Immunohistochemical identification and fiber type specific localization of protein kinase C isoforms in equine skeletal muscle

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Objective—To investigate whether protein kinase C (PKC) isoforms are expressed in equine skeletal muscle and determine their distribution in various types of fibers by use of immunofluorescence microscopy.

Animals—5 healthy adult Dutch Warmblood horses.

Procedure—In each horse, 2 biopsy specimens were obtained from the vastus lateralis muscle. Cryosections of equine muscle were stained with PKC isoform (α, β1, β2, δ, ε, or ζ)-specific polyclonal antibodies and examined by use of a fluorescence microscope. Homogenized muscle samples were evaluated via western blot analysis.

Results—The PKC α, β1, β2, δ, ε, and ζ isoforms were localized within the fibers of equine skeletal muscle. In addition, PKC α and β2 were detected near or in the plasma membrane of muscle cells. For some PKC isoforms, distribution was specific for fiber type. Staining of cell membranes for PKC α was observed predominantly in fibers that reacted positively with myosin heavy chain (MHC)-IIa; PKC δ and ε staining were more pronounced in MHC-I-positive fibers. In contrast, MHC-I negative fibers contained more PKC ζ than MHC-I-positive fibers. Distribution of PKC β1 was equal among the different fiber types.

Conclusions and Clinical Relevance—Results indicated that PKC isoforms are expressed in equine skeletal muscle in a fiber type-specific manner. Therefore, the involvement of PKC isoforms in signal transduction in equine skeletal muscle might be dependent on fiber type. (*Am J Vet Res* 2004; 65:69–73)

Protein kinase C (PKC) plays a major role in cell signaling.[1] In skeletal muscle, different atypical PKC isoforms have been identified and linked to carbohydrate metabolism. Although the involvement of PKC isoforms in carbohydrate metabolism has been a matter of discussion,[2] results of recent studies[3,4] indicate their involvement in insulin signaling and thus indirectly in cellular glucose uptake and metabolism.

On the basis of the manner of activation, the PKC family can be categorized into 3 distinct classes, each consisting of different isoforms. The classical isoforms of PKC are α, β1, β2, and γ, which are calcium- and diacylglycerol-dependent for their activation and use phosphatidyl serine as a cofactor. Other PKC isoforms (ie, δ, ε, η, and θ) are calcium-independent and only require diacylglycerol for their activation and use phosphatidyl serine as a cofactor. In addition, PKC ζ and λ (also known as ι) belong to the class of atypical PKC isoforms because these only require phosphatidyl serine as a cofactor. The PKC family has a wide variety of substrates in numerous cell types, including receptors, G proteins, enzymes, cytoskeletal and nuclear proteins, and proto-oncogene products.[1] Identification of which isoform is linked to which substrate is only partly elucidated.

In skeletal muscle, PKC isoforms are linked to **glucose transporter protein-4 (GLUT-4)** translocation and glucose regulation by insulin signaling.[5-8] In addition, PKC has been associated with diabetes mellitus and insulin resistance[9-13] and could play a role in excessive glycogen accumulation in equine muscle, such as that associated with polysaccharide storage myopathy in horses.[14] Interestingly, Valberg et al[15] found that type 2 muscle fibers were primarily affected in polysaccharide storage myopathy in horses.

To the authors' knowledge, PKC isoforms have been identified biochemically in skeletal muscle,[16,17] but their exact localization within muscle fibers has not been described in detail. This is surprising because the subcellular localization of PKC isoforms can provide information concerning their possible function in the physiologic functions of muscle. The purpose of the study reported here was to investigate whether PKC isoforms are expressed in equine skeletal muscle and determine their distribution in different types of fibers by use of immunofluorescence microscopy. We hypothesized that PKC isoforms are localized to specific types of muscle fibers.

Materials and Methods

Animals—Five healthy Dutch Warmblood horses owned by Utrecht University were used in this study. The mean age ± SD of the horses was 6.8 ± 1.8 years. There were 2 mares and 3 geldings with a mean weight of 633 ± 48 kg. The study was approved by the Committee on Animal Welfare of the Faculty of Veterinary Medicine, Utrecht University.

