense oligonucleotides resulted in a significant decrease in cell adhesion and spreading (Haller et al., 1998). In this report, we show that α, δ and εPKC are expressed in cultured skeletal muscle cells, and we demonstrate that an increase in activity of these isozymes is detectable after cell plating on FN. Activation of αPKC, δPKC and εPKC following cellular adhesion has been previously reported (Chun et al., 1996; Miranti et al., 1999). In C2C12 muscle cells, α, γ and λPKC are expressed, but only αPKC was found to be activated upon cell adhesion to FN (Adams et al., 1999). Activated εPKC has been shown to restore spreading of cells in which integrin signaling had been disrupted (Berrier et al., 2000). Chun et al. reported that εPKC becomes activated upon cell attachment (Chun et al., 1996). These studies and our present report reveal that εPKC plays an important role in cell attachment and spreading. The early activation of εPKC in response to integrin binding (Fig. 2) seems to be a critical early event in integrin signaling that promotes cell spreading and cell survival. However, it is also clear that other PKC isozymes are necessary in the signaling cascade, and the later activation of both α and δPKC (Fig. 2) as well as their ability to promote muscle cell spreading (Fig. 5) suggest that they have, perhaps, overlapping functions in the downstream signaling cascade.

The specific function of the homologous PKC isozymes is determined by their subcellular localization (Disatnik et al., 1994; Ron and Kazanietz, 1999; Dempsey et al., 2000). Upon activation, each PKC isozyme translocates to a specific site where it is anchored by a specific RACK, a receptor for activated C kinase (Mochly-Rosen, 1995). Wrenn and Herman (Wrenn and Herman, 1995) have recently demonstrated PKC isozyme translocation upon integrin occupation. Other investigators have demonstrated αPKC localization at focal adhesion sites and have suggested that this isozyme may mediate cell spreading by targeting an unknown substrate at this site (Liao and Jaken, 1993; Haller et al., 1998). Our results also reveal that αPKC is found at focal adhesion sites at a time when cells are beginning to spread. Other studies of the cellular localization of PKC isozymes have suggested an important role in integrin function (Chun et al., 1996). In mammary epithelial cells, αPKC and β1 integrin were found to colocalize, and αPKC was shown to regulate the cellular distribution of β1 integrin, demonstrating a critical role for αPKC in dynamic control of integrin function (Ng et al., 1999). Liliental and Chang (Liliental and Chang, 1998) recently reported a direct association of RACK1 with the integrin β subunit cytoplasmic domain. They showed that the interaction of RACK1 with integrins in vivo requires activation of PKC, which promotes cell spreading and integrin-dependent cell adhesion. These results suggest a direct linkage between integrins and PKC through RACK1 and further implicate PKC in integrin-mediated cell signaling.

We previously proposed a model suggesting a positive feedback loop of integrin engagement, signaling and activation in which we showed the role of PKC (Disatnik and Rando, 1999). The results in this report support the model that integrin engagement with FN mediates the activation of εPKC, which leads to cell attachment and spreading, and these processes involve the organization of actin stress fibers as well as the recruitment of focal adhesion proteins to focal adhesion sites. We show that cell attachment initiates a low level of FAK phosphorylation and a transient activation of αPKC and δPKC. FAK and PKC signaling pathways induce an 'inside-out' signaling, creating a positive feedback loop (Disatnik and Rando, 1999). Further activation of integrins promotes an increase of FAK phosphorylation and finally cell spreading. Here, we demonstrate the importance of εPKC activation in cell attachment that precedes a downstream cascade of biochemical changes involving the activation of α and δPKC. Furthermore the data presented here link the upstream initiators of integrin and PKC signaling with the downstream processes of focal adhesion formation, stress fiber dynamics and finally cell spreading. We provide evidence that MARCKS is a key signaling molecule downstream of the PKC pathway that may mediate these cellular changes. These results add our understanding of the cellular components involved in the integrin-signaling cascade, regulating cellular adhesion, attachment and spreading.

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