Protein kinase C\(\delta\) promotes proliferation and induces malignant transformation in skeletal muscle

Gabriella Czifra a,*, Attila Szöllösi a, Zsuzsanna Nagy a, Miklós Boros a, István JuháSZ b, Andrea Kiss c, Ferenc Erdődi c, Tamás Szabó d, Ilona Kovács e, Miklós Török e, László Kovács a, Peter M. Blumberg f, Tamás Biró a

a DE-MTA “Lendület” Cellular Physiology Research Group, Department of Physiology, Medical Faculty, University of Debrecen, Research Center for Molecular Medicine, Debrecen, Hungary
b Department of Dermatology, Medical Faculty, University of Debrecen, Research Center for Molecular Medicine, Debrecen, Hungary
c Department of Medical Chemistry, Medical Faculty, University of Debrecen, Research Center for Molecular Medicine, Debrecen, Hungary
d Department of Pediatrics, Medical Faculty, University of Debrecen, Research Center for Molecular Medicine, Debrecen, Hungary
e Department of Pathology, Gyula Kenézy Hospital, Debrecen, Hungary
f Laboratory of Cancer Biology and Genetics Center for Cancer Research, National Cancer Institute, Bethesda, MD, USA

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Abstract

In this paper, we investigated the isoform-specific roles of certain protein kinase C (PKC) isoforms in the regulation of skeletal muscle growth. Here, we provide the first intriguing functional evidence that nPKC\(\delta\) (originally described as an inhibitor of proliferation in various cell types) is a key player in promoting both in vitro and in vivo skeletal muscle growth. Recombinant overexpression of a constitutively active nPKC\(\delta\) in C2C12 myoblast increased proliferation and inhibited differentiation. Conversely, overexpression of kinase-negative mutant of nPKC\(\delta\) (DN-nPKC\(\delta\)) markedly inhibited cell growth. Moreover, overexpression of nPKC\(\delta\) also stimulated in vivo tumour growth and induced malignant transformation in immunodeficient (SCID) mice whereas that of DN-nPKC\(\delta\) suppressed tumour formation. The role of nPKC\(\delta\) in the formation of rhabdomyosarcoma was also investigated where recombinant overexpression of nPKC\(\delta\) in human rhabdomyosarcoma RD cells also increased cell proliferation and enhanced tumour formation in mouse xenografts. The other isoforms investigated (PKC\(\alpha\), \(\beta\), \(\epsilon\)) exerted only minor (mostly growth-inhibitory) effects in skeletal muscle cells. Collectively, our data introduce nPKC\(\delta\) as a novel growth-promoting molecule in skeletal muscles and invite further trials to exploit its therapeutic potential in the treatment of skeletal muscle malignancies.

Keywords: Skeletal muscle • C2C12 myoblasts • rhabdomyosarcoma • protein kinase \(\delta\) • PKC isoenzymes • recombinant overexpression • proliferation • differentiation • tumourigenesis

Introduction

The protein kinase C (PKC) system is a central intracellular signalling pathway regulating various cellular processes such as proliferation, differentiation, apoptosis and tumourigenesis [1–4]. Up to date, at least 11 PKC isoenzymes were identified which can be classified to the calcium- and phorbol ester-dependent ‘conventional’ (PKC\(\alpha\), \(\beta\), \(\beta\)I and \(\gamma\); cPKCs); the calcium-independent ‘novel’ (PKC\(\delta\), \(\epsilon\), \(\eta\) and \(\theta\); nPKCs); the calcium- and phorbol ester-independent ‘atypical’ (PKC\(\zeta\), and \(\lambda\); aPKCs); and PKD groups. These isoforms isozyme-specifically and very often differentially regulate the given cellular mechanism [3, 5, 6]. Furthermore, not only may some PKC isoforms be active whereas others not for a given response but different PKC isoforms may have antagonistic effects on the same cellular event [7–9].
Various PKC isoforms were shown to control certain cellular functions in skeletal muscles as well. For example, nPKCα were implicated in mediating the complex effect of insulin to control muscle homoeostasis [10], whereas cPKCα and nPKCδ were shown to participate in the effect of tumour necrosis factor-α to inhibit insulin signalling [11, 12]. In addition, nPKCζ and aPKCκ were found to positively regulate glucose and monocarboxylate transport [13–15] while aPKCδ and λ were documented to play a role in the regulation of exercise-related changes in metabolic and gene-regulatory responses of human skeletal muscle [16].

We, however, possess extremely limited information about the isoform-specific involvement of the PKCs in the regulation of physiological and pathological in vitro and in vivo growth of skeletal muscle cells [17, 18]. cPKCα was introduced as a central promoter of cellular growth of cultured avian myoblasts [19, 20] while nPKCδ was suggested to promote differentiation of mouse [21] and human [22] skeletal muscles. PKC isoforms are suggested to function as oncogenes in rhabdomyosarcoma (RMS), the most common and lethal skeletal muscle sarcomas in children. Indeed, the phosphorylation levels of cPKCα, nPKCδ, nPKCζ and aPKCζ are up-regulated in alveolar and embryonal RMS as well [23].

We have previously shown [24] that nPKCδ – which isoform was previously suggested to inhibit proliferation, induces apoptosis and/or promotes differentiation [9] – plays a pivotal and exclusive role in mediating the in vitro growth-promoting effect of insulin-like growth factor-I (IGF-I) both in human skeletal muscle cultures and in the mouse C2C12 skeletal muscle myoblast cell line (which is very often used to model growth and differentiation of this tissue [25, 26]).

Therefore, as a continuation of the above study, in the present work – using combined molecular biology (recombinant overexpression), pharmacology (inhibitors), as well as in vitro assay (tumourigenesis in SCID mice) – our goal was to further dissect the role of nPKCs in the regulation of in vitro and, or further importance, in vivo growth of the cells. In addition, we also intended to define the specific roles of several other PKC isoforms in skeletal muscle growth. We report here for the first time that nPKCδ functions as a novel signaling molecule to promote in vitro and in vivo cell growth as well as to induce malignant transformation of skeletal muscle myoblasts.

