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Identification of protein kinase D as a novel contraction-activated kinase linked to GLUT4-mediated glucose uptake, independent of AMPK

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Abstract

Contraction-induced glucose uptake is only partly mediated by AMPK activation. We examined whether the diacylglycerol-sensitive protein kinase D (PKD; also known as novel PKC isoform μ) is also involved in the regulation of glucose uptake in the contracting heart. As an experimental model, we used suspensions of cardiac myocytes, which were electrically stimulated to contract or treated with the contraction-mimicking agent oligomycin. Induction of contraction at 4 Hz in cardiac myocytes or treatment with 1 μM oligomycin enhanced (i) autophosphorylation of PKD at Ser916 by 5.1- and 3.8-fold, respectively, (ii) phosphorylation of PKD's downstream target cardiac-troponin-I (cTnI) by 2.9- and 2.1-fold, respectively, and (iii) enzymatic activity of immunoprecipitated PKD towards the substrate peptide syntide-2 each by 1.5-fold. Although AMPK was also activated under these same conditions, in vitro phosphorylation assays and studies with cardiac myocytes from AMPKα2$^{-/-}$ mice indicated that activation of PKD occurs independent of AMPK activation. CaMKKβ, and the cardiac-specific PKC isoforms α, δ, and ε were excluded as upstream kinases for PKD in contraction signaling because none of these kinases were activated by oligomycin. Stimulation of glucose uptake and induction of GLUT4 translocation in cardiac myocytes by contraction and oligomycin each were sensitive to inhibition by the PKC/PKD inhibitors staurosporin and calphostin-C. Together, these data elude to a role of PKD in contraction-induced GLUT4 translocation. Finally, the combined actions of PKD on cTnI phosphorylation and on GLUT4 translocation would efficiently link accelerated contraction mechanics to increased energy production when the heart is forced to increase its contractile activity.

Keywords: PKD; AMPK; Glucose uptake; GLUT4 translocation; Cardiac myocytes

1. Introduction

The heart predominantly consists of specialized muscle cells, cardiac myocytes, which contract constantly in a coordinated fashion. To generate energy for a proper electro-mechanical activity, cardiac myocytes utilize long-chain fatty acids (LCFA) and glucose [1,2]. In rat cardiac myocytes it was demonstrated that electrically induced contraction increases the rate of glucose uptake, coinciding with the translocation of the glucose transport protein GLUT4 from intracellular storage compartments to the sarcolemma [3,4]. Just like contraction, oligomycin, an inhibitor

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CaMKK, calcium/calmodulin-dependent kinase kinase; cTnI, cardiac troponin-I; ERK, extracellular signal-regulated protein kinase; PKC, protein kinase C; PKD = Protein kinase D; PMA, phorbol-12-myristate-13-acetate.

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of mitochondrial F_1/F_0 ATPase, also stimulates GLUT4-mediated glucose uptake: the effect of oligomycin on glucose uptake is non-additive to that of contraction, indicating that both treatments use the same mechanism to induce GLUT4 translocation [4]. In addition, we have previously demonstrated in cardiac myocytes that, upon electrical stimulation or treatment with oligomycin, the intracellular AMP/ATP ratio increases, resulting in AMPK activation [4]. This simultaneous activation of AMPK and induction of GLUT4 translocation by contraction and contraction-mimetic agents have led to the general notion that AMPK is involved in contraction-induced glucose uptake in heart and skeletal muscle [5,6].

The activity of AMPK is not only regulated by the intracellular AMP/ATP ratio, but also by phosphorylation at Thr172 by AMPK kinases (AMPKK's) [7]. Recently two AMPKK's have been identified, namely LKB1 and CaMKKβ [8–10]. In the heart, AMPK can be activated during exercise, hypoxia and ischemia [11]. The main downstream target of AMPK is acetyl-CoA carboxylase (ACC) [12]. Active AMPK phosphorylates ACC at Ser79 thereby inactivating ACC which results in an increase in LCFA oxidation. AMPK is a protein consisting of three different subunits, the catalytic α-subunit and the regulatory β- and γ-subunits. Although two isoforms (α1 and α2) of the catalytic subunit are present in the heart, the α2 subunit is predominant [12,13].

Recently, it was shown that in heart from transgenic mice overexpressing a dominant negative AMPKα2 mutant, contraction was still able to stimulate glucose uptake [14]. This demonstrates that contraction-induced glucose uptake can only be partly ascribed to AMPK. Interestingly, in H-2K skeletal muscle cells expressing dominant negative AMPKα2, a cell-permeable diacylglycerol (DAG) analogue, phorbol 12-myristate 13-acetate (PMA), was able to stimulate glucose uptake [15], suggesting that a protein kinase sensitive to DAG is involved. In L6 skeletal muscle cells it has been demonstrated that the DAG-sensitive protein kinase D3 (PKD3) directly contributes to basal glucose uptake [16]. Taken together, these results suggest that PKD, in addition to AMPK, could also mediate contraction-induced glucose uptake.

Previously, PKD has been classified as a member of the PKC family [17], and has been frequently referred to as PKC-μ. The PKC family consists of three subfamilies, i.e., conventional, novel and atypical PKCs [18]. Conventional PKCs require diacylglycerol (DAG) and Ca^{2+} for their activation, whereas novel PKCs also require DAG but are Ca^{2+}-independent, and atypical PKC's require neither DAG nor calcium [18,19]. PKD possesses a DAG binding site, and was therefore subclassified as a novel PKC isoform, i.e., PKC-μ. However, the catalytic domain of PKD is more closely related to that of the Ca^{2+}-calmodulin-regulated protein kinases (CaMKs) and displays relatively little homology to the catalytic domains of the PKC family [20]. Moreover, compared to other members of the PKC family, PKD possesses an additional pleckstrin-homology domain, a putative transmembrane sequence and lacks a pseudosubstrate region. Therefore, PKD has been positioned into a novel kinase family, comprising three members: PKD-1 (referred to as PKD), PKD2 and PKD3, [21,22].

In non-stimulated mammalian cell lines, PKD was found to be localized to the cytosol and several intracellular membrane compartments including Golgi and mitochondria [22,23]. Treatment of COS cells with phorbol esters induced a persistent translocation of PKD from the cytosol to the plasma membrane, requiring the DAG-binding domain. In addition to phorbol esters, PKD can also be activated by various agonists, most of which bind to G protein-coupled receptors (GPCR) [22]. GPCR-mediated activation of PKD is mediated by members of the PKC family, and involves a phosphorylation of two serine residues within the activation loop, i.e., Ser744 and Ser748 [23–25]. In addition to the transphosphorylation at Ser744/748, PKD is autophosphorylated at Ser916 upon activation [26]. Ser916 autophosphorylation has also been shown to occur upon phorbol ester stimulation, and was found to correlate accurately with catalytic activity of PKD [26,27].

PKD has been found to be present in the heart, where it is also activated by phorbol ester treatment [28]. In addition, GPCRs have been shown to activate PKD in the heart via a PKC-dependent mechanism [29]. The heart expresses several conventional and novel PKC isoforms (PKC-α,-δ, and-ε) [30,31]. It has not yet been investigated which of these PKCs is involved in GPCR-mediated PKD activation.

In the present study, we explored in cardiac myocytes whether PKD is activated by contraction, and whether this is linked to glucose uptake. First, we determined whether electrically induced contraction and treatment of cardiac myocytes with oligomycin-stimulated PKD translocation, Ser916 phosphorylation, as well as PKD enzymatic activity. Subsequently, the positioning of PKD relative to AMPK was studied with in vitro kinase assays and in cardiac myocytes isolated from AMPKα2$^{-/-}$ mice. Thereafter, we attempted to identify upstream kinases involved in oligomycin/contraction-induced PKD activation in cardiac myocytes. Finally, we linked contraction-induced PKD activation to contraction-induced glucose uptake by using pharmacological agents that inhibit selected PKCs as well as PKD. The combined observations reveal that PKD is activated in cardiac myocytes by contraction, independent of AMPK activation. This suggests that there is a PKD-mediated contraction signaling pathway leading to GLUT4 translocation, parallel to AMPK signaling.

