Effect of exercise on protein kinase C activity and localization in human skeletal muscle

Adam J. Rose[1], Belinda J. Michell[2], Bruce E. Kemp[2,3] and Mark Hargreaves[1]

[1]*Centre for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University, Burwood, Victoria 3125, Australia*
[2]*St Vincent's Institute of Medical Research, Fitzroy, Victoria 3065, Australia*
[3]*CSIRO Health Sciences and Nutrition, Parkville, 3052, Australia*

To investigate the effect of exercise on protein kinase C (PKC) activity and localization in human skeletal muscle, eight healthy men performed cycle ergometer exercise for 40 min at 76 ± 1% the peak pulmonary O_2 uptake ($\dot{V}_{O_2peak}$), with muscle samples obtained at rest and after 5 and 40 min of exercise. PKC expression, phosphorylation and activities were examined by immunoblotting and *in vitro* kinase assays of fractionated and whole tissue preparations. In response to exercise, total PKC activity was slightly higher at 40 min in an enriched membrane fraction, and using a pSer-PKC-substrate motif antibody it was revealed that exercise increased the serine phosphorylation of a ∼50 kDa protein. There were no changes in conventional PKC (cPKC) or PKCθ activities; however, atypical PKC (aPKC) activity was ∼70% higher at 5 and 40 min, and aPKC expression and Thr$^{410/403}$ phosphorylation were unaltered by exercise. There were no effects of exercise on the abundance of PKCα, PKCδ, PKCθ and aPKC within cytosolic or enriched membrane fractions of skeletal muscle. These data indicate that aPKC, but not cPKC or PKCθ, are activated by exercise in contracting muscle suggesting a potential role for aPKC in the regulation of skeletal muscle function and metabolism during exercise in humans.

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Corresponding author M. Hargreaves: School of Exercise and Nutrition Sciences, Deakin University, Burwood, Victoria 3125, Australia. Email: mark.hargreaves@deakin.edu.au

The protein kinase C (PKC) enzymes are multifunctional Ser/Thr protein kinases involved in many cellular responses (Dempsey *et al.* 2000). There are at least 10 isoforms of PKC grouped into three classes based upon structural differences and cofactor requirements for activation (for review see Newton, 1995). In human skeletal muscle, nearly all of the PKC isoforms are expressed (Itani *et al.* 2000), and there is evidence that some PKC isoforms have a role in insulin resistance (Cortright *et al.* 2000; Itani *et al.* 2000, 2002; Beeson *et al.* 2003) as well as insulin- (Bandyopadhyay *et al.* 2000; Braiman *et al.* 2001; Beeson *et al.* 2003) and exercise- (Chen *et al.* 2002; Beeson *et al.* 2003) mediated glucose transport.

PKC activation occurs during contractions of rat skeletal muscle, as demonstrated by the translocation of PKC activity to a membrane-enriched fraction from a cytosolic fraction (Richter *et al.* 1987; Cleland *et al.* 1989). More recently, it has been demonstrated that there is higher activity of the atypical isoforms of PKC in skeletal muscle during exercise in mice (Chen *et al.* 2002) and in humans (Beeson *et al.* 2003; Nielsen *et al.* 2003). Given that PKC may be involved in the exercise-induced activation of glucose transport (Cleland *et al.* 1990; Wojtaszewski *et al.* 1998) and hormone-sensitive lipase (Donsmark *et al.* 2003) in skeletal muscle, the aim of the present study was to examine the effect of exercise on PKC activity in human skeletal muscle.

Methods

Experimental protocol

Healthy, active but untrained, men ($n = 8$; 24 ± 5 years; body mass index, 23 ± 2 kg m^{-2}; mean ± S.D.) were recruited, and written and verbal information about the purpose, nature and potential risks relating to the experimental procedures was given to the subjects before they provided consent to participate. The protocol was reviewed and approved by the Deakin University Human Research Ethics Committee. One to two weeks prior to testing, subjects completed an incremental exercise test to volitional exhaustion on an electromagnetically braked cycle ergometer (Lode, Groningen, the Netherlands) to determine their peak pulmonary O_2 uptake ($\dot{V}_{O_2peak}$), which averaged 51 ± 2 ml kg^{-1} min^{-1} (mean ± S.E.M.).

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Expired air was analysed by O_2 and CO_2 analysers (AEI Technologies, Pittsburgh, PA, USA) and expired volume by a turbine ventilometer (Flow transformer K 520, KL Engineering, Australia). The gas analysers were calibrated against gases of known composition prior to each test.

Subjects were asked to refrain from exercise as well as caffeine, nicotine and alcohol ingestion for at least 24 h prior to the study. Subjects were provided with a standardized meal (~80% carbohydrate) for the evening prior to testing and reported to the laboratory in the morning after an overnight fast (plasma glucose, 4.9 ± 0.1 mM). Subjects rested for at least 20 min in the supine position before a muscle sample was obtained from the vastus lateralis by percutaneous needle biopsy under local anaesthesia and immediately frozen in liquid N_2. Subjects exercised for 40 min at $76 \pm 1\%$ $\dot{V}_{O_2peak}$ with biopsies taken at 5 and 40 min of exercise and frozen within 20 s after the last contraction. Muscle samples were stored in liquid N_2 until analysis.