Materials and methods

Antibodies for Western blotting

All primary antibodies against PKC isoforms were developed in rabbits and were shown to react specifically with the given PKC isoforms [9, 24, 27]. Anti-PKCα, β, δ, ε were from Sigma-Aldrich (St. Louis, MO, USA), whereas anti-PKCζ was from Santa Cruz BioTech (Santa Cruz, CA, USA). Specificities of anti-PKC antibodies were also tested by applying isoform-specific blocking peptides, which blocked the immunostaining in all cases [9]. Monoclonal mouse antibody against the intermediate filament protein desmin was from DAKO (Glostrup, Denmark), p44/42 MAP kinase (ERK 1/2) and phospho-p44/42 MAP kinase (phospho-ERK 1/2) antibodies were from Cell Signaling Technology (Beverly, MA, USA). In addition, monoclonal rabbit α-actin antibody (Sigma-Aldrich) was employed as internal control.

Generation of PKC constructs

Protein kinase C constructs were engineered as described previously [9, 24, 27–31]. Briefly, the cDNA sequences of PKCα, β, δ, ε, and α of the kinase (dominant)-negative (DN-nPKCα) mutant of nPKCs were subcloned into a metallothionein promoter-driven eukaryotic expression vector (MTH) [32]. The vector sequence encodes a C-terminal PKCα-derived 12 amino acid tag (cMTH) and attaches it to the end of the PKC proteins. As we previously described [29, 30], this epitope tag does not affect the functional properties of the given isoform.

Cell culture and transfection of cells

The C2C12 myoblasts (obtained from the American Type Culture Collection, ATCC No. CRL-1717) were cultured in DMEM (Sigma-Aldrich) supplemented with 15% (v/v) foetal calf serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 50 U/ml penicillin, 50 μg/ml streptomycin, 1.25 μg/ml Fungizone (both from PAA Laboratories GmbH, Austria). Human RMS-derived RD cells (obtained from the American Type Culture Collection, ATCC No. CCL-136) were maintained in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) foetal bovine serum (Invitrogen, Paisley, UK), 2 mM Glutamine (Sigma-Aldrich), 50 μg/ml penicillin and 50 μg/ml streptomycin (both from TEVA). Medium was changed every other day and cells were sub-cultured at 90% confluence at 37°C in a humidified atmosphere with 5% CO2.

For transfection, C2C12 or RD cells were seeded in 6-well tissue culture dishes and at 60–70% confluence and were transfected by either the empty pmMTH vector (control cells) or by the vectors encoding the cDNA sequences of PKCα, β, δ, ε or DN-nPKCδ [9, 27, 29, 30]. Transfections were performed with a Lipofectamine anionic detergent (Invitrogen, Paisley, UK), 2 mM Glutamine (Sigma-Aldrich), 50 μg/ml penicillin and 50 μg/ml streptomycin (both from TEVA). Medium was changed every other day and cells were sub-cultured at 90% confluence at 37°C in a humidified atmosphere with 5% CO2.

The samples were subjected to SDS-PAGE (7.5%–15%) gel in serum-free DMEM solution using 2 μg cDNA according to the protocol suggested by the manufacturer. Cells were selected in DMEM containing 750 μg/ml G418 (Genetin, Invitrogen) for 12–18 days, then single colonies were isolated. PKC overexpressing cells were cultured in supplemented DMEM containing 500 μg/ml G418. Experiments were routinely carried out on pools of transfected cells, but the results were confirmed on at least three individual clones for each isoform. The efficacy of recombinant overexpression was monitored by Western blotting and PKC kinase assays (see below and in Fig. 1).
cells were analysed for kinase activity by measuring 32P incorporation into Histone IIIS (H-III, from Sigma-Aldrich) or the 20 kD light chain of smooth muscle myosin (MLC20), isolated from turkey gizzard, as substrates. The assay mixture contained 20 mM TRIS-HCl (pH 7.5), 20 mM MgCl2, 1 mM CaCl2, 25 μM [γ-32P]-ATP (600–1000 cpm/pmol) and 0.2 mg/ml H-III or MLC20. The reaction was started by the addition of [γ-32P]-ATP (Izinta Ltd., Budapest, Hungary) and assays were incubated at 30°C. Aliquots were spotted on P81 phosphocellulose paper and washed three times in 500 ml of 0.5% phosphoric acid, then with acetone. Incorporation of 32P into the proteins was determined by counting the dried P81 papers in a scintillation counter. Data represent triplicate determinations.

PKC activity (kinase) assay

The PKC activity of transfected C2C12 cells was determined as described before [9, 24]. Briefly, cells were lysed in the lysis buffer described above and the kinase activity of the cell lysates was determined using Histone IIIS (H-III, from Sigma-Aldrich) or the 20 kD light chain of smooth muscle myosin (MLC20), isolated from turkey gizzard, as substrates. The assay mixture contained 20 mM TRIS-HCl (pH 7.5), 20 mM MgCl2, 1 mM CaCl2, 25 μM [γ-32P]-ATP (600–1000 cpm/pmol) and 0.2 mg/ml H-III or MLC20. The reaction was started by the addition of [γ-32P]-ATP (Izinta Ltd., Budapest, Hungary) and assays were incubated at 30°C. Aliquots were spotted on P81 phosphocellulose paper and washed three times in 500 ml of 0.5% phosphoric acid, then with acetone. Incorporation of 32P into the proteins was determined by counting the dried P81 papers in a scintillation counter. Data represent triplicate determinations.