2. Materials and methods

2.1. Materials

[γ-^{32}P]-ATP and 2-deoxy [1-^{3}H]-glucose were obtained from GEHealthcare (Piscataway, NJ, USA). STO-609 and antibodies directed against PKC-α, PKC-δ, PKC-ε, PKD/PKCμ, phospho-AMPK (Thr172), phospho-ERK (Thr202/Tyr204), phospho-PKC-α (Thr638), phospho-PKC-δ (Ser643), phospho-PKD/PKC-μ (Ser916) and phospho-troponin-I (Ser23/24) were obtained from Cell Signaling (Beverly, MA, USA). Phospho-ACC (Ser79) antibody was obtained from Upstate (Charlotteville, VA, USA). The antibody directed against AMPKα1 was a gift from Prof Grahame Hardie (University of Dundee, UK). Murine PKD anti-peptide polyclonal antibody was raised against the synthetic peptide EEREMKALSERVSIL [32]. Oligomycin, phorbol-12-myristate-13-acetate, staurosporine, Gö6976 and Gö6983 were purchased from Sigma-Aldrich (St. Louis, NJ, USA). GST-PKD was prepared and purified as described [28]. Recombinant AMPK was a kind gift of Prof Uwe Schlattner and Dr

Dietbert Neumann (Zürich, Switzerland) and CamKKβ was kindly provided by Dr David Carling (London, UK).

2.2. Isolation and treatment of adult rat cardiac myocytes

Cardiac myocytes were isolated from male Lewis rats (200–250 g) using a Langendorff perfusion system and a Krebs Henseleit bicarbonate medium equilibrated with a 95% O_2/5% CO_2 gas phase (medium A) at 37 °C as previously described [33]. After isolation, the cells were washed twice with medium A supplemented with 1 mM $CaCl_2$ and 2% (w/v) fatty acid-free BSA (medium B) and then suspended in 15 ml medium B. The isolated cells were allowed to recover for approximately 45 min at room temperature. At the end of the recovery period, cells were washed and suspended in medium B. Only when more than 80% of these cells has a rod-shaped appearance and excluded trypan blue were they used for subsequent studies. The isolated cardiac myocytes were equilibrated for 15 min at 37 °C with continuous shaking. Thereafter, cardiac myocytes were incubated for 20 min at 37 °C with continuous shaking either with 0.5% DMSO (control), 1 μM oligomycin, electrically stimulated (at 200 V with a frequency of 4 Hz) or 1 μM PMA. The figure legends indicate where cardiac myocytes were pre-incubated with specific inhibitors or where the duration of incubation differed.

2.3. Isolation and treatment of adult mouse cardiac myocytes

Cardiac myocytes were isolated from 6 months old male wild-type mice and AMPKα2$^{-/-}$ mice using a Langendorff perfusion system as recently described [34]. For all the experiments the viability of cardiac myocytes was 60–80% (data not shown). The isolated mouse cardiac myocytes were incubated for 20 min at 37 °C with continuous shaking, and either with 0.5% DMSO (control), 1 μM oligomycin or 1 μM PMA.

2.4. Fractionation of adult rat cardiac myocytes into particulate and cytosolic fractions

Adult rat cardiac myocytes (2 ml, 15–20 mg wet weight/ml) were incubated in medium B for 15 min with either 1 μM oligomycin or 1 μM PMA, and divided in two: 0.5 ml for obtaining the total protein fraction, and 1.5 ml for fractionation into particulate and cytosolic fractions. Then, both aliquots were immediately centrifuged at 4000 rpm for 4 min at 4 °C. The pellets were resuspended in ice-cold homogenizing buffer (20 mM Tris HCl, pH 7.4, 0.33 M sucrose, 5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF and 0.005% Aprotinin), 0.3 ml for obtaining the total protein fraction (which was then immediately frozen in liquid nitrogen and stored at −80 °C), and 0.5 ml for allowing further fractionation. Thereafter, the 0.5-ml suspension was frozen in liquid nitrogen and thoroughly homogenized by three cycles of freeze/thawing. Homogenates were then centrifuged at 18,000 ×*g* for 20 min at 4 °C to collect particulate (pellet) and cytosolic (supernatant) fractions. The pellet was resuspended in 0.3 ml ice-cold homogenizing buffer. Both fractions were stored at −80 °C.

2.5. Fractionation of adult rat cardiac myocytes into plasma membrane and intracellular membrane fractions

Cardiac myocytes (2.25 ml; 20–25 mg wet mass/ml) were incubated in medium B for 15 min without further additions or with 1 μM oligomycin or with 1 μM staurosporin 15 min prior to oligomycin addition. At the end of the incubation, the total cell suspension was quickly transferred to a tightly fitting 5-ml Potter-Elvejhem glass homogenizer on ice containing 1 ml of cold H_2O, after which NaN_3 was added to a final concentration of 5 mM in order to stop ATP-dependent vesicular trafficking events such as GLUT4 translocation. Immediately thereafter, cell suspensions were homogenized with 10 strokes of the Potter-Elvejhem homogenizer. Subsequently, fractionation was carried out as described previously [4]. Plasma membrane (PM) and intracellular membrane fractions (light-density microsomes; LDM) were stored at −80 °C.

2.6. Cardiac myocyte AMPK assays

Cardiac myocytes treated at various times with 1 μM oligomycin were centrifuged, pelleted and resuspended in lysis buffer. 300 μl of supernatant fraction was precipitated with 10% (w/v) polyethylene glycol 6000 and resuspended in 100 μl of "lopamix" (100 mM Tris HCl, pH=7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 3,5% glycerol, 50 mM NaF and 5 mM NaPPi). Aliquots (corresponding to 50 μg protein) were incubated for 10 min at 30 °C with 0.1 mM Mg[γ-^{32}P]-ATP (specific activity 1000 cpm/pmol), kinase assay mix, 0.2 mM AMP and 0.2 mM SAMS peptide, in a final volume of 50 μl [35]. Reactions were stopped by spotting 20 μl aliquots on phosphocellulose p81 papers. The papers were washed four times with 2% phosphoric acid, once with acetone and dried. The amount of radioactivity incorporated into the SAMS peptide was determined by scintillation counting.

2.7. PKD immunoprecipitation and assay of PKD activity in cardiomyocytes

Isolated cardiac myocytes were incubated for 15 min at 37 °C with or without 1 μM oligomycin or 1 μM phorbol myristate acetate. Thereafter the cells were pelleted and resuspended in ice-cold lysis buffer (50 mM Tris HCl (pH=7.4), 2 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol, 1:25 cocktail of protease inhibitors (Complete) and 1% (w/v) Triton X-100). Immunoprecipitation and PKC activity were determined as according to Brooks et al. [28]. Briefly, cell suspensions were centrifuged at 13,000 rpm for 30 min at 4 °C. Supernatant fractions (600 μl) were immunoprecipitated with the murine PKD anti-peptide polyclonal antibody (1:100) [32] overnight at 4 °C on a rotating wheel. Thereafter, 40 μl of Protein A Sepharose bead suspension was added and incubations were continued for 1 h at 4 °C on a rotating wheel. The beads were collected (6000 rpm x 2 min) and washed twice with lysis buffer, twice with kinase assay buffer (30 mM Tris, pH 7.4, 15 mM $MgCl_2$), and finally 30 μl of kinase assay buffer containing 0.1 mM Mg[γ-^{32}P]-ATP (specific activity 1000 cpm/pmol) and 5 μl 2 mg/ml syntide-2 were added to each condition to start the reaction. After 20 min at 30 °C, aliquots (20 μl) were spotted on phosphocellulose papers, and radioactivity incorporation into syntide-2 was quantified as described above.

2.8. Immunoblotting

Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed with the relevant primary antibodies. The protein bands were visualized using enhanced chemiluminescence and immunoblot intensities were analyzed by using densitometry.

2.9. In vitro protein kinase studies

To test whether AMPK could be activated by PKD, purified recombinant AMPK α2β1γ1 (1.5 μg) was incubated in the presence or absence of purified recombinant CaMKKβ (0.1 U) or PKDSer916E (0.3 U). The incubations contained 1 mM $MgCl_2$, 0.1% (v/v) β-mercaptoethanol in 50 mM Hepes (pH 7.4) and 300 μM MgATP, and were conducted for 40 min at 30 °C. The activity of PKD-or CamKKβ-activated AMPK was assayed by incorporation of [γ-^{32}P] into SAMS peptide as described above.

To test whether PKD could be activated by AMPK, purified recombinant PKD (5 μg) was incubated in the presence or absence of purified recombinant α1 T172E β1γ1 AMPK (0.2 U) as described above. The activity of PKD was then measured by incorporation of [γ-^{32}P] into syntide-2 peptide.