Analytical techniques

All chemicals were purchased from Sigma-Aldrich (St Louis, MI, USA) unless otherwise stated. Muscle samples were homogenised as outlined previously (Rose & Hargreaves, 2003) in a homogenization buffer containing (mM): Tris-HCl 50 (pH 7.5), sucrose 250, EDTA 1, EGTA 1, phenylmethylsulfonyl fluoride 1, dithiothreitol 1, sodium flouride 5, sodium pyrophosphate 5 and benzamidine 1, with 10% glycerol, 5 μl ml^{-1} protease inhibitor cocktail and Nonidet P-40 (NP-40; 1%), and mixed well at 4°C. To examine protein localization and PKC activity, muscle samples (40–50 mg) were homogenised in 1 : 8 volumes of homogenization buffer not containing NP-40 until no visible particles remained. These samples were spun at 350 000 g for 30 min and the resultant supernatant comprised the cytosolic fraction. The pellet was resuspended and mechanically disrupted in homogenization buffer containing NP-40 and after 30 min on ice, the pellet fraction was subjected to centrifugation at 100 000 g for 60 min, and the supernatant (particulate fraction) removed. For all samples, aliquots were taken for total protein assay (Pierce BCA, Rockford, IL, USA) and the remaining extract was stored at −80°C until required for analysis.

In preliminary experiments equal amounts of protein from both fractions were immunoprobed using antibodies for proteins specific to skeletal muscle subcellular organelles/structures. These included antibodies against sarcolemmal proteins: phospholemman (PLM; antibody provided by Professor Randall Moorman, University of Virginia, VA, USA) and Na^+,K^+-ATPase-α1 (NKAα1; B. Fambrough, Johns Hopkins University, MD, USA); t-tubule protein: dihydropyridine receptor-α1 (DHPRα1; Santa Cruz Biotechnology, CA, USA); sarcoplasmic reticulum protein: Ca^{2+}-ATPase-1 (SERCA1; Santa Cruz Biotechnology); mitochondrial protein: cytochrome C-oxidase subunit I (COXI; Molecular Probes, OR, USA); and general marker: actin. The particulate fraction represents a preparation in which membranous structures are enriched, and the cytosolic fraction is devoid of membranes (Fig. 1).

PKC activity was measured in skeletal muscle extracts (10–20 μg protein, 5 μl) in kinase assay buffer containing (mM): Hepes 10 (pH 7.2), $MgCl_2$ 5, EGTA 1, sodium pyrophosphate 0.1, ATP 0.1 (0.2 Ci mmol^{-1} 5′[γ^{32}P]ATP; Amersham Biosciences, Uppsala, Sweden); with 40 μM epidermal growth factor (EGF) receptor substrate peptide (H_2N-KR**T**LRR-OH), 1.2 mM $CaCl_2$,

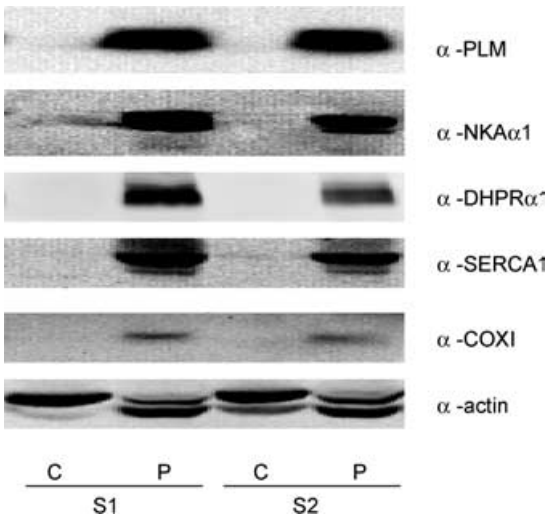

Figure 1. Immunoblots of marker proteins for subcellular membrane structures in human skeletal muscle after fractionation
Muscle samples ($n = 2$; S1, S2) were fractionated as described in the Methods, and equal amounts of protein (35 μg) were subjected to immunoblotting procedures and probed with antibodies specific to skeletal muscle subcellular/membrane structures. Blots were deliberately overexposed to detect the slightest immunoreactivity. Prefix α, anti; S, sample; P, particulate; C, cytosolic.

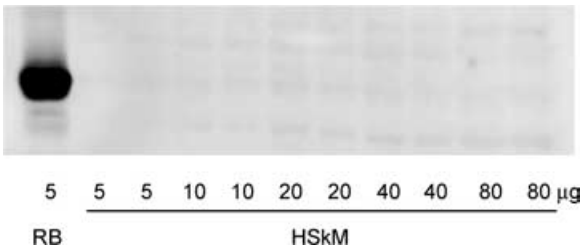

Figure 2. Protein kinase Cβ_{II} is not expressed in human skeletal muscle
Rat brain (RB) and human skeletal muscle (HSkM) proteins were subjected to immunoblotting procedures and probed using a polyclonal anti-PKCβ_{II} antibody.

