

Activity of novel protein kinase C and distribution of protein kinase C θ in subcellular fractions of normal and Duchenne muscular dystrophic muscle

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Phospholipid-dependent, Ca²⁺-independent isoenzymes termed novel protein kinase C or nPKC, include PKC δ , ϵ , η , θ and μ . Status and role of nPKC and PKC θ in Duchenne muscular dystrophic (DMD) condition is unknown. In the present study, we have shown that most of the nPKC isoforms are translocated to the membrane fraction of DMD tissue specimen. It is well established that translocation plays a key role in signal transduction by individual PKC isoforms. In our experiment, the increased association of nPKC isoform PKC θ to membrane was further confirmed by Western blot. Increased expression of PKC θ mRNA was identified by dot blot analysis. The above results suggest that, the alterations in nPKC location and increased expression of PKC θ observed is a result of modification of PKC-mediated signal transduction and cell function.

The mutant gene responsible for Duchenne muscular dystrophy (DMD) has been identified, as dystrophin. The function of dystrophin is not fully understood though it has been implicated in submembranal architecture, as well as in calcium regulation. In the absence of dystrophin, the pathological events of DMD in response to calcium and calcium-dependent protein kinases suggest a key role of protein kinase C (PKC) in muscle cell death. Activation of PKC in U937 leukaemic cells was accompanied by marked alterations in the expression of protein governing apoptosis. These alterations were largely absent in the PKC-deficient U937 subclone TUR¹. This evidence suggests key role of PKC in cell signaling machinery.

Molecular cloning has revealed that PKC is a multigene family encoding for closely related serine/threonine specific protein kinases. Alignment of the predicted amino acid sequence led to identification of 4 conserved (C1 to C4) and 5 variable regions (V1 to V5). On the basis of structural difference, 4 subfamilies of isoforms have been identified that include classical isoforms (c) α , β I, β II and γ ; novel isoforms (n) ϵ , δ , θ , and n/L ; atypical isoforms (a) ζ and λ ; and mu isoform, (m) μ /PKD.

Novel and atypical subfamilies lack C2 region, which contains the Ca²⁺-sensitive site. The classical PKC (cPKC) and novel PKC (nPKC) isoforms are activated by diacylglycerol, phospholipid and phorbol esters. However, calcium activates cPKC also, while atypical PKC (aPKC) isoforms are activated by phospholipids only. The conventional mechanism for activation of Ca²⁺-dependent or independent PKC enzymes involves receptor-mediated activation of phospholipase C (PLC) and production of second messenger, diacylglycerol (DAG); DAG binds to the cystein rich C1 region in the NH₂-terminal regulatory domain of the kinase. This leads to recruitment of the kinase to the membrane and initiation of its activation via complex mechanisms involving Ca²⁺-binding (for Ca²⁺-dependent PKCs) and regulatory phosphorylation events^{2,3}.

Individual PKC isoforms differ in their cofactor requirements and tissue expression⁴. The specific combination of isoenzymes expressed in a given cell may, therefore, determine the outcome of the PKC-dependent response of that particular cell⁵. Protein kinase C θ (PKC θ), a Ca²⁺-independent, phorbol ester stimulated and phospholipid-dependent nPKC isoform, which is selectively expressed in T-lymphocytes and skeletal muscle, has several unique properties that attest to its important role in T-cells and skeletal muscle⁶. The biological activity of PKC enzymes is closely regulated by their subcellular localization². In the present study, the status of novel

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Abbreviations used:

PMSF, Phenyl methyl sulfonyl fluoride; TPA, 12-O-tetradecanoyl phorbol-13-acetate; NF- κ B, nuclear factor of κ B; NP-40, Nonidet P-40.

kinases, especially PKC θ has been assessed in dystrophic muscle in order to know its importance in the pathogenesis.

Materials and Methods

Tissue sample

Educated consent from parents of patients under the scrutiny of the ethical committee of Nizam's Institute of Medical Sciences (NIMS), Hyderabad was followed. Skeletal muscle tissues from 6 DMD patients were obtained from diagnostic muscle biopsies. Normal human control muscle biopsies were received from 6 patients undergoing orthopedic surgery not related to neuromuscular disorders. Control and disease patients were matched by age and gender.