2.10. Statistics

Data are presented as means±S.E.M. Differences between cardiac myocyte preparations were tested with a non-parametric Mann–Whitney *U*-test. The paired *t*-test was used to define differences between treatments within a cardiomyocytes preparation. $p<0.05$ indicates a statistical significance.

3. Results

3.1. Effect of oligomycin and contraction on PKD activity in rat cardiac myocytes

Autophosphorylation of PKD at Ser916 is considered to be an accurate indicator of activity of this protein kinase

[26,27,36]. We first determined the optimal conditions for oligomycin treatment of cardiac myocytes (Fig. 1A). Treatment of cardiac myocytes with oligomycin at 1 μM already increased Ser916 phosphorylation by 3.8-fold, which slightly increased to 4.9-fold above basal at 30 μM oligomycin (Fig. 1A). Incubation of cardiac myocytes at higher oligomycin concentrations resulted in decreased cell viability [4]. When examining Ser916 phosphorylation as function of incubation time of cardiac myocytes with oligomycin, already after 5 min, Ser916 phosphorylation reached the maximal level, after which it remained constant until at least 20 min (Fig. 1A). Electrical stimulation at 4 Hz enhanced Ser916 phosphorylation in cardiac myocytes to 5.1-fold, a similar order of magnitude compared to oligomycin treatment (Fig. 1B). As a positive control for PKD activation, we used the phorbol ester species phorbol-12-myristate-13-acetate (PMA; 1 μM, 15 min), which had a more potent effect on Ser916 phosphorylation (16.5-fold; Fig. 1B). Ser916 phosphorylation did not further increase when oligomycin was added together with PMA (Ser916 phosphorylation in cardiac myocytes treated simultaneously with oligomycin and PMA: 13.7±4.6-fold above basal; $n=3$). When examining phosphorylation of cTnI, a direct downstream target of PKD [37], oligomycin treatment, electrically induced contraction, and PMA treatment stimulated Ser23/24 phosphorylation by 2.1-, 2.9- and 2.4-fold, respectively (Fig. 1B).

We have previously shown that both oligomycin treatment and electrostimulation induce AMPK activation in cardiac myocytes [4], which was confirmed in the present study by the simultaneous phosphorylation of AMPK-Thr172 and ACC-Ser79 upon oligomycin treatment (12.2-fold and 18.7-fold, respectively) and after electrostimulation (5.8-fold and 5.0-fold, respectively; Fig. 1B). In contrast, PMA treatment had no effect on phosphorylation of AMPK or ACC.

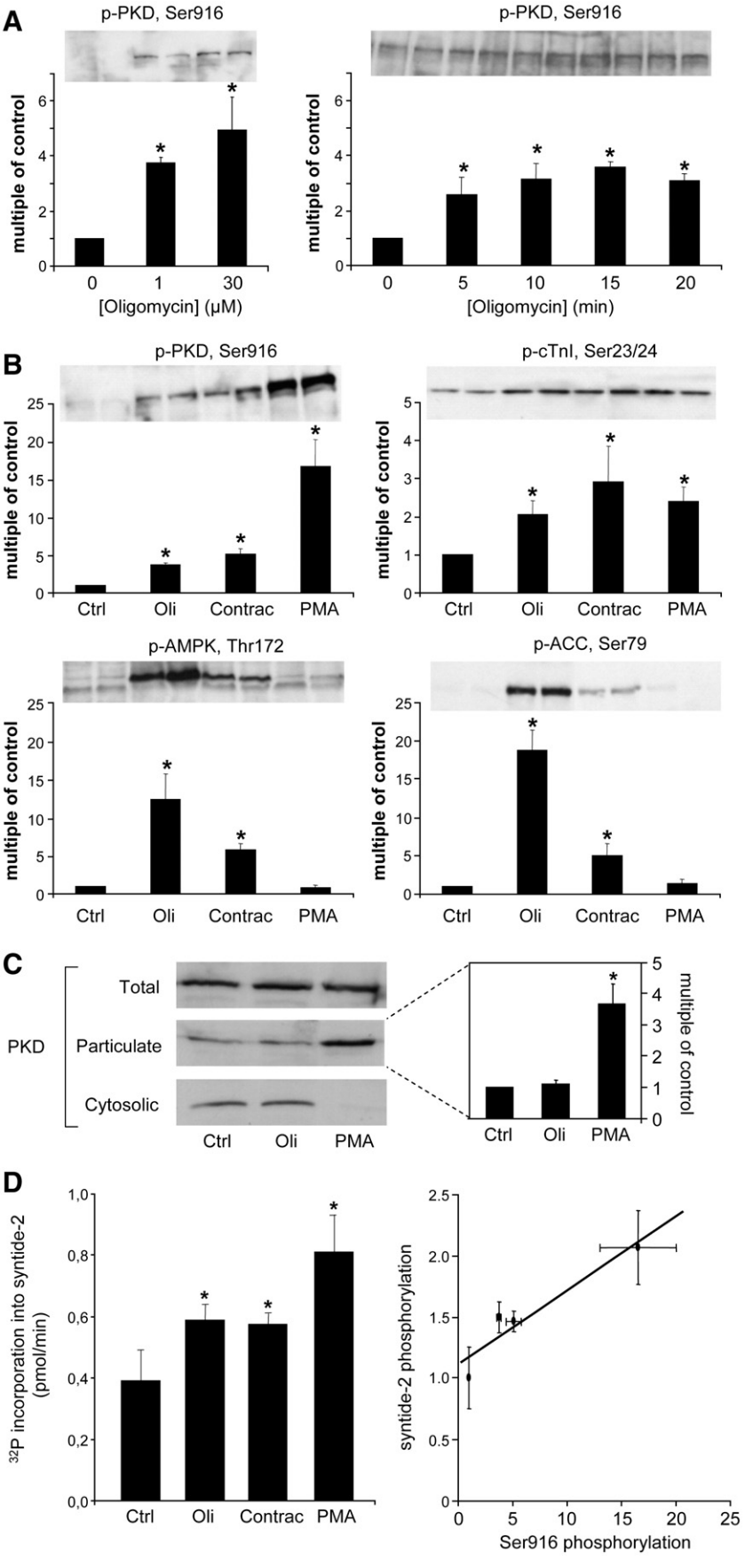
Besides by phosphorylation, PKD, just like PKC's, is activated by binding to intracellular membranes [20–22]. Therefore, we investigated whether the contraction-mimetic agent oligomycin-induced translocation of PKD to cellular membranes. For this purpose, cardiac myocytes were incubated for 15 min with 1 μM oligomycin or, for comparison, 1 μM PMA, and then fractionated into a cytosolic and a particulate fraction. Under non-stimulated conditions (control) PKD is present both in the soluble cytoplasm and bound to subcellular membranes. PMA treatment resulted in an entire disappearance of PKD from the cytosolic fraction and a concomitant 3.2-fold increase in the particulate fraction, indicating that PMA induces a complete translocation of PKD from the soluble cytoplasm to subcellular membranes of cardiac myocytes (Fig. 1C). An estimation of the amount of membrane-bound PKD relative to total cellular PKD in non-stimulated cells cannot be made by comparing PKD Western signals between the different fractions, because the ratio of PKD over total protein in each fraction is likely to be different. But since the amount of membrane-bound PKD in PMA-treated cells is equal to the total cellular PKD content, it can be deduced that the amount of membrane-bound PKD in non-stimulated cells is 0.3-fold of that of PMA-treated cells (inset of Fig. 1C). In contrast to PMA, oligomycin treatment did not affect the subcellular distribution of PKD, maintaining the ratio of membrane-bound over total PKD at 0.3 (Fig. 1C).

Translocation of PKD, PKD autophosphorylation, and phosphorylation of the cellular PKD substrate cTnI each are indirect indications of PKD activation. Therefore, we have also directly measured PKD enzymatic activity. For this, cardiac myocytes were treated with the various stimuli, followed by PKD immunoprecipitation, and an in vitro kinase assay with syntide-2 as peptide substrate. The three treatments each resulted in increased [^{32}P]ATP incorporation into syntide-2 (oligomycin and contraction: both 1.5-fold; PMA: 2.0-fold; Fig. 1D). In addition, the changes in PKD enzymatic activity were proportional to the increases in Ser916 phosphorylation (Fig. 1D).