10 μM phorbol-12-myristate-13-acetate (PMA), and 20 μg ml^{-1} phosphatidylserine in a final reaction volume of 25 μl. The EGF receptor peptide was chosen as it has the minimal requirements for conventional, novel and atypical PKC substrates including basic residues at -2 and $+3$, as well as a hydrophobic residue at the $+1$ position (Nishikawa *et al.* 1997). The reaction procedure was conducted as outlined previously (Rose & Hargreaves, 2003), except that the reaction time was 5 min.

To directly measure isoform-specific PKC activity, PKC isoforms and isotypes were immunoprecipiated (IP) from muscle extracts (500 μg) incubated with anti-PKCθ or -PKCζ/ι antibodies (2 μg; Santa Cruz Biotechnology) in a final volume of 600 μl with gentle mixing for 2 h at 4°C. Following this 40 μl of 50% (v:v) of protein-A-sepharose (Amersham Biosciences) was added and further incubated with mixing for 2 h at 4°C. To immunopurify conventional PKC (cPKC), extracts (500 μg) were incubated with agarose-conjugated PKC-MC5 antibodies (20 μl; Santa Cruz Biotechnology) in a final volume of 600 μl with gentle mixing for 2 h at 4°C. The beads were washed and resuspended (40 μl) in buffer containing (mM): Tris 10 (pH 7.2), sodium pyrophosphate 1, EGTA 1. cPKC and PKCθ activities were assayed for 10 min as described above. PKCζ/ι activity was assayed as described by Bandyopadhyay *et al.* (1997*b*) with minor

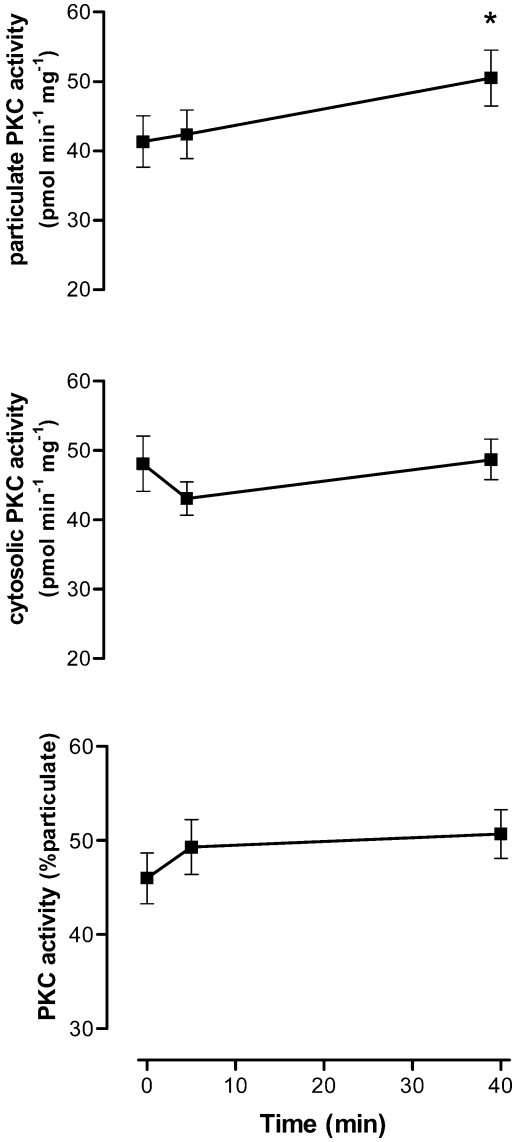

Figure 3. Protein kinase C (PKC) activity of skeletal muscle extracts at rest and after exercise
Cytosolic and particulate skeletal muscle extracts were assayed *in vitro* for PKC activity. Data are mean ± S.E.M., $n = 8$. Significantly different from 0: $^*P < 0.05$.

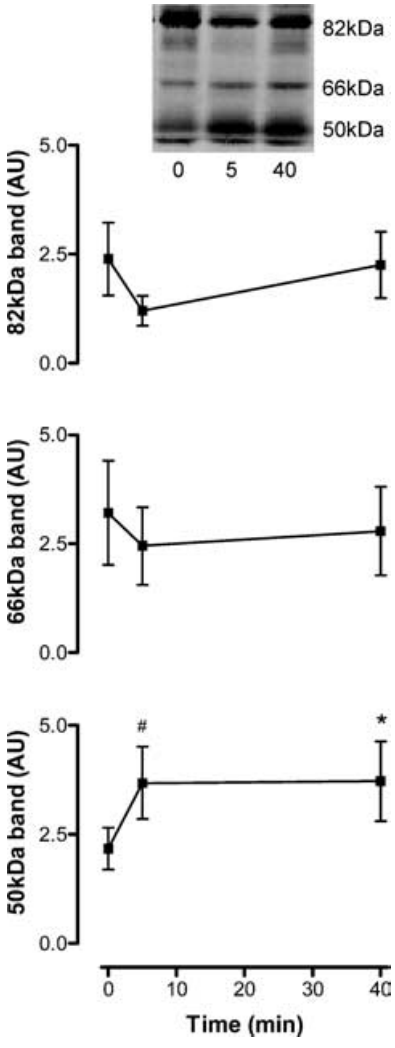

Figure 4. Exercise increases the serine phosphorylation of a 50-kDa protein, but not 66- or 82-kDa proteins, in contracting human skeletal muscle
Solubilised protein from crude skeletal muscle extracts was subjected to immunoblotting procedures and probed using a polyclonal phospho-serine PKC substrate motif antibody. Data are mean ± S.E.M., $n = 8$. Significantly different from 0: $^*P < 0.05$, $\#P < 0.01$. Insert shows a representative blot from one subject.