Preparation of subcellular fractions

Muscle biopsy samples (~200 mg wet wt) from control and DMD patients were homogenized in 5 volumes of cold homogenizing buffer A [20 mM Tris-HCl buffer, pH 7.5 containing 250 mM sucrose, 1 mM EGTA, 2 mM EDTA, 50 mM 2-mercapto ethanol, 2 mM PMSF, 10 mM benzamidine, 10 μ l/ml sodium orthovanadate (100 mM) and 5% glycerol] using a polytron homogeniser (Kinematic, Leuzerne) setting at 5 to 15 sec burst (4 $\times$). The supernatant was ultra centrifuged for 60 min at $1,00,000 \times g$ and the resulting supernatant (cytosolic fraction) was removed and stored at 4°C. The pellet was resuspended in the homogenization medium containing 1% NP-40, gently mixed for 45 min on ice and centrifuged at $1,00,000 \times g$ for 30 min. The resulting supernatant was the soluble particulate fraction.

Nuclear fraction was isolated from normal and dystrophic muscle biopsy specimens as described⁷, with certain modifications. Muscle specimens (200 mg wet wt) were minced in 5 ml ice-cold homogenization buffer B (10 mM HEPES buffer, pH 7.5 containing 5 mM KCl, 10 mM MgCl₂, 5 mM 2-mercaptoethanol and 0.32 mM sucrose) and homogenized by polytron homogenizer (Kinematic, Luzerene) set at 5 to 30 sec burst homogenization for 3 times. The homogenate was passed through four layers of cheese-cloth and filtered using a 100 mesh, 150-micron standard sieve (Fischer Scientific). The filtrate was centrifuged at $1000 \times g$ for 10 min. The crude nuclear pellet was resuspended by gently triturating with 3.5 ml of ice-cold homogenization buffer B containing 2.4 M sucrose and centrifuged at

$80,000 \times g$ for 90 min in a sorval AH-650 rotor. Resultant nuclear pellet was gently rinsed with wash buffer and nuclei were re-pelleted by centrifugation at $5000 \times g$ for 10 min. Isolated nuclei were examined under phase contrast microscope (10 $\times$).

Novel protein kinase C assays

PKC was partially purified from the cytosolic and particulate fractions of muscle⁸. Protein concentrations were determined by Folin-Lowry method.

nPKC assays were carried out in cytosolic and particulate fractions of DMD and control muscle⁹. The reaction conditions for assay contained 20 mM Tris-HCl pH 7.5, 4 mM MgCl₂, 100 μ M ATP, 20 μ Ci/ml of [γ ³²P] ATP, 160 μ g/ml histone HHS, 80 nM TPA, 50 μ g/ml cardiolipin (CL) and 1 mM EGTA. Aliquots of each sample (50 μ l) were incubated with above reaction mixture (final volume, 250 μ l) and the reaction was initiated by adding [γ ³²P] ATP. Each fraction was incubated for 5 min at 30°C. The reaction was stopped by adding 1 ml of ice cold TCA (20% v/v) and 10 mM potassium monophosphate, followed by addition of BSA (500 μ g) as a carrier protein. The mixture was centrifuged at $2500 \times g$ for 15 min. The resulting pellet was solubilised with 1 N NaOH followed by reprecipitation with TCA. Finally, the pellet was dissolved in 1 N NaOH and its radioactivity was counted by liquid scintillation.

Immunoblot analysis of PKC θ

Protein samples were prepared by solubilising the nuclear and particulate fractions with SDS and reducing agents such as 2-mercaptoethanol and electrophoretically separated on 8% SDS-PAGE¹⁰. Following size separation, proteins were transferred to nitrocellulose membrane by electroblotting¹¹. The blot was probed with polyclonal rabbit PKC θ antibody raised against carboxyl terminus of PKC θ (Santa Cruz Biotechnology, USA) followed by anti rabbit IgG HRPO conjugate. Final detection was done by enhanced chemiluminescence (Amersham Pharmacia Biotech, USA). The blot was subjected to autoradiography and developed after brief exposure.