3.2. Positioning of PKD relative to AMPK: in vitro kinase studies

Because AMPK and PKD are activated simultaneously by either oligomycin or contraction, the question arises whether, or not, the kinases are components of the same signaling pathway. In an initial attempt to address this question we investigated whether purified PKD and purified AMPK were able to activate each other directly in in vitro kinase assays. Firstly, we determined whether PKD was able to directly activate AMPK.

Fig. 1. Effect of oligomycin and contraction on PKD translocation, phosphorylation and activity in cardiac myocytes. (A) Isolated adult rat cardiac myocytes were treated with varying oligomycin concentrations during 15 min (left panel; $n=5$), or with 1 μM oligomycin at varying incubation periods (right panel; $n=3$). Cell incubations were terminated by pulse centrifugation and subsequent dissolution in sample buffer (see Materials and methods). Then, the degree of PKD-Ser916 phosphorylation was determined by Western blotting using a phosphospecific antibody, followed by densitometry using Quantity-One (Biorad) software. A representative Western blot is displayed. In B, C, and D, cardiac myocytes were treated with 1 μM oligomycin (Oli), 4 Hz electrostimulation (Contrac), or with 1 μM PMA during 15 min. (B) For assessment of phosphorylation of PKD-Ser916 ($n=9$), cTnI-Ser23/24 ($n=5$), AMPK-Thr172 ($n=10$) and ACC-Ser79 ($n=10$), cardiac myocytes were pelleted, dissolved in sample buffer, after which Westerns were performed with phosphospecific antibodies. Gels were loaded with equal quantities of protein for each cell lysate (20 μg per lane). Values are expressed as multiple of "Control". Representative Western blots are displayed. Note that the lower band in the p-AMPK blot likely represents albumin, which is present abundantly in the cardiac myocyte lysates. Albumin, like AMPK, has a molecular mass of ~60 kDa. (C) For assessment of PKD translocation from cytosol to membranes, cardiac mycoytes were separated into total, particulate and cytosolic fractions ($n=3$). In these fractions the protein content was determined, after which 10 μg for each fraction was subjected to Western blotting using a specific antibody directed against PKD1/2 (see Materials and methods). Representative Western blots are presented. The amount of PKD in the particulate fraction was quantified using densitometry (see inset). (D) For assessment of PKD activity, cardiac myocytes were lysed and used for immunoprecipitation of PKD (see Materials and methods). PKD activity is measured by the incorporation of [γ-^{32}P]-ATP into syntide-2 and is expressed as pmol/min (left panel). The relationship between PKD activity, measured as syntide-2 phosphorylation (data derived from left panel, but calculated to multiple of Control, which is set at 1), and PKD autophosphorylation at Ser916 (data derived from B, and expressed as multiple of Control, which is set at 1) is displayed in the right panel. All data are expressed as means±S.E.M.; n refers to the number of experiments carried out with different cardiac myocyte preparations. ∗Significantly different from non-treated cardiac myocytes ($p<0.05$).


A
p-PKD, Ser916
multiple of control
[Oligomycin] (μM)
p-PKD, Ser916
multiple of control
[Oligomycin] (min)
B
p-PKD, Ser916
p-cTnI, Ser23/24
p-AMPK, Thr172
p-ACC, Ser79
Ctrl
Oli
Contrac
PMA
C
PKD
Total
Particulate
Cytosolic
multiple of control
D
32P incorporation into syntide-2 (pmol/min)
syntide-2 phosphorylation
Ser916 phosphorylation

For measurement of AMPK activity, we determined Thr172 phosphorylation of AMPK with a phosphospecific antibody, as well as the rate of incorporation of ^{32}P into the SAMS peptide. As a positive control for AMPK activation in these in vitro kinase assays, Ca^{2+}/calmodulin-dependent protein kinase kinase-β (CaMKKβ), a well-established upstream activating AMPKK, was able to strongly activate AMPK as measured by the SAMS assay as well as Thr172 phosphorylation (Fig. 2A). However, full length constitutively active PKD (PKDSer916E) had no effect on AMPK activity or on Thr172 phosphorylation (Fig. 2A). Secondly, we determined whether AMPK was able to directly activate PKD by measuring PKD activity with syntide-2 as substrate and by phosphorylation at Ser916. Constitutively active AMPK (AMPKThr172E) had no effect on PKD activity. In addition, PKD could not be activated by treatment with CaMKKβ (Fig. 2B).

3.3. Is PKD a downstream target of AMPKα2?

The lack of effect of AMPK on PKD activity, and vice-versa, does not rule out the possibility that both kinases are operating within one signaling pathway. To more decisively solve this issue, we investigated PKD activation in cardiac myocytes from AMPKα2$^{-/-}$ mice (for initial characterization of this knockout mouse model in which the AMPKα2 catalytic subunit gene was inactivated, the reader is referred to [38]). In these cardiac myocytes, the total amount of AMPKα1 did not appear to be different from that of wild-type cardiac myocytes (Fig. 3A), indicating that the absence of the α2-subunit in mice is not compensated by an increase in expression of the α1-subunit. In cardiac myocytes from wild-type mice, oligomycin treatment during 15 min resulted in an increase in AMPK-Thr172 phosphorylation by 8.3-fold (±0.6, $n=4$) (Fig. 3A), but oligomycin did not increase AMPK-Thr172 phosphorylation in cardiac myocytes from AMPKα2$^{-/-}$ mice, confirming the phenotype of this knockout model. Moreover, oligomycin-induced ACC phosphorylation was markedly, but not completely blunted in cardiac myocytes from AMPKα2$^{-/-}$ mice (i.e., residual oligomycin-induced ACC-Ser79 phosphorylation: 35%±7, $n=4$), suggesting that in the absence of the AMPKα2 isoform, the α1 subunit or possibly other kinases could contribute towards ACC phosphorylation. By contrast, PMA did not affect either AMPK or ACC phosphorylation (Fig. 3A).

To determine whether PKD could be downstream of AMPKα2, we determined whether oligomycin and, for comparison PMA, was able to activate PKD in AMPKα2$^{-/-}$ cardiac myocytes. Treatment of cardiac myocytes from wild-type mice for 15 min with oligomycin or PMA markedly increased PKD activity by 1.7-fold, 2.1-fold, respectively, and in cardiac myocytes from AMPKα2$^{-/-}$ mice both compounds increased PKD activity by 2.0-fold and 2.1-fold, respectively (Fig. 3B). Taken together, the data suggest that AMPKα2 is unlikely to be involved in oligomycin-induced PKD activation.

3.4. Search for protein kinases upstream of PKD in contraction signalling

- Protein kinases C-α,-δ and-ε: it has been reported that in several cell lines, PKD is activated in a PKC-dependent manner, and novel PKC isoforms especially have been implicated in PKD activation. Features of PKC activation are its translocation to subcellular membranes possibly in combination with

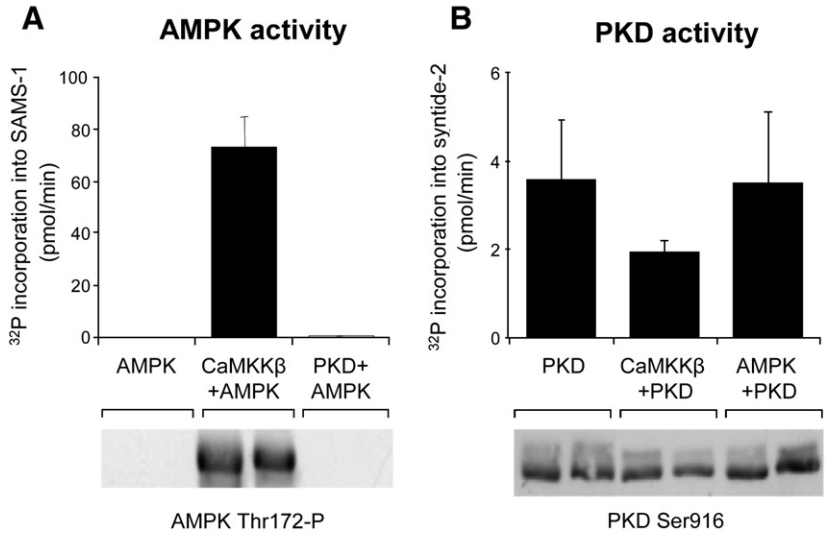


Fig. 2. In vitro effect of recombinant PKD on recombinant AMPK activity and vice-versa. (A) In vitro effect of CaMKKβ and PKD-Ser916E on AMPKα2 β1γ1 activity and Thr172 phosphorylation. Active CaMKKβ or PKD were incubated for 40 min at 30°C min with AMPKα2 β1γ1. Thereafter, AMPKα2 activity was determined by incorporation of [γ-^{32}P]-ATP into SAMS peptide within 5 min (=initial activation). In addition, phoshhorylation of AMPK at Thr172 was measured by Western blotting using a phosphospecific antibody. A representative Western blot is presented. AMPK activity is expressed in pmol/mg protein per min and presented as means±S.E.M., $n=3$. (B) In vitro effect of AMPK on initial PKD activity. Activated AMPK α1-T172E β1γ1 was incubated for 40 min at 30 °C with full length PKD. Thereafter, PKD activity was determined by incorporation of [γ-^{32}P]-ATP into syntide-2 within 5 min (=initial activation). In addition, phopshorylation of PKD at Ser916 was measured by Western blotting using a phosphospecific antibody. A representative Western blot is presented. AMPK activity is expressed in pmol/mg protein per min and presented as means±S.E.M., $n=3$. *Significantly different from basal ($p<0.05$).