Determination of cellular proliferation

Proliferation of C2C12 myoblasts was measured by a colorimetric bromo-deoxyuridine (BrdU) assay kit (Boehringer Mannheim, Mannheim, Germany) and by analysing standard growth curves [9]. In those BrdU assays where the effects of PKC acting agents were tested on cellular proliferation, cells were plated in 96-well multititre plates (5000 cells/well density) in quadruplicate and 4 hrs later were treated with different concentrations of the agents and further incubated for the time indicated. Cells were then incubated with 10 μM BrdU for 4 hrs, and the cellular incorporation of BrdU (as the indicator of cellular proliferation) was determined colorimetrically according to the manufacturer’s protocol. When BrdU assays were employed to investigate growth properties of PKC transfectants, cells were seeded at a density of 1000 cells/well and the BrdU incorporation was determined after the indicated days of culture, as described above.

Proliferation of RD cells was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. Cells were plated in 96-well multititre plates (1000 cells per well density) in quadruplicates and were cultured for 1–4 days. Cells were then incubated with 0.5 mg/ml MTT for 2 hrs and the concentration of formazan crystals was determined colorimetrically according to the manufacturer’s protocol [32].

To assess doubling times and maximal cell numbers of PKC overexpressers, 104 cells/well were plated in 12-well plates in triplicate in complete DMEM. Fresh medium was added every other day, and the cells in triplicate were harvested by trypsinization as indicated (usually on a daily basis) and counted using a haemocytometer. In the determination of the average doubling time, the 24 hr timepoint was used as the starting point to avoid artefacts because of the initial lag period after plating [9, 29, 30]. The following equation was used to calculate doubling time: τ = D/log2(N/N0) where τ is the doubling time, D is the number of days of culturing, N and N0 are the number of cells at the end and the beginning of the experiments, respectively. To determine the maximal cell density, cells were grown in 12-well plates to confluence and kept post-confluent for 3 additional days with daily medium changes and then counted as described above.
Xenograft experiments

Severe combined immunodeficiency (SCID) mice were bred and maintained in the animal facility of the Department of Dermatology (University of Debrecen) in accordance with the animal-welfare ordinance. The studies were performed under the current regulations and standards of the Institutional Research Ethics Committee of the University of Debrecen, Hungary. Cells were harvested by trypsinization and washed twice with DPBS. Cell pellets [2 × 10^6 viable cells (C2C12 cells) and 4 × 10^6 viable cells (RD cells)] were re-suspended in culture medium and injected in a single subcutaneously site on the right flank of SCID mice (0.2 ml/injection) and observed over a period of 30 days [9]. Animals were finally killed and the averaged three-dimensional size and histological characteristics of the developed tumours (five animals for each group) were analysed.

Immunohistochemistry

The histological parameters were determined on formalin-fixed, paraffin-embedded, and haematoxylin-eosin-stained sections of the developed tumours [9]. Immunohistochemical images were captured and digitalized using an RT Spot Colour CCD camera (Diagnostic Instruments Inc.) integrated on a Nikon Eclipse 600 fluorescence and light microscope (Nikon). Digitalized images were then analysed using image J (NIH, Bethesda, MD, USA) image analysis software. The averaged number of cell divisions was measured by counting the number of nuclei showing clear signs of mitosis in ten individual visual fields at high magnification using a light microscope. Results obtained in each tumour of the same group were then averaged and the mean values were calculated.

In addition, to assess the number of proliferating cells, formalin-fixed, paraffin-embedded sections were immunostained against the nuclear marker Ki67 [9] using a streptavidin-biotin-complex (SABC) three-step immunohistochemical technique (DAKO, Hamburg, Germany). First, the inhibition of endogenous peroxidase activity was performed with 3% H_2O_2 in 100% methanol (both from Sigma-Aldrich). Then, non-specific binding was blocked by 1% bovine serum albumin (Sigma-Alrich) in PBS buffer (pH 7.5). After washing various concentrations of the anti-Ki-67 monoclonal mouse primary antibody (DAKO), an optimal 1:50 dilution was employed. The sections were then incubated in a humid chamber using a biotin-coupled antinouse secondary antibody (1:100, DAKO) followed by streptavidin conjugated with horseradish peroxidase (1:600, DAKO). To reveal the peroxidase activity, VIP SK-4600 (Vector, Burlingame, CA, USA) was employed as a chromogene. The tissue samples were finally slightly counterstained with methyl green (DAKO) and mounted with Aquatex (Merck, Wien, Austria). The averaged number of proliferating (Ki67 positive) cells was measured by counting the total number of Ki67 positive cells at five randomly placed, equal areas of interest and the values were normalized to the total number of cells measured at the fields.

Statistical analysis

The data are expressed as mean ± SEM. Significance differences were assessed by a two-tailed un-paired t-test (P < 0.05 values were defined as significance).
PKC inhibitors differentially modify whereas the kinase-negative mutant of nPKC\(\delta\) (DN-nPKC\(\delta\)) inhibits cellular proliferation of C2C12 myoblasts

The above findings strongly suggested that in C2C12 myoblasts (i) the cPKC\(\alpha\) and \(\beta\) isoforms inhibit proliferation and promote differentiation; (ii) the nPKC\(\delta\), in contrast, markedly stimulates cell growth but inhibits differentiation; and (iii) the nPKC\(\epsilon\) plays an insignificant role in regulating the above processes. To further investigate these proposals, we measured the effects of certain PKC inhibitors on proliferation of control C2C12 myoblasts. In addition, similar to as previously reported [24], we constructed such C2C12 myoblasts which stably overexpress the kinase-negative mutant of nPKC\(\delta\) (DN-nPKC\(\delta\)) and measured the effects of this recombinant modification on the cell growth of the cells.