modifications. Assays were performed in kinase assay buffer at 30°C with 40 μM [S^{25}]-PKC(19–31) substrate peptide (H_2N-RFARKG**S**LRQKNV-OH), and 40 μg ml^{-1} phosphatidylserine in a final reaction volume of 25 μl. Assays were conducted as previously described (Rose & Hargreaves, 2003). The assay time and PKC concentration were within a linear range for lysate and IP assays (data not shown). Preliminary experiments demonstrated that exercise did not alter the efficiency of immunoprecipitation of any of the PKC isoforms investigated (data not shown).

PKC abundance and phosphorylation were determined in equal amounts of protein in muscle extracts and IP samples using immunoblot analysis as previously described (Rose & Hargreaves, 2003) with anti-PKCα (H7), anti-PKCβII (C-18), anti-PKCδ (C-20), anti-PKCε (C-15), anti-PKCθ (C-19), anti-PKCζ/ι (C-20) (Santa Cruz Biotechnology), anti-p$T^{410/403}$-PKCζ/ι and anti-pS-PKC substrate motif (R/K-X-S^{PO_4}-Hyd-R/K; Cell Signalling Technology, Beverly, MA, USA) antibodies.

To provide a control sample for assays, L6 skeletal myotubes were treated with PMA (0.1 μM; PKC activity) or insulin (1 μM; PKCζ/ι activity). All media solutions were purchased from Invitrogen (Carlsbad, CA, USA). L6 myotubes were prepared as previously described (Rose & Hargreaves, 2003). For PMA treatment, cells ($n = 3$ dishes per treatment) were treated with PMA (0.1 μM) and control cells were treated with vehicle (0.1% ethanol) for 30 min. The cells were lysed in homogenization buffer without NP-40 and collected after 10 min on ice. PKC activity in the cytosolic and particulate fractions was determined as described above. The percentage of PKC activity in the particulate fraction relative to total PKC activity was $46 \pm 5\%$ in vehicle and $64 \pm 3\%$ in PMA-treated myotubes ($n = 3$, $P < 0.05$). Furthermore, a significant translocation of PKCα from the cytosolic fraction to the particulate fraction was shown by immunoblot analysis ($P < 0.01$; data not shown) as observed in other studies (Braiman *et al.* 1999). In addition, PMA induced increases (0.7- to 1.3-fold, $P < 0.05$) in the density of four out of five bands detected when particulate extracts were analysed by immunoblotting with a pSer-PKC substrate motif antibody (data not shown). These data indicate that the PKC assay and immunoblot analyses of fractionated cell lysates are sensitive measures of PKC activity and localization.

Myotubes ($n = 3$ dishes per treatment) were treated with or without insulin (1 μM) for 5 min and cells were lysed with homogenization buffer containing NP-40 and collected after 10 min on ice. IP PKCζ/ι activity was determined as described above. An increase in aPKC activity (control, 12.3 ± 1.3 pmol mg^{-1}min^{-1}; insulin, 28.3 ± 1.3 pmol mg^{-1}min^{-1}; $P < 0.001$) was

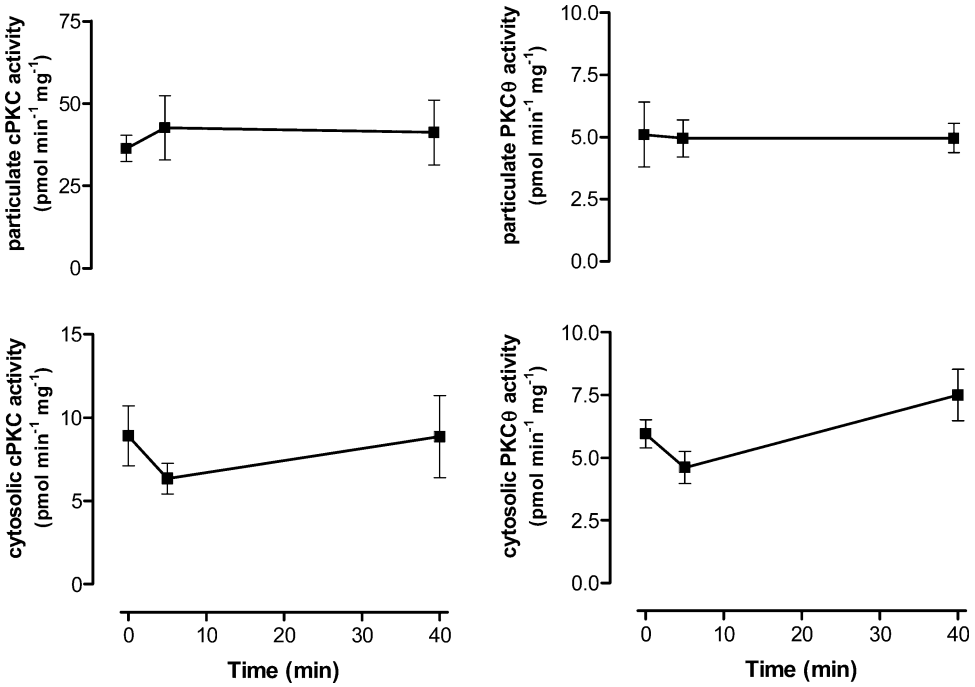

Figure 5. Conventional protein kinase C (cPKC) and PKCθ activity of skeletal muscle fractions at rest and after exercise