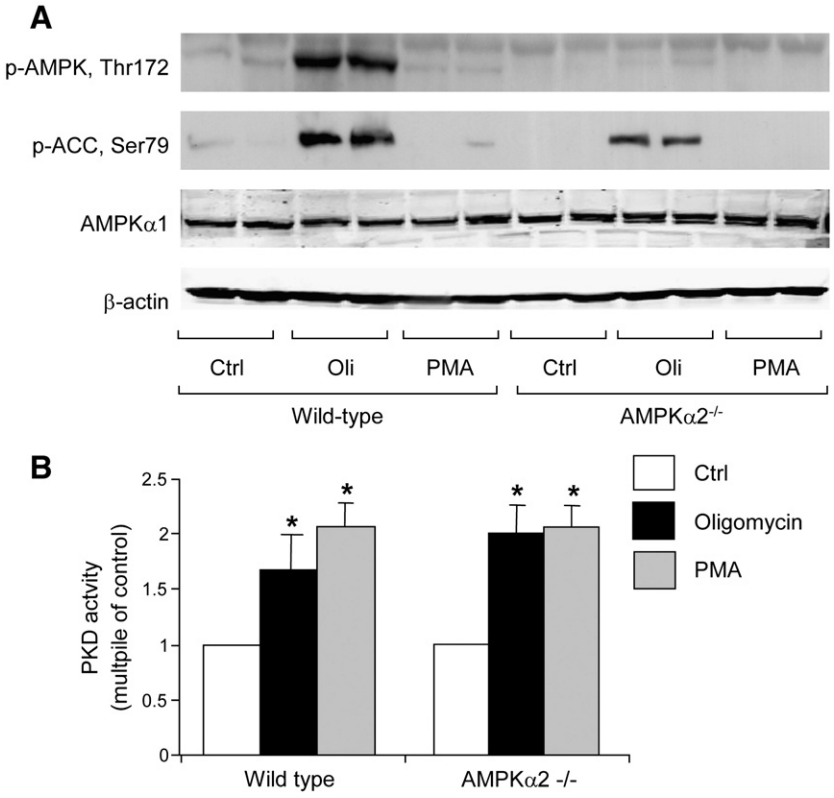


Fig. 3. Effect of oligomycin and PMA on PKD activity, p-AMPK (Thr172) and p-ACC (Ser79) in cardiomyocytes from wild-type and AMPKα$^{-/-}$ mice. Adult cardiac myocytes were isolated from wild-type mice (n=4) and AMPKα2$^{-/-}$ mice (n=4). These cardiac myocytes were either treated for 20 min at 37 °C without or with 1 μM oligomycin (Oli) or 1 μM PMA. (A) Upon pelleting of cardiac myocytes and their subsequent dissolution in sample buffer, phosphorylation of AMPK at Thr172 and ACC at Ser79 and the total amount of AMPKα1 was determined by Western blotting. In addition, total protein content of AMPKα1 and β-actin (loading control) was determined. (B) Upon PKD immunoprecipitation of lysed cardiac myocytes, PKD activity was measured by incorporation of [γ-^{32}P]-ATP into syntide-2. PKD activity in non-stimulated cardiac myocytes from wild-type mice amounted to 0.30±0.06 pmol/min. PKD activity in non-stimulated cardiac myocytes from AMPKα2$^{-/-}$ mice was not significantly different from that in non-stimulated cardiac myocytes from wild-type mice. Data are expressed relative to control cardiac myocytes, and presented as means±S.E.M. *Significantly different from control wild-type cardiac myocytes. **Significantly different from oligomycin-treated wild-type cardiac myocytes.

phosphorylation of activation loop Ser/Thr residues. First, we tested whether the major conventional and novel PKC isoforms that are present within the heart [30,31] are subject to membrane translocation in response to oligomycin. In these cardiac myocyte incubations, PMA was used as a positive control for PKC activation. During the incubation period, the total protein content of PKCα, δ and ε in cardiac myocytes was unaltered upon treatment with either oligomycin or PMA compared with untreated cardiac myocytes (Fig. 4A). PMA treatment caused a complete shift in the content of PKC-α,-δ and-ε from the cytosolic to the particulate fraction (Fig. 4A). However, oligomycin treatment had no effect on the distribution of PKC-α,-δ and-ε between particulate and cytosolic fractions (Fig. 4A).

We also tested whether commercially available phosphospecific antibodies against the major cardiac conventional/novel PKCs could provide an indication for oligomycin-induced PKC activation. Thus, we examined phosphorylation of PKC-α at Thr638 and phosphorylation of PKC-δ at Ser643. Although phosphorylation of these sites does not seem to be directly involved in activation [39,40], phosphorylation of Thr638 and Ser643 might still reflect activation due to subsequent poorly understood autophosphorylation events. PMA treatment increased Ser643 phosphorylation of PKC-δ (1.7-fold±0.2; n=3), but not Thr638 phosphorylation of PKC-α. Oligomycin treatment had no effect on phosphorylation at either of these sites (Fig. 4B).

- CaMKKβ: because of the marked sequence homology of PKD with members of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) family [20], we investigated whether PKD could be downstream of CaMKKβ. Therefore, we treated isolated rat cardiomyocytes with STO-609, a specific CaMKK inhibitor [41], at a relevant concentration of 3 μM [42]. However, STO-609 did not affect oligomycin-induced PKD-Ser916 phosphorylation (Fig. 4C). In another attempt to assess the involvement of CaMKK in activation of PKD via Ser916 phosphorylation, cardiac myocytes were incubated with compounds that cause a rise in cytosolic Ca^{2+}. The sarcoplasmatic Ca^{2+}-releasing agent thapsigargin was used at 1 μM, a concentration at which CaMKK is activated in cell lines [43]. Under this condition, PKD-Ser916 phosphorylation was not observed (Fig. 4C). However, there was also no detectable PKD-Ser916 phosphorylation in the presence of 1 μM of the Ca^{2+} ionophore A23187, at which concentration CaMKK-