As seen in Figure 3, G66976, an inhibitor of the cPKC isoforms [33] (i.e. the cPKC\(\alpha\) and \(\beta\) in C2C12 cells) stimulated the proliferation of the cells in a dose-dependent manner (Fig. 3A). In contrast, the nPKC\(\delta\) inhibitor Rottlerin [34] dose-dependently inhibited cellular growth (Fig. 3B). Furthermore, confirming our previous results [24], the overexpression of DN-nPKC\(\delta\) resulted in a dramatically suppressed cellular proliferation rate (Fig. 2A) and prolonged doubling time (Table 1; actually, cell cultures of DN-nPKC\(\delta\) overexpressers never reached confluence; hence, the saturation density of these cultures was not measurable). Although confidence in the interpretation is limited because of possible effects of G66976 and Rottlerin on systems other than PKC [33, 35], these findings may further argue for that cPKC\(\alpha\) and \(\beta\) are negative while nPKC\(\delta\) is indeed a positive regulator of proliferation in C2C12 myoblasts.

Cells overexpressing nPKC\(\delta\) induce malignantly transformed, large tumours in SCID mice

We then investigated the behaviour of PKC overexpressing cells in assays for tumour formation and in vivo growth. SCID mice (four in each group) were injected with cell suspensions of C2C12 myoblasts overexpressing different PKC isoforms and, after 30 days, the developed tumours (Fig. 4A) were characterized. As revealed on haematoxylin-eosin-stained sections, control (empty vector-transfected) C2C12 cells formed small tumours with expansive growth properties at the periphery and with signs of rhabdoid differentiation at the centre of the tumour (Fig. 4B). The injection of C212 cells overexpressing cPKC\(\alpha\), \(\beta\) or nPKC\(\delta\) isoforms, when compared to the control ones, generally did not change the major histological characteristics of the tumours. Namely, these small tumours maintained the expansive (i.e. non-infiltrative, benign) growth characteristics and histological features of peripheral proliferation and rhabdoid differentiation. In addition, we found only minor differences in the average size of the tumours, the number of dividing cells, and the percentage of Ki67\(+\) (hence proliferating) cells on the histological sections of tumours (these values were somewhat smaller in those tumours which were induced by cells overexpressing cPKC\(\alpha\) and \(\beta\), when compared to the control ones; Table 1).
Of great importance, however, cells overexpressing nPKCd induced the development of extremely large tumours (often with superficial exulceration and bleeding) which, in numerous cases, resulted in significant weight loss and eventually death of the animals within the 30-day investigation period (Fig. 4A, Table 1). Histologically, these tumours were characterized by markedly high cell division rate (as reflected by the elevated number of mitosis and Ki67+ cells), infiltrating (hence malignant) growth properties resulting in destruction of various layers of different cell types of the skin, and complete lack of rhabdoid differentiation (Fig. 4B). Therefore, these tumours could be diagnosed as malignant RMSs. Finally, it was also important to observe that C2C12 myoblasts overexpressing the DN-nPKCd failed to induce any tumour when injected intradermally to SCID mice (Table 1).

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Table 1 In vitro and in vivo growth analysis of C2C12 cells overexpressing various PKC isoforms

| Isoform       | In vitro growth analysis | In vivo tumour growth analysis |
|---------------|-------------------------|-------------------------------|
|               | Doubling time (hrs)     | Saturation density (10^5 cells/cm²) | Averaged tumour size (mm) | Number of cell division | Percentage of Ki67 positive cells |
| Control       | 29.4 ± 3.6             | 1.2 ± 0.1                      | 6 x 6 x 3.5              | 2 ± 0.3                | 18.2 ± 3.8                     |
| cPKCd         | 33.5 ± 3.8             | 1.1 ± 0.3                      | 5 x 5 x 3                | 1.8 ± 0.3              | 15.2 ± 2.9                     |
| cPKCc         | 31.8 ± 4.9             | 1.1 ± 0.1                      | 4.5 x 5 x 3.5            | 1.5 ± 0.5              | 13.8 ± 4.6                     |
| nPKCd         | 28.5 ± 4.3             | 1.3 ± 0.2                      | 6 x 5 x 3                | 2.2 ± 0.8              | 20.5 ± 5.9                     |
| *nPKCd*       | 11.6 ± 3.8             | 3.6 ± 0.5                      | 23 x 18 x 11             | 25 ± 1.5               | 88.5 ± 6.2                     |
| *DN-nPKCd*    | 73.6 ± 5.9             | Not measurable                 | Did not induce tumours   |                         |                               |

*All data obtained with the nPKCd and DN-nPKCd are significantly (P < 0.05) different from those of the control cells (see text for further details). Various parameters were analysed as described under ‘Materials and methods’. Data are expressed as mean ± SEM, except for averaged tumour size, where the three-dimensional sizes of three–four tumours per group were averaged and the mean values are shown.

Fig. 3 Effects of PKC inhibitors on cellular proliferation of C2C12 myoblasts. C2C12 cells were seeded at densities of 5000 cells/well in 96-well microtitre plates, treated with various concentrations of (A) Go6976, inhibitors of the cPKCs or (B) Rottlerin, inhibitor of nPKCd, and then BrdU assays were performed at certain culturing days. Points represent the mean ± SEM of quadruplicate determinations in one representative experiment. Two other experiments yielded similar results. * marks significant (P < 0.05) differences compared to the daily-matched untreated control.