Cytosolic and particulate extracts were prepared and cPKC (left panel) and PKCθ (right) were immunoprecipitated from skeletal muscle extracts (500 μg) and assayed for PKC activity *in vitro*. Data are mean ± S.E.M., $n = 6$.

measured with insulin treatment when compared with control cells, as observed in other studies (Bandyopadhyay *et al.* 1997*a*).

Calculations and statistics

Kinase activity was calculated as described by Goueli *et al.* (2001). The molecular weights of immunoreactive proteins were estimated by fitting their relative mobility in a curve generated by plotting the relative mobility against the log of the molecular weights of protein standards (Precision Plus Protein Standards, Bio-Rad, CA, USA). Arbitrary units for protein (total or phosphorylated) abundance were expressed as a ratio of sample band intensity relative to an internal control band intensity. The relative PKC activity and abundance between fractions are expressed as a ratio of particulate units and total (particulate + cytosolic) units. Statistical testing was done with unpaired *t* tests (MS Excel) or one-way ANOVA with repeated measures with *post hoc* (Student–Newman–Keuls) testing performed when differences were significant as appropriate (GraphPad Prism, v.2.01). Data are expressed as mean ± S.E.M. and differences were considered to be significant when $P < 0.05$.

Results

PKC isoform expression in human skeletal muscle

In the present study, PKCα, PKCδ, PKCε (data not shown), PKCθ and PKCζ/ι were expressed in human skeletal muscle. These findings are similar to observations made by Itani *et al.* (2000). Despite being able to obtain a strong signal in rat brain, PKCβ_{II} was not detected in human skeletal muscle (Fig. 2), which is in contrast to the findings of others (Itani *et al.* 2002).

Effect of exercise on PKC activity

With 40 min ergometer exercise a slight increase in total PKC activity was observed in the particulate fraction only ($P < 0.05$; Fig. 3). No change was detected with 5 min of exercise in cytosolic or particulate PKC ($P > 0.1$; Fig. 3). As there were no changes in the ratio of PKC activity between fractions (Fig. 3), this suggests that there was no translocation of total PKC activity, and thus total PKC abundance, between the fractions. Rather, there may be an increase in the intrinsic activity of PKCs in membrane structures. To obtain an index of PKC activity, whole tissue extracts from basal and exercise samples were subjected to immunoblotting with a phospho-PKC substrates motif antibody. There were three consistently strongly immunoreactive bands detected with this antibody, when reacted with proteins from human skeletal muscle (Fig. 4). While there were no changes in the serine phosphorylation (pS) of the 82- and 66-kDa proteins, there was a 73 ± 18% and 75 ± 25% increase in pS of a 50-kDa protein at 5 and 40 min, respectively ($P < 0.05$; Fig. 4), indicating that there may be an increase in PKC activity towards this protein substrate/s.

To examine whether select PKC isoforms were activated by exercise in contracting skeletal muscle, PKCs were immunopurified from tissue lysates and assayed for activity. There were no differences in cPKC or PKCθ activity in particulate or cytosolic fractions when basal and exercise samples were compared at both time points (Fig. 5). In contrast, there were 65 ± 26% and 80 ± 38%