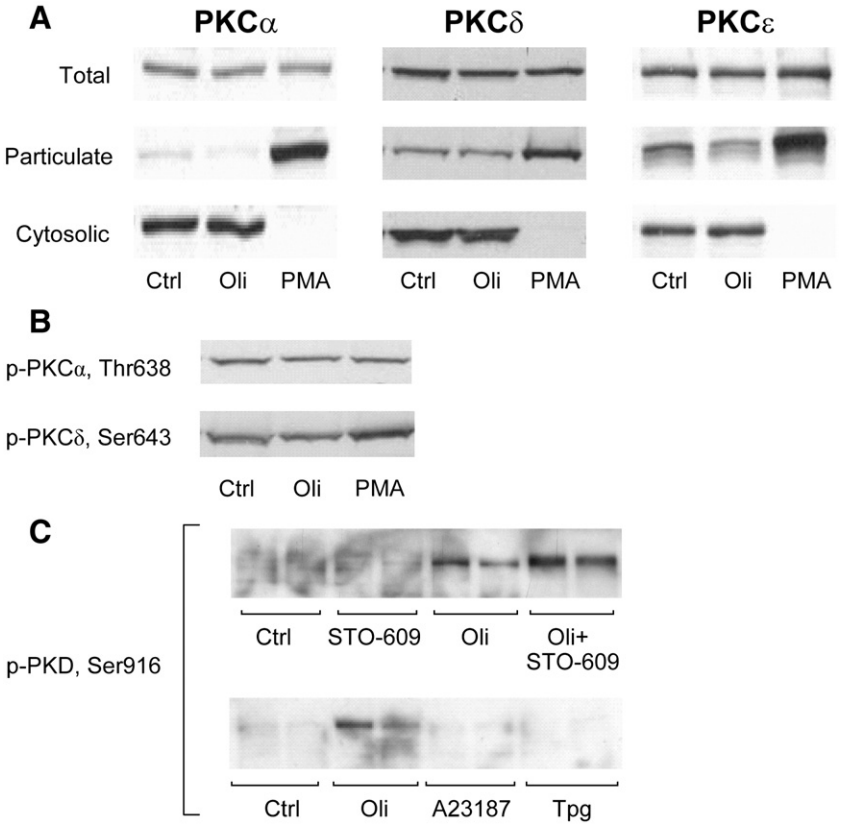


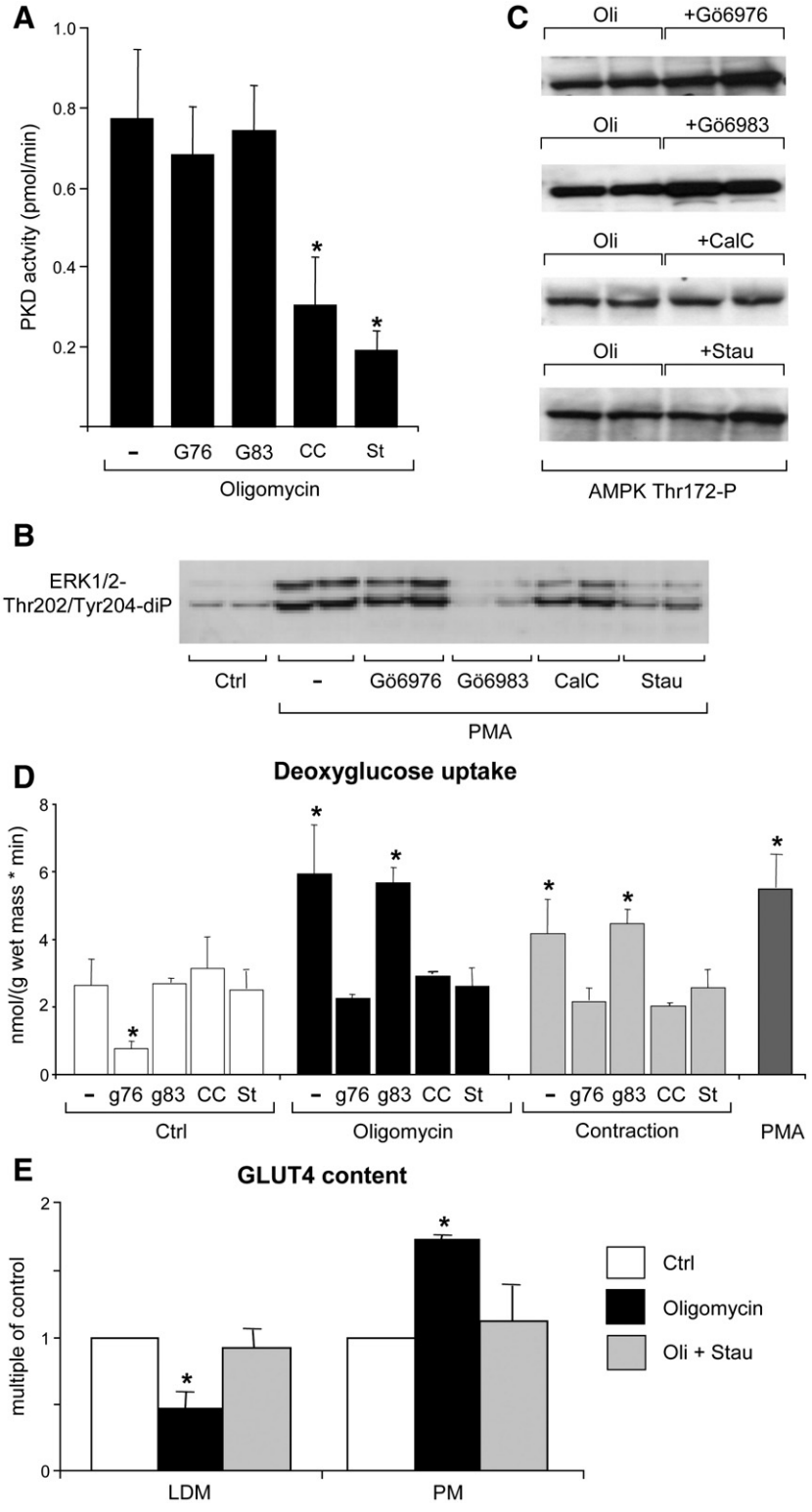
Fig. 4. Possible upstream activating kinases of PKD. (A) Effect of oligomycin and PMA on translocation of the α-, δ- and ε-isoforms of PKC. Isolated adult rat cardiac myocytes were incubated for 15 min at 37 °C without additions or with 1 μM oligomycin (Oli) or 1 μM PMA. Then, cardiac myocytes were separated into total, particulate and cytosolic fractions (see Materials and methods). In these fractions the protein content was determined, after which 10 μg for each fraction was subjected to Western blotting using antibodies against PKCα,-δ and-ε. Representative Western blots out of 3 independent experiments are presented. (B) Possible role of CaMKKβ in PKD-Ser916 phosphorylation. Isolated adult rat cardiac myocytes were pre-incubated for 30 min at 37 °C without additions or with 3 μM STO-609 (=a specific CaMKKβ inhibitor). Thereafter, cardiac myocytes were incubated without additions or with 1 μM oligomycin, 1 μM A23187 or 1 μM thapsigargin (Tpg) for 20 min at 37 °C. Gels were loaded with equal quantities of protein for each cell lysate (20 μg per lane). PKD-Ser916 phosphorylation was determined by Western blotting. Representative Western blots out of 3 independent experiments are presented.

related effects have been observed in HeLa cells and in mouse embryonic fibroblasts [42]. In cardiac myocyte incubations from the same experiment, PKD was strongly phosphorylated at Ser916 in the presence of PMA. Based on these observations it is unlikely that Ca^{2+} signaling and CaMKKs play a role in contraction-induced PKD signaling.

Fig. 5. Effects of protein kinase inhibitors on contraction/oligomycin-stimulated glucose uptake and GLUT4 translocation in cardiac myocytes. (A) Effects of PKC/PKD inhibitors on PKD enzymatic activity in immunoprecipitates from oligomycin-treated cardiac myocytes. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions or with various kinase inhibitors i.e., 1 μM staurosporine (Stau), 2 μM calphostin-C (CalC), 10 μM Gö6976 (Gö76) or 10 μM Gö6983 (Gö83). Thereafter, cardiac myocytes were treated with 1 μM oligomycin for 15 min at 37 °C. Then, cardiac myocytes were lysed and used for immunoprecipitation of PKD (see Materials and methods). PKD activity is measured by the incorporation of [γ-^{32}P]-ATP into syntide-2 and is expressed as pmol/min. (B) Effects of PKC/PKD inhibitors on ERK phosphorylation in PMA-treated cardiac myocytes. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions or with the various kinase inhibitors, as described above. Thereafter, cardiac myocytes were treated without additions or with 1 μM PMA for 15 min at 37 °C. For assessment of ERK phosphorylation, cardiac myocytes were pelleted, dissolved in sample buffer, after which Westerns were performed with a phosphospecific antibody against ERK1/2-Thr202/Tyr204. (C) Effects of PKC/PKD inhibitors on AMPK phosphorylation in oligomycin-treated cardiac myocytes. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions or with the various kinase inhibitors, as described above. Thereafter, cardiac myocytes were treated with 1 μM oligomycin for 15 min at 37 °C. For assessment of AMPK phosphorylation, cardiac myocytes were pelleted, dissolved in sample buffer, after which Westerns were performed with a phosphospecific antibody against AMPK-Thr172. In case of the assessments of both ERK- and AMPK phosphorylation, gels were loaded with equal quantities of protein for each cell lysate (20 μg per lane), and representative Western blots out of 3 independent experiments are displayed. (D) Effects of PKC/PKD inhibitors on contraction- and oligomycin-stimulated glucose uptake; comparison with PMA-stimulated glucose uptake. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions or with the various kinase inhibitors, as described above. Thereafter, cardiac myocytes were either treated with DMSO, 1 μM oligomycin, or 1 μM PMA for 15 min at 37 °C. Initial rates of ^{3}H-deoxyglucose uptake were determined and expressed as nmol/(g cellular wet mass * min). Data are presented as means±S.E.M., $n=5$. (E) Effect of PKC/PKD inhibitors on oligomycin-induced GLUT4 translocation. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions or with 1 μM staurosporine (Stau). Thereafter, cardiac myocytes were treated with DMSO (Ctrl) or 1 μM oligomycin for 15 min at 37 °C. Then, cardiac myocytes were separated into a plasma membrane (PM) fraction and a light-density microsomal (LDM) fraction. In these fractions the protein content of GLUT4 was determined by Western blotting. Data are presented as means±S.E.M., $n=4$. *Significantly different from non-treated (control) cardiomyocytes ($p<0.05$).