Biopsy specimens—From each horse, 2 percutaneous needle biopsy specimens were obtained by use of a modified Bergström needle with a diameter of 7 mm after induction of local anesthesia with lidocaine hydrochloride plus adrenalin.[a] The biopsy specimens were obtained from the vastus lateralis

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muscle at a point located 15 cm ventral from the center of the tuber coxa and 10 cm caudal from the cranial border of the vastus lateralis muscle; the specimens were obtained from 1 incision at a depth of 5 cm. The vastus lateralis muscle was selected for this study because training has shown to induce glycogen disposal and changes in metabolic enzyme activities in this muscle.[18] In addition, the triceps brachii and the pectoralis descendens muscles were evaluated in the same manner (data not shown). For these muscles, biopsy procedures were identical to those for the vastus lateralis muscle. Biopsy specimens from the triceps brachii were obtained at a point located 5 cm above the olecranon and 3 cm cranial of the caudal border of the triceps brachii muscle; the specimens were obtained from 1 incision at a depth of 4 cm. Biopsy specimens from the pectoralis descendens muscle were obtained at a point 20 cm caudal from the imaginary line through the shoulder joints in the middle of the muscle; the specimens were obtained from 1 incision at a depth of 4 cm. For immunohistochemical studies, 1 biopsy sample was frozen in isopentane that was cooled to melting point in liquid nitrogen. The other sample was frozen immediately in liquid nitrogen and used for biochemical analysis.

Homogenization of muscle specimens—Frozen muscle was homogenized in ice-cold homogenization buffer (20mM Tris-HCl, 10mM EDTA, 1% Triton-X100, and protease inhibitor cocktail[b]; pH, 7.4) by use of a tissue homogenizer and centrifuged (11,000 × *g* for 30 minutes at 4°C). The supernatant was diluted with an equal volume of sample buffer, boiled for 4 minutes, and centrifuged (10,000 × *g* for 5 minutes at 20°C). Processed samples were stored at –20°C until electrophoresis was performed.

Evaluation via western blot analysis—Homogenized muscle samples were separated via 10% polyacrylamide gel electrophoresis as described by Laemmli[19] and transferred to nitrocellulose for 1 hour (100 V, 4°C) in blotting buffer (25mM Tris, 192mM glycine, and 20% methanol). Nitrocellulose sheets were blocked with 2% nonfat dry milk and 0.05% Tween-20 in PBS solution for 30 minutes. Incubation of the nitrocellulose sheets with anti-PKC specific antibodies[c] (diluted 1:500 in 0.2% nonfat dry milk in PBS solution) was performed for 16 hours at 20°C with gentle shaking. Blots were washed 3 times (10 min/wash) in PBS solution containing 0.05% Tween-20 and incubated for 2 hours with horseradish peroxidase-conjugated swine antibodies raised against rabbit IgG[d] or horseradish peroxidase-conjugated goat antibodies raised against goat IgG[d] diluted 1:5,000 in 2% non fat dry milk in PBS solution containing 0.05% Tween-20. Blots were washed 6 times (15 min/wash) in PBS solution with 0.05% Tween-20 and for 10 minutes with PBS solution. Chemiluminescence was performed with a commercially available kit,[e] visualized with a phosphoimager,[f] and analyzed by use of a computer software program.[f]

Immunocytochemical evaluation of frozen muscle tissue sections—Muscle biopsy specimens were cryosectioned (5 µm), thaw-mounted on glass slides, air-dried at 20°C for 3 hours, and stored at –80°C. Prior to staining, sections were thawed and dried for 30 minutes. Sections were treated with PBS solution containing 0.5% Triton-X100 for 5 minutes; washed with PBS solution for 5 minutes; and probed for 2 hours with rabbit polyclonal PKC,[c] mouse **myosin heavy chain (MHC)**,[g] and mouse laminin[g]-specific antibodies diluted 1:25 in PBS solution containing 1% bovine serum albumin. After washing 3 times (5 min/wash) with PBS solution, the sections were incubated with goat polyclonal Alexa-conjugated antibodies[h] raised against rabbit IgG, mouse IgM, and mouse IgG diluted 1:100 (Alexa350) or 1:200 (Alexa488 and Alexa568) in PBS solution for 45 minutes. Finally, sections were washed 3 times with PBS solution and mounted with an antifade reagent.[i]