nPKCd promotes cellular growth of human RMS cells

The above data strongly argued for the key role of nPKCd in promoting proliferation and inducing malignant transformation of myoblasts. To further assess these phenomena, in the next steps of our experiments, we have stably transfected human RMS-derived RD cells either with empty MTH (pEMTH) vector or with vectors encoding the active (nPKCd) or dominant-negative (DN-nPKCd) isoform. Similar to as described in Figure 1, Western blot analyses revealed that the levels of the overexpressed nPKCd was several-fold higher compared to the control (empty vector expressing) cells (Fig. 5A). nPKCd antibody is unable to differentiate between the endogenous and the ectopically overexpressed nPKCd isoform.
isoform and it also recognizes DN-nPKC<sub>d</sub>. Therefore, to make the differentiation possible, we have performed another Western blot analysis, but now using an anti-PKC<sub>e</sub> antibody which, besides the endogenous PKC<sub>e</sub>, also recognizes the e-tag of the recombinantly overexpressed nPKC<sub>d</sub> and DN-nPKC<sub>d</sub>. As seen in Figure 5A, the densitometry analysis of the various immunoreactive bands revealed that whereas the PKC<sub>e</sub>-specific signals of the nPKC<sub>d</sub> and the DN-nPKC<sub>d</sub> samples were very similar and significantly stronger than measured in the control samples, the nPKC<sub>d</sub>-specific immunoreactivity was significantly higher only in the PKC<sub>d</sub> but not in the DN-nPKC<sub>d</sub> group. These results strongly suggest that, upon DN-nPKC<sub>d</sub> transfection and overexpression, the level of the endogenous nPKC<sub>d</sub> was markedly suppressed.

We then investigated the effect of overexpression of the nPKC<sub>d</sub> mutants on the in vitro proliferation of RD cells. As revealed by growth curve analysis (Fig. 5B), the overexpression of nPKC<sub>d</sub> significantly increased the proliferation of RD cells compared to control (empty vector-transfected) cells. Further, DN-nPKC<sub>d</sub> overexpresser RD cells exhibited a significantly suppressed growth rate when compared to control.

nPKC<sub>d</sub> is involved in IGF-I-induced ERK 1/2 activation in RMS cells

Insulin-like growth factor-I is reported to be a significant growth factor in skeletal muscle biology and physiology [24, 36–38]. To uncover the potential mechanism by which nPKC<sub>d</sub> modulates the proliferation and tumourigenicity of RMS cells, we also evaluated the role of nPKC<sub>d</sub> in modulating the IGF-I-induced activation of the Ras-MAPK signalling pathway. Cells were treated with IGF-I (Fig. 6) as indicated, and the activation of the possibly most important downstream molecule related to Ras, ERK 1/2 kinase, was examined by Western blot. We show that the overexpression of nPKC<sub>d</sub> enhanced the IGF-I-induced ERK 1/2 phosphorylation (Fig. 6) compared to the cells overexpressing the dominant-negative mutant (DN-nPKC<sub>d</sub>) or the empty vector (control) suggesting the involvement of nPKC<sub>d</sub> in mediating the growth-promoting effect of IGF-I (similar to as we have previously shown for human skeletal muscle cells and C2C12 myoblasts [24]).

Role of nPKC<sub>d</sub> on tumourigenesis of human RD cells

To establish the relevance of nPKC<sub>d</sub> in RMS tumourigenesis, we also investigated the role of the nPKC<sub>d</sub> isoform in the in vivo tumour formation of RD cells. For this, tumours were induced in SCID mice (five in each group) by injecting RD cells overexpressing either nPKC<sub>d</sub>, DN-nPKC<sub>d</sub> or the empty vector (control). As expected, injection of all RMS-derived RD cell types resulted in tumour development in
immunodeficient mice (Fig. 7A). Histologically, these tumours were diagnosed as malignant RMSs with high cell division rates (number of mitosis) and infiltrating (malignant) growth properties, very often destructing the neighbouring adipose and skeletal muscle tissues (Fig. 7B and Table 2). Among them, tumours induced by nPKCδ overexpressers were characterized by the largest three-dimensional size and the highest percentage of Ki67 positive (i.e. proliferating) cells within the sarcomas; the latter value was significantly different from those measured in tumours induced by control or DN-nPKCδ overexpressing cells. Interestingly, features of tumours induced by DN-nPKCδ overexpressers did not significantly differ from those of the control RD cells. These differential features of the various cells on tumourigenesis were also proven by immunohistochemical analysis of the expression of the proliferation marker Ki67.

Discussion

In this study, we provide the first evidence that certain cPKC and nPKC isoforms play differential and antagonistic roles in regulating the in vitro proliferation and differentiation of C2C12 myoblasts as well as in vivo tumour growth induced by these cells. Using molecular
biological (recombinant overexpression) methods combined with pharmacological modifications (inhibition) of the PKC isoform activities, we have shown that the 'conventional' cPKC\(\alpha\) and \(\beta\) act as negative regulators of cellular growth and, moreover, their activities stimulate differentiation of the cells (Figs 2 and 3). Interestingly, in cultured avian myoblasts, Capiati et al. [18, 19] have elegantly proven that cPKC\(\alpha\) played a central role in promoting cellular growth which findings contradict with our current data. Moreover, we have previously reported that this isoform did not participate in the growth-inhibitory action of the PKC activator phorbol esters in human cultured skeletal muscle cells [28]. These data, therefore, suggest that the regulatory role of cPKC\(\alpha\) to affect skeletal muscle proliferation possesses marked species dependence.

Our most remarkable data in this investigation were obtained with nPKC\(\delta\). This isoform was also very often implicated in the regulation of cellular proliferation and differentiation of numerous cell types [3, 5–8]. However, in most studies (for example, in human keratinocytes [9, 31] and fibroblast [29, 30]) the isoform was suggested to stimulate differentiation and apoptosis and to inhibit proliferation, whereas, up to the start of the current study, nPKC\(\delta\) was shown to stimulate proliferation and induction of differentiation [5, 7–9]. In the current study, however, we found that this isoform plays an insignificant role in regulating proliferation, differentiation, and the tumour inducing properties of C2C12 myoblast (Figs 2 and 4, Table 1). As we and others have failed to identify this isoform in C2C12 cells [24], which finding was also confirmed in this study (Fig. 1A), the lack of effect of recombinant overexpression of the constitutively active (Fig. 1B) nPKC\(\delta\) on cellular growth of the myoblasts is most probably because of the lack of the signalling – substrate system related to this isoenzyme.