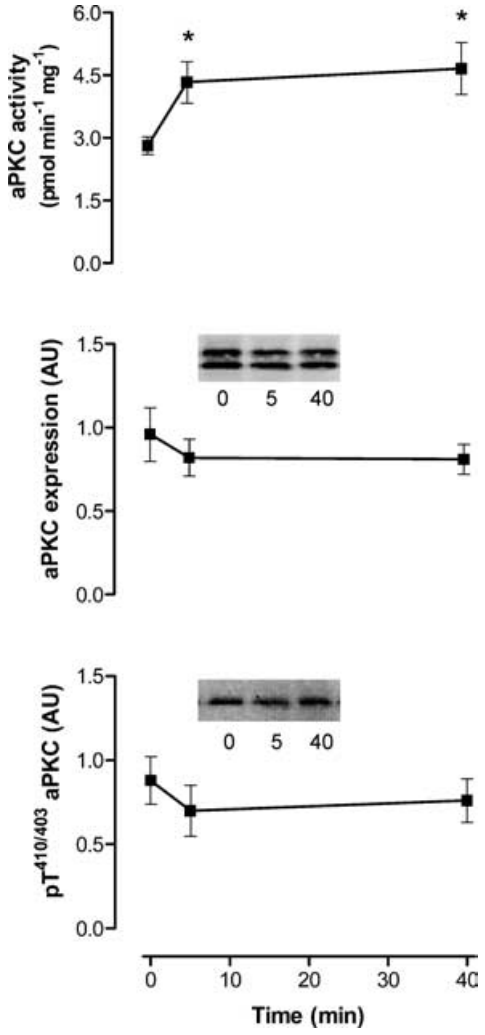

Figure 6. Atypical protein kinase C (aPKC or PKCζ/ι) activity, expression and Thr$^{410/403}$ phosphorylation of contracting skeletal muscle before and during exercise in humans
aPKC was immunoprecipitated from skeletal muscle tissue extracts (500 μg) and assayed *in vitro* (top panel). Equal amounts of solubilised protein from crude skeletal muscle tissue extracts were also subjected to immunoblotting procedures and probed using a polyclonal PKCζ/ι (middle panel) and phospho-Thr$^{410/403}$-PKCζ/ι (bottom panel) antibodies. Data are mean ± S.E.M., $n = 8$. Significantly different from 0: $^*P < 0.05$. Inserts show representative blots from one subject.

increases in aPKC activity immunopurified from whole tissue lysates at 5 and 40 min, respectively (Fig. 6). These increases in aPKC activity occurred despite no changes in aPKC protein expression or phosphorylation at threonine residue 410/403 (Fig. 6) measured from the same tissue extracts.

Effect of exercise on PKC isoform localization

The relative PKC isoform abundance between skeletal muscle membrane and cytosol fractions from basal and exercise samples were determined by immunoblotting using isoform-specific antibodies. There were no differences in the abundance of PKCα, aPKC, PKCδ and PKCθ, within cytosolic or particulate fractions when comparing basal and exercise samples (Figs 7 and 8). Nor were there any differences in the fraction ratio (% in the particulate extract) induced by exercise. These data suggest that exercise does not induce translocation from the cytosol to membranes of these PKC isoforms in human skeletal muscle.

Discussion

The major finding of this study was that atypical PKC (aPKC) activity increased early (5 min) after the commencement of exercise in contracting skeletal muscle, and remained elevated after 40 min of exercise. This result is similar to findings of others that show higher activity of aPKC in contracting skeletal muscle during

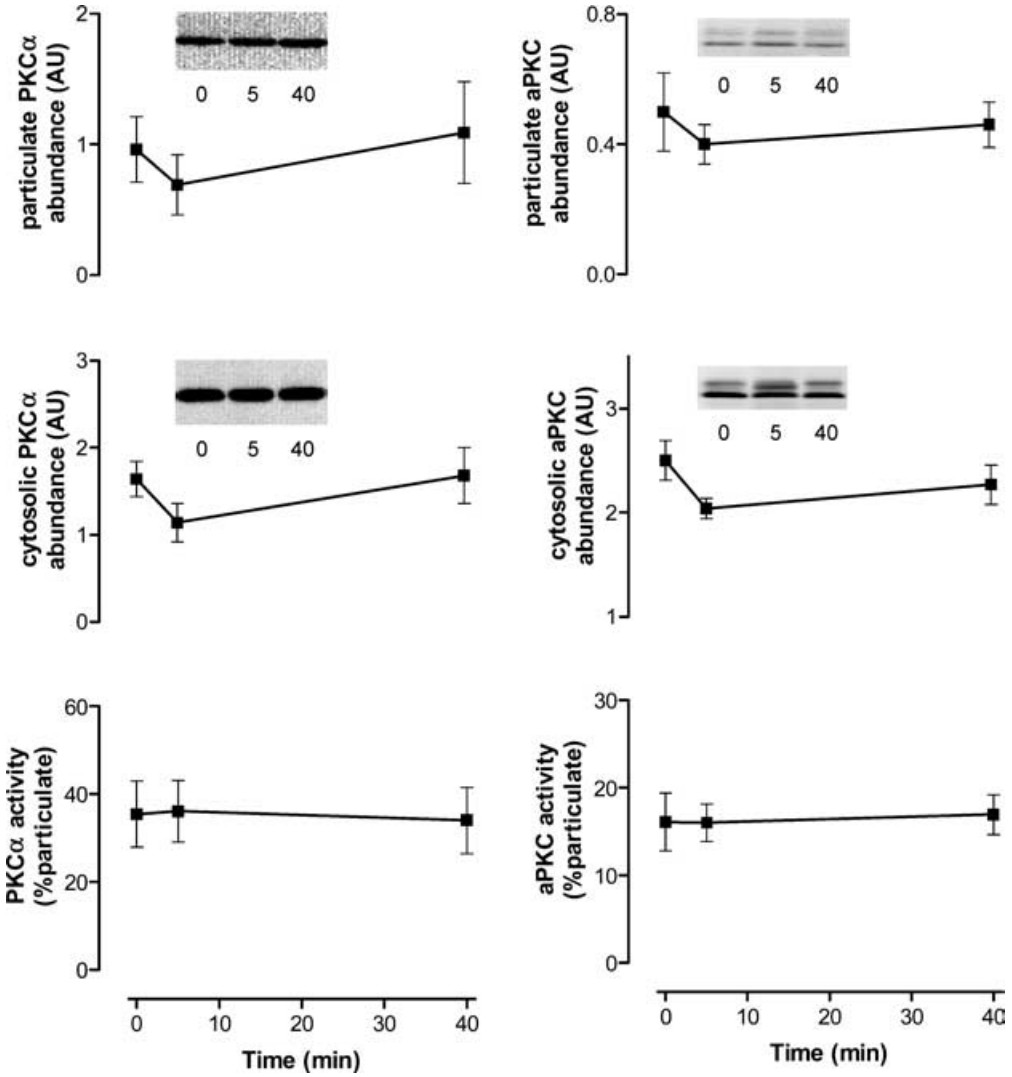

Figure 7. Effect of exercise on isoform-specific protein kinase C (PKC) localization in contracting skeletal muscle during exercise in humans
Equal amounts of protein from skeletal muscle extracts were subjected to immunoblotting procedures and probed using PKCα (left panel) and PKCζ/ι (right panel) antibodies. Data are mean ± S.E.M., $n = 7$. Inserts show representative blots from one subject.