Image analysis—Images from all sections were captured by use of a fluorescence microscope[j] that was coupled to a digital CCD camera and processed with image software.[k] Exposure time of the fluorescent sections was constant for each antibody stain evaluated. Images were corrected for autofluorescence and background staining by use of data obtained from images of stained serial sections in which the primary antibody was omitted. From each biopsy specimen, 100 fibers were evaluated. The presence of cytosol or membrane staining in fibers was scored subjectively via visual assessment of the abundance of PKC isoforms. To exclude bias from assessments, procedures were performed by different researchers (biopsy procedures [EdGR and IDW], histochemical techniques and first assessments [MMEvG], and second assessments [HAK, GS, and EvB]). Subsequently, fields of serial sections were matched via visual assessment to determine fiber types on the basis of MHC composition. Laminin staining was used to identify individual fibers and membranes.

Results

Results of specific immunofluorescence staining of equine muscle sections with anti-human PKC antibodies illustrated the general application of these antibodies and possible conservation of PKC among different species. Assessment of equine muscle homogenates via western blot analysis confirmed specific binding of PKC antibodies (**Fig 1**). Native PKC isoforms were detected as bands of approximately 80 kd, whereas posttranslational-modified PKC isoforms appeared as bands between approximately 36 and 49 kd.[20,21] The lower bands of PKC isoforms β1, β2, and δ were comprised of the phosphorylated, constitutively active catalytic domain of PKC at approximately 49 kd and a smaller dephosphorylated inactive catalytic domain.

Five hundred fibers were analyzed; of these, 26% were identified as MHC-I-positive, 58% were MHC-IIa-positive, and 16% yielded negative results for MHC-I and MHC-IIa. Immunofluorescence staining of equine muscle sections with anti-PKC α revealed membrane fluorescence illumination in a subset of fibers (**Fig 2**). Triple-staining of serial sections with anti-MHC-I, anti-MHC-IIA, and antilaminin antibodies indicated that membrane staining of PKC α was restricted to approximately 80% of the MHC-IIA-positive fibers. Compared

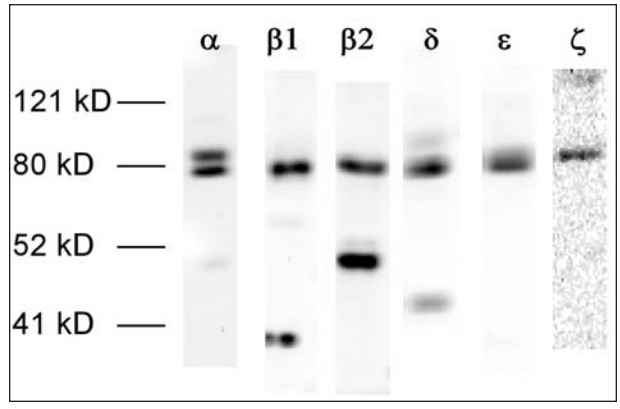

Figure 1—Results of western blot analysis of a homogenate of a specimen of skeletal muscle from a horse. Staining was performed with isoform-specific polyclonal protein kinase C (PKC) antibodies and peroxidase-conjugated secondary antibodies. Equal amounts of protein were applied. The position of the molecular weight marker in kilodaltons is indicated on the left.