Fig. 7 Effect of overexpression of nPKC\(\delta\) and DN-nPKC\(\delta\) on tumourigenicity of rhabdomyosarcoma cells. (A) Representative images of tumours (indicated by arrows) induced by nPKC\(\delta\), DN-PKC\(\delta\) or empty vector-transfected RD cells at day 30; scale bar: 10 mm. (B) After 30 days, animals were killed, the developed tumours were excised and haematoxylin-eosin staining was performed on formalin-fixed paraffin-embedded sections. Note that the tumour (TU) induced by nPKC\(\delta\) overexpresser RD cells infiltrated and destroyed the subcutaneous adipose tissue (AT) and skeletal muscle (SM) layers; scale bar: 100 \(\mu\)m.

Table 2 In vivo growth analysis of RD cells overexpressing nPKC\(\delta\) or DN-nPKC\(\delta\)

| Isoform       | In vivo tumour growth analysis | Percentage of Ki67 positive cells |
|---------------|-------------------------------|----------------------------------|
| Control       | Averaged tumour size (mm)     | Number of cell division          | Percentage of Ki67 positive cells |
|               | 22 × 14.6 × 12.6               | 14.6 ± 3.0                       | 82.75 ± 8.13                     |
| nPKC\(\delta\) | 25 × 18 × 15.2                | 16.6 ± 1.5                       | 97.6 ± 4.32*                     |
| DN-nPKC\(\delta\)| 21.25 × 16 × 12             | 17.1 ± 4.4                       | 78.31 ± 7.28                     |

*Data obtained with the nPKC\(\delta\) is significantly (\(P < 0.05\)) different from those of the control and DN-PKC\(\delta\) cells (see text for further details). Various parameters were analysed as described under ‘Materials and methods’. Data are expressed as mean ± SEM, except for averaged tumour size, where the three-dimensional sizes of five tumours per group were averaged and the mean values are shown.
proliferation (acting as a prosurvival factor) only in certain breast cancer cell lines [39].

Of great importance, our current findings introduce nPKCδ as a novel significant player in skeletal muscle biology positively controlling cellular growth. These statements were supported by the following data: (i) overexpression of the constitutively active nPKCδ stimulated whereas the kinase inactive DN-nPKCδ mutant inhibited in vitro growth of C2C12 myoblasts (Fig. 2A); (ii) overexpression of nPKCδ suppressed the expression of the differentiation marker desmin (Fig. 2B); (iii) the inhibition of PKCδ activity by Rottlerin inhibited cellular proliferation of the control C2C12 cells (Fig. 3B); (iv) [PKCδ overexpressor C2C12 cells, when injected to immunodeficient mice, initiated the development of large and, of great importance, malignantly transformed RMSs (in contrast to control myoblasts which induced benign tumours of much smaller size) (Fig. 4 and Table 1); and (v) DN-nPKCδ overexpressor myoblasts did not induce tumours in SCID mice. Moreover, the above argument is also supported by our previous report presenting that nPKCδ plays a central role in mediating the mitogenic effect of IGF-I, one of the key autocrine – paracrine growth factors in skeletal muscle physiology and pathology [40], both in human and C2C12 skeletal muscle cells [24].

Protein kinase C isoforms have been implicated in the pathogenesis of numerous human malignancies including breast, colon, lung, prostate, pancreatic, liver and hematopoietic ones [41, 42]. RMS is a group of aggressive muscle tumours and the most common soft tissue sarcomas in children [43]. The poor clinical outcome fostered trials for a better understanding of the tumourigenic mechanisms so that new therapeutic targets can be identified [44, 45]. Although only few reports are available on describing the expression profile of the PKC family in RMS, involvement of individual PKC isoforms and their role as therapeutic targets are beginning to be explored [46–48]. As we found that C2C12 myoblasts overexpressing nPKCδ induced malignant tumours in immunodeficient mice (Fig. 4), we sought to define the exact functional role of this isoform in RMS tumourigenesis. Importantly, overexpression of nPKCδ further enhanced the already highly accelerated cell proliferation of human RMS-derived RD cells, compared to control cells or DN-nPKCδ overexpressers (Fig. 5B).

Insulin-like growth factor-I is known as a potent mitogenic factor for RMS, expressions of IGF-I receptor have reportedly been elevated in the disease [49–51]. Although we have previously identified that nPKCδ is involved in the IGF-I induced ERK 1/2 activation in C2C12 [24], here we provide the first evidence that nPKCδ also contributes to signalling downstream of IGF-I in RD cells by modifying the level of IGF-I induced phosphorylation of ERK 1/2. Indeed, overexpression of nPKCδ increased the activation of ERK 1/2 induced by IGF-I stimulation compared to the p38MTH vector or DN-nPKCδ overexpressor cells (Fig. 6).

Furthermore, overexpression of nPKCδ further increased xenograft tumour growth as well as the proliferation rate of the developed tumours (Ki67 positivity; Fig. 7 and Table 2). Interestingly, the size of tumours induced by cells overexpressing the inactive DN-nPKCδ did not differ from the control tumours suggesting that other factors than nPKCδ may also be involved in promoting the aggressive growth of RMS-derived cells. Nevertheless, these data (again) strongly suggest that nPKCδ may play a central role in RMS tumourigenesis.

Comparison of the current data with our previous experimental findings [9] revealed another intriguing phenomenon. In human epidermal keratinocytes, using identical molecular biological and pharmacological methods, we found that the overexpression of cPKCβ and nPKCδ stimulated cellular differentiation and inhibited cellular proliferation and tumour growth. Conversely, the activity of cPKCβ and nPKCδ increased both in vitro and in vivo growth of cells and inhibited differentiation. As our current investigation on skeletal muscle cells resulted mostly opposite findings (cPKCβ inhibited growth, nPKCδ played minor role in the regulation of proliferation, nPKCδ markedly enhanced cellular and tumour growth), these data strongly suggest that certain PKCs not only isoform-specifically regulate cellular proliferation and differentiation but their effect exert a marked cell-type dependence as well.