3.5. Effect of PKC inhibitors on deoxyglucose uptake into cardiac myocytes

PKD has been previously classified as a member of the novel PKC family [17]. It shares extensive homology with regulatory domains of novel PKCs. Specific inhibitors against PKD have not yet been identified or generated. In order to link oligomycin/contraction-induced activation of PKD to oligomycin/contraction-induced glucose uptake and GLUT4 translocation, we used a set of PKC inhibitors that exhibit different selectivity towards



PKC isoforms and PKD. Staurosporine is among the most potent PKC inhibitors, and is known to inhibit the catalytic domain of all three classes of PKCs and also PKD with high affinity [44]. Gö6976 and Gö6983 have been documented to inhibit conventional PKCs, but only Gö6976 was reported to have an additional inhibitory effect on PKD [17]. This differential inhibitory action of these staurosporine-derived compounds towards PKD has been exploited to investigate the involvement of PKD in a given cellular process (e.g., see [45,46]). In contrast with staurosporine and the Gö-compounds, calphostin-C inhibits PKCs not at their catalytic domain, but at their regulatory subunit, by competing at the binding site for phorbol esters and diacylglycerol [47].

Prior to investigating the effects of various PKC inhibitors on oligomycin/contraction-stimulated deoxyglucose uptake, we determined the extent to which these PKC inhibitors were able to block (i) PKD activation, (ii) PKC activation and/or (iii) AMPK activation.

(i) PKD activation: PKD enzymatic activity was measured in in vitro kinase assays on immunoprecipitates from oligomycin-treated cardiac myocytes with syntide-2 as peptide substrate. Calphostin-C (−60%) and staurosporine (−75%) markedly inhibited oligomycin-induced PKD activation, but Gö6976 and Gö6983 were without effect (Fig. 5A).

(ii) PKC activation: both conventional and novel PKC isoforms have been reported to be involved in phorbol ester-induced ERK activation (e.g., see Ref. [48]). As shown in Fig. 5B, PMA treatment of cardiac myocytes resulted in a marked (>10-fold) increase in p44/p42 ERK1/2 phosphorylation at Thr202 and Tyr204. This dual ERK phosphorylation was potently blocked by both Gö6983 (−78%±5; $n=3$) and staurosporine (−55%±3; $n=3$), modestly inhibited by calphostin-C (−26%±8, $n=3$), and not affected by Gö6976.

(iii) AMPK activation: none of the four inhibitors affected oligomycin-induced AMPK-Thr172 phosphorylation (Fig. 5C), adding novel evidence contributing to the presumed specificity of the used PKC inhibitors.

Basal [^{3}H]deoxyglucose uptake into cardiac myocytes was not affected by treatment with staurosporine, calphostin-C or Gö6983, while treatment with Gö6976 caused a large inhibition (−72%; Fig. 5D). Oligomycin treatment (15 min) and contraction (15 min) increased the rate of [^{3}H]deoxyglucose uptake into cardiac myocytes by 2.1-fold and 1.5-fold, respectively (Fig. 5D). Staurosporine, calphostin-C and Gö6976 each completely blocked [^{3}H]deoxyglucose uptake induced by either oligomycin or contraction. In contrast, oligomycin/contraction-induced [^{3}H]deoxyglucose uptake was unaffected by Gö6983 (Fig. 5D). Like oligomycin treatment, PMA enhanced [^{3}H]deoxyglucose uptake into cardiac myocytes, i.e., by 1.6-fold (Fig. 5D).

Given that staurosporine inhibited both oligomycin- and contraction-induced glucose uptake into cardiac myocytes and simultaneously inhibited PKD activation by each of these treatments, we investigated whether the role of PKD in contraction-induced glucose uptake could be extended to contraction-induced GLUT4 translocation. Subcellular fractionation of cardiac myocytes treated with oligomycin resulted in a 1.8-fold increase in GLUT4 content of the PM fraction concomitant with a 53% decrease in the LDM fraction (Fig. 5E), confirming that oligomycin induces the translocation of GLUT4 from an intracellular membrane compartment to the sarcolemma [3]. Pre-incubation of cardiac myocytes with staurosporin completely prevented oligomycin-induced GLUT4 translocation (Fig. 5E). Taken together, these observations point towards an important role of PKD in GLUT4-mediated glucose uptake into cardiac myocytes.

4. Discussion

PKD is a newly identified family of DAG-activated Ser/Thr protein kinases that play a role in multiple cellular processes in a variety of mammalian cell types. These processes include Golgi organization, cell proliferation and apoptosis [21]. The present study is the first to explore the role of PKD in signaling and glucose metabolism in heart. The major observations in this study are (i) an increase in contraction activates PKD in cardiac myocytes independently of AMPK signaling, and (ii) PKD activation is linked to contraction-induced GLUT4 translocation and GLUT4-mediated increase in glucose uptake. These observations identify a role for PKD in cardiac energy metabolism.

4.1. Contraction activates PKD in cardiac myocytes independently of AMPK

Contraction activates multiple signaling pathways, mainly arising from a rise in calcium oscillations and a reduction in cellular energy status. A number of key protein kinases, among which CaMKs, AMPK, extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (MAPK), are activated by an increase in contractile activity [49]. However, it was not known whether PKD is activated in the contracting heart.

Previously, we designed a system of cardiac myocytes in suspension to study the effect of controlled contractions by electric field stimulation on metabolism [3]. We showed that at a contraction rate of 4 Hz, intracellular AMP content rises, and consequently, AMPK and ACC are phosphorylated [3]. In these same experiments, the mitochondrial F1/F0 ATPase inhibitor oligomycin was also able to activate AMPK and induce ACC phosphorylation. In the present study, we confirmed the activation of AMPK by contraction and by oligomycin treatment, after which we made the novel observation that both treatments also induced PKD activation. Namely, contraction, oligomycin and PMA treatment each (i) induced PKD autophosphorylation at Ser916 (Fig. 1B), (ii) increased the enzymatic activity of PKD (Fig. 1D), and (iii) increased phosphorylation of the cellular PKD substrate cTnI (Fig. 1B). Importantly, the degree of Ser916 phosphorylation was proportional to the level PKD enzymatic activity (Fig. 1D),

confirming the validity of these measurements [26,27]. Specifically, contraction/oligomycin treatment induced a modest increase in both parameters of PKD activity, whereas PMA exerted a stronger effect (Fig. 1D). Since the effects of oligomycin and PMA treatment on Ser916 phosphorylation were non-additive, it is likely that the portion of PKD that is phosphorylated by contraction/oligomycin treatment of cardiac myocytes is a subpopulation of PMA-phosphorylated PKD. Since the maximal oligomycin-induced Ser916 phosphorylation amounts to 4.9-fold above basal (Fig. 1A) and the PMA-induced Ser916 phosphorylation amounts to 16.5-fold (Fig. 1B), it can be calculated that the oligomycin-phosphorylated PKD comprises 0.3-fold of the PMA-phosphorylated PKD (maximal phosphorylation state). We also found that contraction/oligomycin did not induce PKD translocation to subcellular membranes, while PMA-induced a complete translocation of PKD to subcellular membranes (Fig. 1C). The content of membrane-bound PKD in non-stimulated and in contraction/oligomycin stimulated cardiac myocytes was calculated to be 0.3-fold of that of PMA-treated cells (reflecting total cellular PKD, see results section 3.1). The remarkable similarity of the ratio of membrane-bound PKD in oligomycin-treated versus PMA-treated cells with the ratio of Ser916 phosphorylated PKD in oligomycin-treated versus PMA-treated cells (i.e., both 0.3) may indicate that contraction/oligomycin specifically induces phosphorylation of membrane-bound PKD, while PMA treatment will likely result in a uniform phosphorylation of both cytoplasmic and membrane-bound PKD in cardiac myocytes.