Dot blot analysis of PKC θ expression

Isolation of total RNA was carried out from muscle biopsy specimens of normal and DMD patients¹², the amount of RNA isolated was 120 μ g per 200 mg wet wt muscle. Northern blot analysis was carried out as

described¹³. Serially diluted (10 to 0.312 μ g) total RNA was spotted on nitrocellulose membrane and the amount of specific mRNA was determined by hybridizing with radio labeled nPKC θ gene specific DNA probe. The intensity of hybridization signal on autoradiogram of normal and dystrophic muscle was quantified by scanning densitometer.

Results and Discussion

Novel protein kinase C assay

The nPKC activity in cytosolic fractions of normal and dystrophic muscles was 223 ± 9 and 92 ± 8 pico moles/min/mg of protein, respectively. There was 2.4 fold decrease in nPKC activity in dystrophic muscle (Fig. 1A). Decreased activity of PKC in group 1, 2 and 4 of DMD cytosolic fraction in the absence of Ca^{2+} confirms decreased activity of nPKC. The nPKC activity in particulate fraction of control and dystrophic muscles was 106 ± 12 and 307 ± 11 pico moles/min/mg of protein, respectively. A 3-fold increase in nPKC activity has been observed in particulate fraction of dystrophic muscle compared to control (Fig. 1B). Increased activity of PKC in group 1, 2 and 4 of DMD particulate fraction, in the absence of Ca^{2+} confirms increased activity of nPKC.

The above results suggest that, in healthy condition, nPKC isoforms are mostly present in cytosol of muscle. As the muscular dystrophy progresses, most of the nPKC isoforms are located in the membrane fraction. It implies that muscular dystrophy is characterized by the increased presence of nPKC isoforms. Immuno-cytochemical staining of skeletal muscle demonstrated that a large fraction of the cellular PKC θ is found in the cell membrane, while the ubiquitously expressed PKC α was found in the

cytosol (data not shown). Translocation plays a key role in signal transduction by individual PKC isoforms¹⁴. Therefore, the alterations in nPKC location observed in our experiments could result in modification of PKC-mediated signal transduction and cell function.

Densitometric analysis of PKC θ immunoblot

The increased association of novel isoforms to membrane in DMD was further confirmed by Western blot analysis. Novel protein kinase represented by PKC θ , a predominant isoform from muscle was monitored in solubilised muscle particulate or nuclear fraction electrophoresed on polyacrylamide gel. The specificity of the primary antibodies used in quantification of PKC θ was evident from the immunoblot showing a single 80 kD protein band (Fig. 2A, B).

Densitometric analysis of immunoblot shows that PKC θ (Fig. 3) was translocated highly to the particulate fractions of dystrophic muscle (~62%) than in normal muscle. In contrast, protein levels of PKC α were high in cytosolic fractions of normal muscle (~60%) than the dystrophic muscle (data not shown). In case of nuclear fraction, PKC θ content was high in dystrophic condition (~66%) than in normal muscle. Analysis of PKC θ expression at protein level confirmed previous findings about its unique expression profile in skeletal muscle^{6,14-16}. Nuclear localization of PKC θ in dystrophic muscle also indicates distinct role for it in regulation of gene expression, the transcription factors NF- κ B and AP-1 being the primary targets of PKC θ ¹⁷. It was also evident from denervated muscle cells when stimulated, that nuclear PKC is activated within