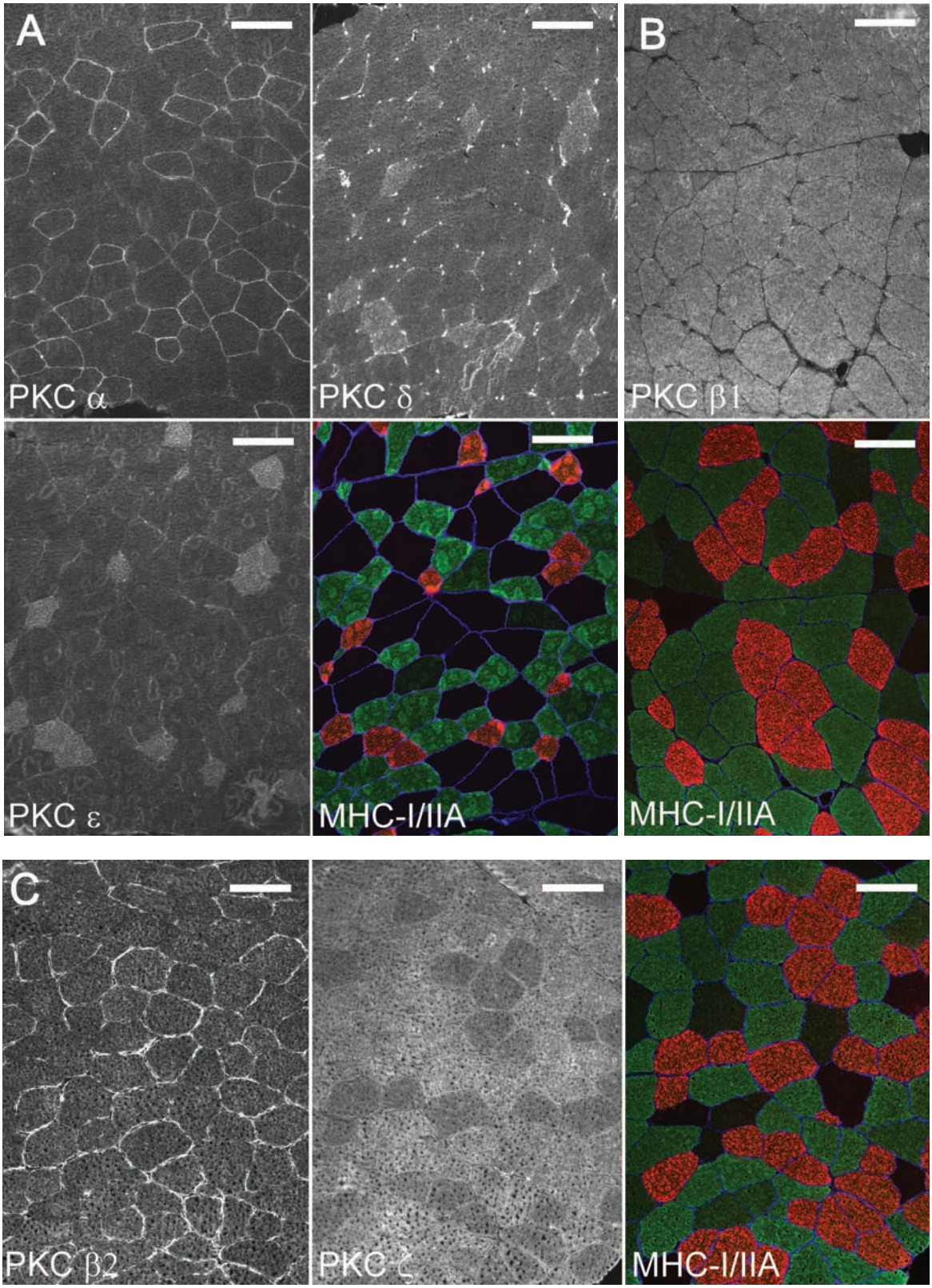

Figure 2—Immunofluorescence photomicrographs of serial sections of equine vastus lateralis muscle stained with polyclonal PKC antibodies and fluorescence-labeled secondary antibodies illustrating PKC isoform distribution in skeletal muscle of horses. A—Notice that PKC α expression is high near the plasma membrane of type 2a fibers (ie, green myosin heavy chain ([MHC])-IIA-stained fibers). Expression of PKC δ and ε is higher in type 1 fibers (ie, red MHC-I-stained fibers) than in type 2a and 2b fibers (ie, black, unstained fibers). Bar = 100 μm. B—Notice that PKC β1 is distributed equally over all fiber types. Bar = 100 μm. C—Notice that PKC β2 is present near the plasma membrane of all fiber types and PKC ζ is predominant in both type 2a and 2b fibers. In the images of MHC-I/IIA, laminin staining is blue. Bar = 100 μm.

with the MHC-I-positive fibers, the MHC-I-negative fibers had greater anti-PKC ζ antibody staining. Immunofluorescence staining against PKC δ and ε was more intense in MHC-I-positive fibers than it was in MHC-I-negative fibers. Protein kinase C β1 and β2 were detected in all fibers. Interestingly, PKC β2 was predominantly found in the membrane area of the muscle fibers. The specific distribution of PKC isoforms did not appear to be specific for fibers of the vastus lateralis muscle because fibers of the triceps brachii and pectoralis descendens muscles had similar staining patterns (data not shown).