In conclusion, in this study we present the first evidence that certain cPKC and nPKC isoforms play specific, yet antagonistic roles in regulating the in vitro and in vivo growth of C2C12 muscle cells. In addition, we describe nPKCδ as a novel key player in promoting cellular growth and inducing malignant transformation, which findings introduce this isoform as a promising, therapeutically exploitable, novel target for the treatment of skeletal muscle malignancies.

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Conflicts of interest

No potential conflicts of interest were disclosed.

Author contribution

CG and AS performed the experiments on C2C12 cells, analysed data and co-wrote the manuscript. ZN performed the experiments on RD cells, analysed data and co-wrote the manuscript. IJ provided the SCID mice and managed in vivo experimentation. AK and FE performed the kinase assay experiments. IK and MT conducted pathological review of the histopathology slides and provided assistance with the in vivo experimentation. TSz, LK and PB conceived the hypothesis, provided material and intellectual support. TB conceived of the hypothesis, contributed to obtaining all necessary approvals and clearances to conduct the research, contributed to obtaining grant funding, supervised all aspects of the research, co-wrote the manuscript and approved the final version. All authors revised the manuscript and gave their final approval.
References

1. Nishizuka Y. The molecular heterogeneity of protein kinase C and its implication for cellular regulation. Nature. 1988; 334: 661–5.
2. Nishizuka Y. Intracellular signaling by hydrolisis of phospholipids and activation of protein kinase C. Science. 1992; 258: 607–14.
3. Gutcher I, Webb PR, Anderson NG. The isoform-specific regulation of apoptosis by protein kinase C. Cell Mol Life Sci. 2003; 60: 1061–70.
4. Becker KP, Hannun YA. Protein kinase C and phospholipase D: intimate interactions in intracellular signaling. Cell Mol Life Sci. 2005; 62: 1448–61.
5. Goodnight JA, Mischak H, Mushinski JF. Selective involvement of protein kinase C isoforms in differentiation and neoplastic transformation. Adv Cancer Res. 1994; 64: 159–209.
6. Way KJ, Chou E, King GL. Identification of PKC-isozyme-specific biological actions using pharmacological approaches. Trends Pharmacol Sci. 2000; 21: 181–7.
7. Mischak H, Goodnight JA, Kolch W, et al. Overexpression of protein kinase C-α and γ in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. J Biol Chem. 1993; 268: 6090–6.
8. Brodie C, Kuperstein I, Ács P, et al. Differential role of specific PKC isozymes in the proliferation of glial cells and the expression of the astrocytic markers GFAP and glutamine synthetase. Mol Brain Res. 1998; 56: 108–17.
9. Papp H, Czifra G, Bodó E, et al. Opposite roles of protein kinase C isozymes in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. Cell Mol Life Sci. 2004; 61: 1095–105.
10. Serra C, Federici M, Buongiorno A, et al. Transgenic mice with dominant negative PKC-θ in skeletal muscle: a new model of insulin resistance and obesity. J Cell Physiol. 2003; 196: 89–97.
11. Leitges M, Plomann M, Standaert ML, et al. Knockout of PKCα enhances insulin signaling through PI3K. Mol Endocrinol. 2002; 16: 847–58.
12. Rosenzweig T, Braiman L, Bak A, et al. Differential effects of tumor necrosis factor-alpha on protein kinase C isozymes alpha and delta mediate inhibition of insulin receptor signaling. Diabetes. 2002; 51: 1921–30.
13. Braiman L, Alt A, Kuroki T, et al. Protein kinase Cdelta mediates insulin-induced glucose transport in primary cultures of rat skeletal muscle. Mol Endocrinol. 1999; 13: 2002–12.
14. Braiman L, Alt A, Kuroki T, et al. Activation of protein kinase Cζ induces serine phosphorylation of VAMP2 in the GLUT4 compartment and increases glucose transport in skeletal muscle. Mol Cell Biol. 2001; 21: 7852–61.
15. Narumi K, Kobayashi M, Otake S, et al. Regulation of human monocarboxylate transporter 4 in skeletal muscle cells: the role of protein kinase C (PKC). Int J Pharm. 2012; 428: 25–32.
16. Perrini S, Henriksson J, Zierath JR, et al. Exercise-induced protein kinase C isozyme-specific activation in human skeletal muscle. Diabetes. 2004; 53: 21–4.
17. Ehrhardt J, Morgan J. Regenerative capacity of skeletal muscle. Curr Opin Neurol. 2005; 18: 548–53.
18. Wagers AJ, Conboy IM. Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. Cell. 2005; 122: 659–67.
19. Capizzi LA, Limbozzi F, Tellez-Inon MT, et al. Evidence on the participation of protein kinase C alpha in the proliferation of cultured myoblasts. J Cell Biochem. 1999; 74: 292–300.
20. Capizzi LA, Vaquez G, Tellez Inon MT, et al. Anti-sense oligonucleotides targeted against protein kinase C alpha inhibit proliferation of cultured myoblasts. Cell Prolif. 2000; 33: 307–15.
21. Miles K, Wagner M. Overexpression of nPKC θ is permissive for myogenic differentiation. J Cell Biochem. 2000; 79: 71–9.
22. Chalfant CE, Claraldi TP, Watson JF, et al. Protein kinase Cθeta expression is increased upon differentiation of human skeletal muscle cells: dysregulation in type 2 diabetic patients and a possible role for protein kinase Cθeta in insulin-stimulated glycolcogen synthesis activity. Endocrinology. 2000; 141: 2773–8.
23. Cen L, Amoczky KJ, Hsieh FC, et al. Phosphorylation profiles of protein kinases in alveolar and embryonal rhabdomyosarcoma. Mod Pathol. 2007; 20: 936–46.
24. Czifra G, Toth IB, Marincsák R, et al. Insulin-like growth factor-I-coupled mitogenic signaling in primary cultured human skeletal muscle cells and in C2C12 myoblasts. A central role of protein kinase Cdelta. Cell Signal. 2006; 18: 1461–72.
25. Ostrovsky O, Bengal E, Aronheim A. Induction of terminal differentiation by the c-Jun dimerization protein JDP2 in C2 myoblasts and rhabdomyosarcoma cells. J Biol Chem. 2002; 277: 40043–54.
26. Koleva M, Kappler R, Vogler M, et al. Pleiotropic effects of sonic hedgehog on muscle satellite cells. Cell Mol Life Sci. 2005; 62: 1865–70.
27. Griger Z, Payer E, Kovác I, et al. Protein kinase C-β and -δ isozymes promote arachidonic acid production and proliferation of MonoMac-6 cells. J Mol Med. 2007; 85: 1031–42.
28. Boczan J, Biro T, Czifra G, et al. Phorbol ester treatment inhibits proliferation and differentiation of cultured human skeletal muscle satellite cells by differentially acting on protein kinase C isoforms. Acta Neuropathol. 2001; 102: 55–62.
29. Ács P, Bognár K, Marquez AM, et al. The catalytic domain of protein kinase C chimeras modulates the affinity and targeting of phorbol ester induced translocation. J Biol Chem. 1997; 272: 22148–53.
30. Ács P, Wang QJ, Bognár K, et al. Both the catalytic and regulatory domains of protein kinase C chimeras modulate the proliferation properties of NIH 3T3 cells. J Biol Chem. 1997; 272: 28793–9.
31. Li L, Lorenzo PS, Bognár K, et al. Protein kinase Cδ targets mitochondria, alters mitochondrial membrane potential, and induces apoptosis in normal and neoplastic keratinocytes when overexpressed by and adenoviral vector. Mol Cell Biol. 1999; 19: 8547–58.
32. Bodó E, Biró T, Telek A, et al. A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. Am J Pathol. 2005; 166: 985–98.
33. Martiny-Baron G, Kazanietz MG, Mischak H, et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem. 1993; 268: 9197–9.
34. Gschwendt M, Müller HJ, Kiabassa K, et al. Rottlerin, a novel protein kinase inhibitor. Biochem Biophys Res Commun. 1994; 199: 93–8.
35. Alessi DR. The protein kinase C inhibitors Ro 318220 and GF 109203X are equally potent inhibitors of MAPKAP kinase-1 & p70 S6 kinase. FEBS Lett. 1997; 402: 121–3.
36. Milasincic DJ, Calera MR, Farmer SR, et al. Stimulation of C2C12 myoblast growth by basic fibroblast growth factor and...
insulin-like growth factor 1 can occur via mitogen-activated protein kinase-dependent and -independent pathways. Mol Cell Biol. 1996; 16: 5964–73.