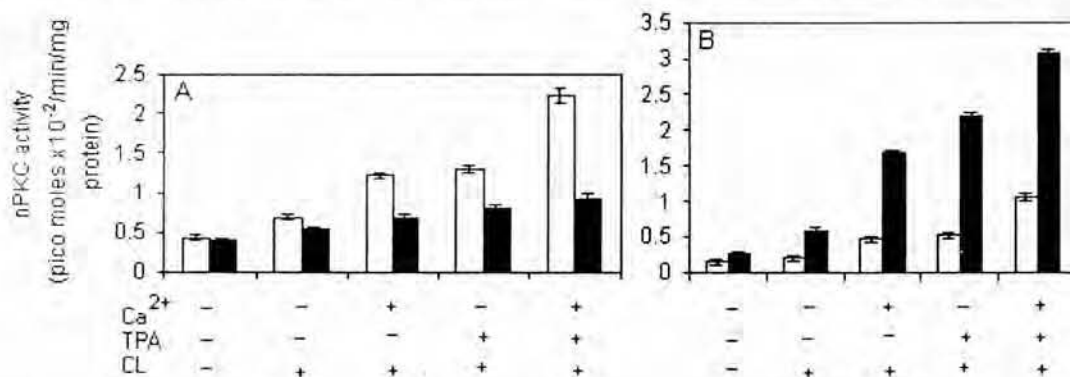


Fig. 1—Effect of Ca^{2+} , phorbol ester and phospholipid on novel PKC activity of (A): cytosol fraction; (B): particulate fraction of Control (□) and DMD (■) muscle [The kinase activity was assayed as described in Materials and Methods. The concentration of the cofactors was 0.6 mM Ca^{2+} ; 50 μ g/ml cardiolipin (CL); and 80 nM TPA. Data are from 6 DMD patients and corresponding 6 control samples and are expressed as mean $\pm$ SEM for the experiments (n=6) done in duplicate. Statistical significance, $p < 0.05$]

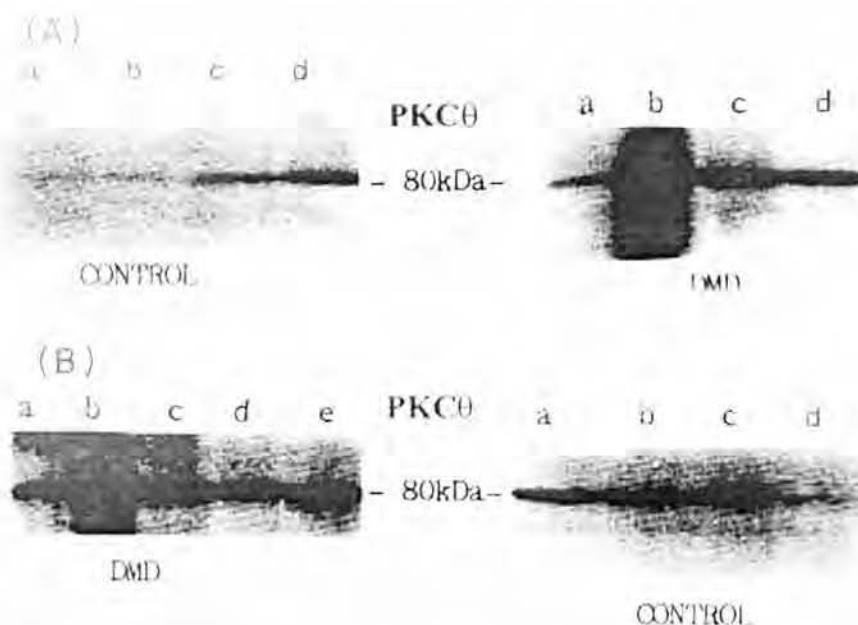


Fig. 2—Immunoblot analysis of PKCθ (A): in the particulate fractions and (B): in the nuclear fractions of control and dystrophic muscle tissues [Solubilised fractions were separated on SDS-PAGE (8% gel), followed by electrophoretic transfer to nitrocellulose membrane. Each membrane was incubated with polyclonal PKCθ antibodies and immuno-reactive band was detected as described in Materials and Methods. The major immuno-reactive band corresponds to 80 kD PKCθ isoform in the particulate and nuclear fractions. a, b, c, d represent the different DMD and control samples subjected to immunoblot analysis]

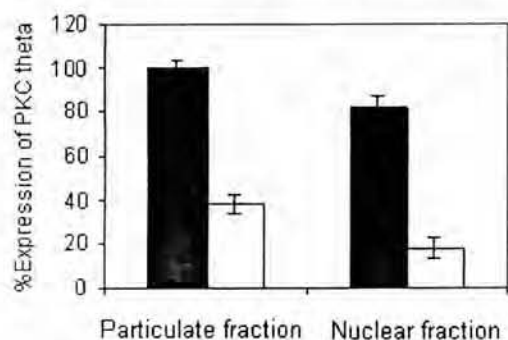


Fig. 3—Densitometric scan of bands in Fig. 2A and B (□), DMD; (■) control. [Particulate and nuclear fractions were separated by SDS-PAGE and transferred onto nitrocellulose membranes, which were then probed with PKCθ antibodies as described in Materials and Methods. Maximal PKCθ expression in the membrane fractions of dystrophic muscle was observed and taken as 100% for the purpose of comparison. Error bars indicate SEM (n=4). Densitometric analysis of the autoradiograms was performed using a computer-interfaced ultrascan X Laser densitometer (LKB, Sweden).