exercise in humans (Beeson *et al.* 2003; Nielsen *et al.* 2003). Furthermore, studies in rodents have also demonstrated a similar time-course for increased aPKC activity in skeletal muscle of exercised animals (Chen *et al.* 2002).

The mechanism for increased aPKC in contracting skeletal muscle is not clear. It has been demonstrated that phosphatidic acid (PA; 5–50 μM) increases aPKC (immunopurified from rodent skeletal muscle) activity *in vitro* (Chen *et al.* 2002). It is important to note that when L6 myotubes were incubated with 2,4-dinitrophenol (DNP), which activated aPKC in these cells via phospholipase D production of PA, incubation with a phospholipase D inhibitor reduced immunopurified aPKC activity (Chen *et al.* 2002), suggesting that the effects of PA are conserved even after the process of immunopurification. Given that PA concentrations are higher in hindlimb muscles of rodents after nerve electrical stimulation *in situ* (Cleland *et al.* 1989), this is a likely mechanism for higher aPKC activity in contracting muscle. The mechanism by which insulin activates aPKC is dependent, at least in part, on phosphorylation of a threonine residue within the activation loop by 3-phosphoinositide-dependent protein kinase-1 (PDK1; Standaert *et al.* 2001). However, there is evidence that activation of aPKC via PLD does not require PDK1-mediated phosphorylation of aPKC (Sajan *et al.* 2002). Furthermore, while the effect of exercise on muscle PDK1 activity has not been investigated, exercise does not activate insulin receptor substrate 1 (IRC1)-associated PI3-kinase (Wojtaszewski *et al.* 1997), and therefore