37. Butler AA, Blakesley VA, Tsokos M, et al. Stimulation of tumor growth by recombinant human insulin-like growth factor-I (IGF-I) is dependent on the dose and the level of IGF-I receptor expression. Cancer Res. 1998; 58: 3021–7.

38. Adams GR. Autocrine/paracrine IGF-I and skeletal muscle adaptation. J Appl Physiol. 2002; 93: 1159–67.

39. McCracken MA, Miraglia LJ, McKay RA, et al. Protein kinase C delta is a prosurvival factor in human breast tumor cell lines. Mol Cancer Ther. 2003; 2: 273–81.

40. Tidball JG. Autocrine/paracrine IGF-I and skeletal muscle adaptation. J Appl Physiol. 2005; 98: 1900–8.

41. Fields AP, Murray NR. Protein kinase C isoforms as therapeutic targets for treatment of human cancers. Adv Enzyme Regul. 2008; 48: 166–78.

42. Ali AS, Ali S, El-Rayes BF, et al. Exploitation of protein kinase C: a useful target for cancer therapy. Cancer Treat Rev. 2009; 35: 1–8.

43. Anderson J, Gordon A, Pritchard-Jones K, et al. Genes, chromosomes, and rhabdomyosarcoma. Genes Chromosom Cancer. 1999; 26: 275–85.

44. Breneman JC, Lyden E, Pappo AS, et al. Prognostic factors and clinical outcomes in children and adolescents with metastatic rhabdomyosarcoma—a report from the Intergroup Rhabdomyosarcoma Study IV. J Clin Oncol. 2003; 21: 78–84.

45. Wachtel M, Schafer BW. Targets for cancer therapy in childhood sarcomas. Cancer Treat Rev. 2010; 36: 318–27.

46. Fields AP, Frederick LA, Regala RP. Targeting the oncogenic protein kinase C iota signaling pathway for the treatment of cancer. Biochem Soc Trans. 2007; 35: 996–1000.

47. Amstutz R, Wachetl M, Troxler H, et al. Phosphorylation regulates transcriptional activity of PAX3/FKHR and reveals novel therapeutic possibilities. Cancer Res. 2008; 68: 3767–76.

48. Kikuchi K, Soundararajan A, Zarzabal LA, et al. Protein kinase C iota as a therapeutic target in alveolar rhabdomyosarcoma. Oncogene. 2013; 32: 286–95.

49. Kalebic T, Blakesley V, Slade C, et al. Expression of a kinase-deficient IGF-I-R suppresses tumorigenicity of rhabdomyosarcoma cells constitutively expressing a wild type IGF-I-R. Int J Cancer. 1998; 76: 223–7.

50. Kim SY, Toretsky JA, Scher D, et al. The role of IGF-1R in pediatric malignancies. Oncologist. 2009; 14: 83–91.

51. Abraham J, Prajapati SI, Nishijo K, et al. Evasion mechanisms to Igf1r inhibition in rhabdomyosarcoma. Mol Cancer Ther. 2011; 10: 697–707.