minutes through phosphorylation of myogenin protein and shuts down myogenin dependent genes including those coding for AchR subunits and for myogenin itself¹⁸. Similarly, in cultured muscle cells activation leads to translocation of PKC to nuclei, which ultimately decreases myogenin D and myogenin mRNAs¹⁹. Both the expression levels and distribution

of PKCθ undergo changes along the process of neuronal differentiation²⁰. This increased association of PKCθ in membrane and nuclear fractions could have implications in specific modification of physiology in dystrophic muscle.

Dot blot analysis of PKCθ expression

Whether increased presence of PKCθ in membrane fractions, was due to increased new synthesis, was probed by dot blot analysis of total RNA using nPKCθ gene probe. From densitometric signal with respect to serially diluted total RNA (Fig. 4) from control and dystrophic muscles it has been observed that PKCθ is expressed 2.25-fold more in dystrophic muscle. The increased expression of PKCθ has been linear within the specific concentration range (2.5 to 0.625 µg) of total RNA. This unique expression of PKCθ in dystrophic muscle suggests that it may play specialized role in dystrophic muscle death through unrevealed signaling pathways. Previous studies have reported the physiological role of PKCθ in normal skeletal muscle¹³, while there have been no reports on PKCθ in dystrophic condition. Involvement of PKC in differentiation of skeletal muscle, especially in myoblast fusion, was suggested from studies with chicken and mouse myoblast^{21,22}. Involvement of

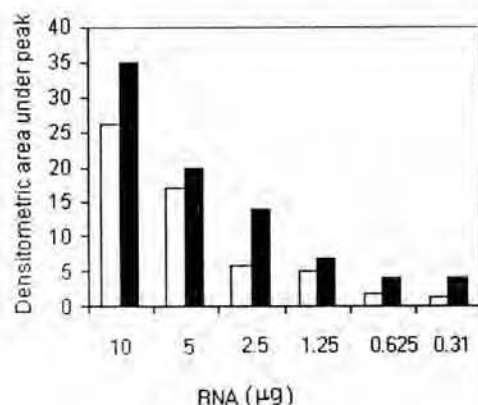


Fig. 4—RNA dot blot of control (□) and dystrophic (■) muscle [The nitrocellulose membrane contained serially diluted mRNA from control and DMD muscle biopsy and hybridized with radiolabelled nPKC θ probe. Densitometric scan was made through each dot. The graphical representation shows the relationship between the amount of RNA in each dot and the area under densitometric peak of each dot. This is a representative of one experiment with DMD and normal muscle]

PKC in insulin-stimulated glucose transport of BC3H-1 myocytes was also reported²³. In our experiment, most of the novel PKC isoforms are translocated to the membrane fraction. The increased association of PKC θ in particulate and nuclear fractions and increased expression of PKC θ gene suggest that the alteration in PKC location observed in our experiment would result in modification of PKC mediated signal transduction and cell function.

A Ca²⁺-independent/cardiophilin stimulated activity of PKC in partially purified enzyme is observed in cytosolic and particulate fractions of normal and DMD muscles. A significant difference observed in the cytosolic and membrane associated activities between DMD and normal, suggests an enhanced association of this novel protein kinase C to membrane in disease. Quantitation by Western blot analysis of the nPKC θ showed that both membrane and nuclear fractions recorded about 60% more of this isoform in DMD muscle. Quantitation of PKC θ mRNA using dot blot analysis of total muscle RNA, revealed that the dystrophic muscle expressed 2.25-fold more of this RNA, suggestive of *de-novo* synthesis of this kinase.

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