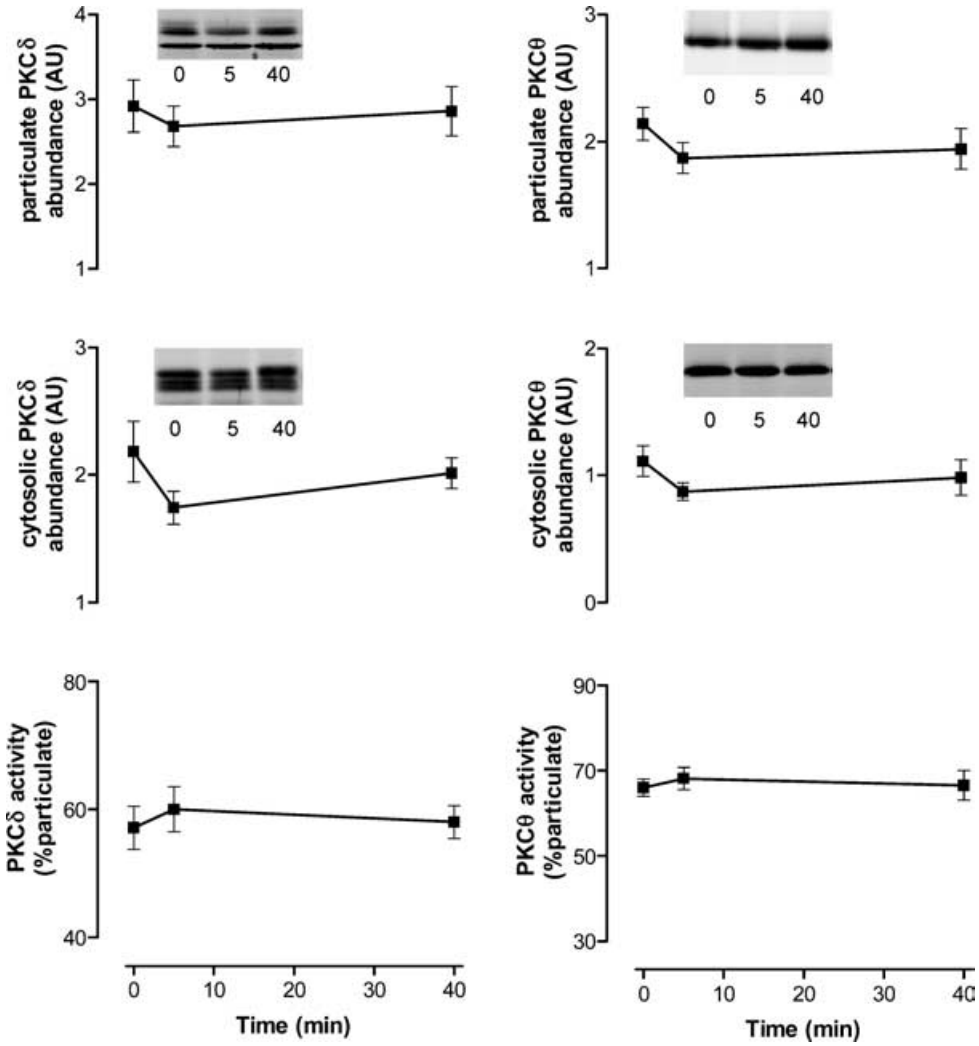

Figure 8. Effect of exercise on isoform-specific protein kinase C (PKC) localization in contracting skeletal muscle during exercise in humans
Equal amounts of protein from skeletal muscle extracts were subjected to immunoblotting procedures and probed using PKCδ (left panel) and PKCθ (right panel) antibodies. Data are mean ± S.E.M., $n = 7$. Inserts show representative blots from one subject.

presumably PDK1, in contracting muscle. In the present study, no change in phosphorylation of these threonine residues of aPKC was observed when comparing exercise and basal samples. Furthermore, there was no translocation of aPKC from the cytosol to membranes with exercise. These findings are consistent with those reported by others (Richter *et al.* 2004). However, the present findings on aPKC phosphorylation and localization are in contrast to a study by Perrini *et al.* (2004), where skeletal muscle aPKC phosphorylation and abundance in membranes were higher after 30 and 60 min of exercise, respectively. While the reason for the discrepancies between the present findings and those by Perrini *et al.* (2004) are not readily apparent, they may be related to sample preparation (phosphorylation) and time-dependent effects (localization).

It has been suggested that aPKC may be involved in the exercise-induced increase in glucose transport (Chen *et al.* 2002). However, there is little evidence linking aPKC and glucose transport in muscle during exercise. Studies using the microbial agent calphostin-C as an inhibitor of PKC (Wojtaszewski *et al.* 1998; Ihlemann *et al.* 1999) or down-regulation of 1,2-diacylglycerol (DAG)-sensitive PKC enzymes by chronic phorbol ester treatment (Cleland *et al.* 1990) have demonstrated a role for PKC in glucose transport during exercise. However, neither of these treatments is likely to affect aPKC (Kobayashi *et al.* 1989; Bandyopadhyay *et al.* 1997*a*), and therefore results of these studies should not be extended to suggest a role for aPKC in contraction-mediated glucose transport. Clearly, further studies are warranted to investigate the potential role of aPKC in exercise-mediated glucose transport in skeletal muscle given that there is strong evidence linking aPKC to insulin-mediated glucose transport and glucose transporter isoform 4 (GLUT4) translocation (Farese, 2002).

In the present study there was a slight increase in general PKC activity within membranes and increased serine phosphorylation of a 50 kDa protein with exercise. While not specific, these data indicate an increase in PKC activity with exercise. Given that the majority of PKC within skeletal muscle consists of conventional and novel isoforms (Itani *et al.* 2000), their activity/localization was investigated. Unlike in previous studies in rodents (Richter *et al.* 1987; Cleland *et al.* 1989), there was no evidence of a translocation of PKC activity or protein from a cytosolic fraction to a particulate/membrane fraction. In particular, there were no alterations in cPKC or PKCθ activities within or between these fractions with exercise. Neither were there any changes in the abundance of PKC isoforms within or between fractions with exercise. The lack of effect of exercise on cPKC activity and PKCα between cytosolic and membrane fractions was unexpected as increases in Ca^{2+} concentration by DNP in L6 myotubes induced increases in cPKC activity and higher cPKC protein abundance in membranes (Khayat *et al.* 1998). Furthermore, exposure of amphibian skeletal muscle to agents that raise intracellular free Ca^{2+} resulted in translocation of cPKC activity towards membranes (Sun & Zhu, 1998). It is unlikely that the different mode of Ca^{2+} stimulation (oscillatory *vs.* constant) is an explanation as Ca^{2+} oscillations are also known to stimulate PKCα translocation (Oancea & Meyer, 1998).

No change was detected in the activity and localization of the other DAG-sensitive PKCs (PKCθ and PKCδ) in skeletal muscle with exercise. Similarly, acute exercise does not affect skeletal muscle PKCδ activity of the Israeli sand rat (Heled *et al.* 2003) or humans (AJ Rose & M Hargreaves, unpublished observations); nor translocation of PKCδ and PKCε in human skeletal muscle (Perrini *et al.* 2004). While not all of the PKC isoforms have been examined, the present results suggest that exercise does not alter the activity of conventional or novel PKCs in contracting human skeletal muscle.

DAG is an important cofactor required for complete activation and translocation of DAG-sensitive PKCs (Newton, 1995). While it has been demonstrated that DAG concentration increases in electrically stimulated rodent hindlimb muscle (Cleland *et al.* 1989) other studies have shown no effect of contraction on skeletal muscle DAG concentration (Turinsky *et al.* 1990). Whether a lack of DAG accumulation accounts for the unresponsiveness of skeletal muscle cPKC and nPKC to exercise in the present study requires further investigation.

In summary, aPKC, but not cPKC or PKCθ, is activated by exercise in contracting human skeletal muscle and thus may play a role in regulating skeletal muscle function and metabolism during exercise in humans.

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