Discussion

Results of the study of this report indicated the presence and localization of different PKC isoforms in equine skeletal muscle. Protein kinase C plays a major role in signaling events in a wide variety of cells. In skeletal muscle cells, different PKC isoforms have been linked to glucose metabolism.[22] Furthermore, it has been shown that PKC isoforms play a role in the insulin signaling pathway.[3,4] Our data indicated the presence and cellular localization of PKC α, β1, β2, δ, ε, and ζ in different skeletal muscle fibers of horses. To identify different fiber types, we applied the MHC staining technique. Although ATPase staining has been used extensively in equine muscle research, Rivero et al[23] recently performed a well-designed study that established a correlation between ATPase staining and MHC staining in equine muscle. Findings of that study confirmed data obtained from a similar study[24] in other species.

In approximately 80% of the MHC-IIa-positive fibers (classified as type 2a fibers), there was intense membrane-localized staining for PKC α. This localization of PKC α is consistent with findings of other immunohistochemical studies in humans[25] and rabbits[26] and denotes an important role for PKC α in receptor-mediated signaling. In addition, Chin et al[27] reported that PKC α reduced the insulin-stimulated increase in antiphosphotyrosine-precipitable phosphatidyl inositol-3 kinase activity in Chinese hamster ovary cells.

Although PKC β2 was also localized in the vicinity of the plasma membrane, PKC β1, δ, ε, and ζ were distributed more homogenously within skeletal muscle fibers. In addition, PKC δ and ε were detected in MHC-I-positive fibers (classified as type 1 fibers). Furthermore, MHC-I-negative fibers (type 2a and 2b fibers) contained more PKC ζ than did MHC-I-positive fibers. This might suggest different roles for PKC isoforms in signaling events related to glucose uptake and insulin signaling.[28,29] One of the major limitations of the aforementioned published studies[3,4,22,27] is the evaluation of whole-muscle homogenates and muscle fractions only. Contamination among membrane and subcellular organelle-specific fractions may well have influenced the results of those studies with regard to PKC isoform distribution.

Nevertheless, it is noteworthy that differential expression of other proteins (ie, uncoupling protein 3[30] and GLUT-4[21]) that are involved in muscle glucose metabolism has been identified in different muscle fibers as well. Thus, it is reasonable to assume that a link between glucose uptake and metabolism and PKC isoform expression is fiber type-specific. Whether the expression of different PKC isoforms in muscle is fiber type-specific is a matter of further investigation.

In conclusion, our data indicated that PKC isoforms are expressed differently in type 1 and type 2 skeletal muscle fibers in horses and suggested a possible role of these isoforms in glucose or insulin signaling events. The role of PKC isoforms in signaling pathways and pathologic processes in muscles of horses remains to be elucidated.

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[a]Alfacaine 2% plus adrenalin (100 ml), Alfasan, Woerden, the Netherlands.
[b]Complete, Roche, Mannheim, Germany.
[c]sc-208/sc-209/sc-210/sc-937/sc-214/sc-216, Santa Cruz Biotechnology, Santa Cruz, Calif.
[d]DAKO, Glostrup, Denmark.
[e]Super Signal West Dura Extended kit, Pierce, Rockford, Ill.
[f]FluorS Imager and QuantityOne software program, Bio-rad Laboratories, Hercules, Calif.
[g]A4.840 and N2.261 (developed by H. M. Blau) and 2E8 (developed by E. Engvall) provided by the Developmental Studies Hydridoma Bank (under the auspices of the National Institute of Child Health & Human Development) and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, Iowa.
[h]Alexa-conjugated antibodies, Molecular Probes, Leiden, the Netherlands.
[i]Mowiol, Calbiochem, San Diego, Calif.
[j]Nikon Eclipse SE800, Uvikon, Bunnik, the Netherlands.
[k]Lucia image software, Laboratory Imaging Ltd, Prague, Slovakia.

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