The simultaneous activation of PKD and AMPK by contraction and oligomycin treatment suggests that these kinases are located within the same signaling pathway. However, two lines of evidence indicate that PKD and AMPK are functioning within separate signaling pathways. First, in in vitro kinase studies, constitutively active PKD was unable to activate AMPK, and conversely, PKD was not activated by constitutely active recombinant AMPK. Secondly, oligomycin-induced PKD activation (1.7-fold in wild-type mice, as assessed with syntide-2) was completely preserved in AMPKα2 null cardiac myocytes. Especially this latter observation strongly indicates that AMPK is not involved in PKD activation due to oligomycin treatment.

Which upstream kinase is then responsible for PKD activation by contraction/oligomycin? In several cell types, PKD is activated by novel PKC's in response to agonists acting via GPCRs [23,25]. However, the novel PKC isoforms that are present in the heart, PKCδ and-ε, as well as the conventional PKCα, are not activated by oligomycin, as evidenced by the lack of translocation and T-loop phosphorylation (Fig. 4). Hence, these novel and conventional PKCs are unlikely to be candidate upstream kinases leading to PKD activation during myocyte contraction. Based on the sequence homology of PKD with members of the CaMK family, another likely candidate for PKD activation would be CaMKK, which is present in the heart as a 67-kDa isoform [50]. However, the specific CaMKK inhibitor, STO-609, did not affect oligomycin- and contraction-induced PKD-Ser916 phosphorylation, strongly arguing against the involvement of CaMKKs in contraction signaling to PKD. One possibility to explain contraction-induced PKD activation could be via reactive oxygen species (ROS), which are elevated during contraction and upon oligomycin treatment [51,52]. Indeed, ROS have been found to be capable of inducing PKD activation [53], possibly via the activation of tyrosine kinases. Specific tyrosine kinases have been claimed to phosphorylate PKD at the autoinhibitory domain, resulting in a release of autoinhibition and allowing further activation [54]. A challenge for future research will be the identification of the immediate upstream kinase responsible for contraction-induced PKD activation.

4.2. Pharmacological inhibitors as tools to link PKD activation to regulation of glucose uptake

Notwithstanding that the signaling mechanisms leading to PKD activation by contraction/oligomycin treatment are yet poorly understood, we have set out to link PKD activation to contraction-induced glucose uptake into cardiac myocytes using several commonly applied PKC/PKD inhibitors.

It must also be stressed that using a pharmacological approach to link signaling processes to metabolic processes harbours potential dangers concerning the presumed specific action of the inhibitors. For instance, Gö6976 has been frequently applied to inhibit PKD in a number of cell types (e.g., see Refs. [17,45,46]). We have used Gö6976 at a relatively high concentration of 10 μM, so as to achieve maximal PKD inhibition but leaving cell viability unaffected (see Materials and methods). Based on the marked inhibitory action of this inhibitor at this applied concentration on contraction/oligomycin-induced glucose uptake into cardiac myocytes, the conclusion is easily drawn that PKD is a key player in contraction-induced GLUT4 translocation. However, Gö6976 also inhibits basal glucose uptake into cardiac myocytes, in accordance with previous observations in L6 myotubes [16], while having no effect on PKD activation in cardiac myocytes. This illustrates that the reported inhibitory actions of pharmacological inhibitors on certain signaling processes cannot be simply extrapolated from one cell type to the other. At 10 μM, Gö6976 also did not affect conventional PKCs (or any other PKC isoform) in cardiac myocytes, based on its inability to inhibit PMA-induced ERK phosphorylation. This is in contrast to the marked inhibitory effect of its structurally closely related analogon Gö6983, when applied at the same concentration. Hence, the efficacy of Gö6983, but not Gö6976, on inhibition of PKC signaling was shown in cardiac myocytes. The inhibitory action of Gö6976 on basal glucose uptake can be explained by a putative blockade of the transport function of GLUT4. This notion was strengthened by the marked Gö6976-mediated inhibition of glucose uptake into giant sarcolemmal vesicles from heart (>−60%) in which signaling and translocation events are absent (data not shown).

Unlike Gö6976, Gö6983, calphostin-C and staurosporine each did not affect basal glucose uptake into cardiac myocytes, while simultaneously calphostin-C and staurosporine potently inhibited the enzymatic activity of PKD. Although calphostin-C and staurosporine are known to affect several PKC isoforms in

addition to PKD, none of the PKC isoforms were activated upon treatment of cardiac myocytes with oligomycin (Fig. 4). Therefore, the effects of calphostin-C and staurosporine on PKCs are irrelevant in this specific condition, making these inhibitors suitable pharmacological tools to link PKD signaling to regulation of glucose uptake and GLUT4 translocation in the contracting heart. Moreover, none of the applied inhibitors affected AMPK-Thr172 phosphorylation. In view that AMPK signaling has been implicated in contraction-induced glucose uptake [5,6], it can be excluded that potential inhibitory effects of these inhibitors on glucose uptake can be attributed to a blockade of AMPK activation in cardiac myocytes.

4.3. PKD activation is linked to contraction-induced GLUT4 translocation

PKD activation by contraction/oligomycin in cardiac myocytes occurred concomitantly with stimulation of glucose uptake, suggesting that there might be a relation between PKD activity and glucose uptake in contracting cardiac myocytes. Under conditions that PKD activation was largely abrogated, i.e., in the presence of calphostin-C or staurosporin, oligomycin- and contraction-induced glucose uptake was completely inhibited. Moreover, oligomycin- and contraction-induced glucose uptake was not inhibited by the conventional PKC inhibitor Gö-6983, which did not alter PKD activity. Hence, these inhibitor studies provide the first pharmacological indications for a possible role for PKD in contraction-induced glucose uptake. On the other hand, it might still be argued that the individual inhibitors might additionally exert non-specific effects not related to PKC/PKD inhibition, although we were able to exclude any effects on AMPK signaling. Theoretically, siRNA approaches to silence PKD in cardiac myocytes could unequivocally proof the role of PKD in contraction-induced glucose uptake, but adult cardiac myocytes are very difficult to transfect, and will loose their characteristic features within a few days of culturing. Therefore, definitive evidence for a role of PKD in contraction-induced glucose uptake awaits in-vivo studies with PKD null mice. Nonetheless, when the individual actions of the applied inhibitors on specific PKC isoforms and PKD on the one hand, and on contraction/oligomycin-induced glucose uptake on the other hand, are integrated, the combined inhibitory action pattern of these inhibitors on contraction/oligomycin-induced glucose uptake do suggest an involvement of PKD herein.

GLUT4 is the major cardiac glucose transporter, which shuttles between the sarcolemma and recycling endosomes, thereby regulating cardiac glucose uptake. Contraction is known to induce GLUT4 translocation to the sarcolemma [3], which we have verified by the increase in plasmalemmal GLUT4 content with a concomitant decrease in intracellular GLUT4 in cardiac myocytes that were fractionated upon oligomycin treatment (Fig. 5C). The observation that this oligomycin-induced GLUT4 translocation, just like oligomycin-induced glucose uptake, was completely inhibited by staurosporine suggests that PKD mediates contraction-induced glucose uptake via the stimulation of GLUT4 translocation. Taken together, we propose that contraction-induced GLUT4-mediated glucose uptake is linked to and possibly dependent on PKD activation.

At present, the molecular mechanisms by which PKD activation could contribute to GLUT4 translocation are unclear. One important clue might be provided by the observation that the magnitude of the effects of oligomycin and PMA on stimulation of glucose uptake is quite similar (oligomycin: 1.8-fold increase; PMA, 1.7-fold increase; Fig. 5), despite the observation that oligomycin is a markedly less potent activator of PKD than is PMA. Combined with the earlier mentioned conclusion that contraction/oligomycin induces the phosphorylation of membrane-bound, but not cytoplasmic, PKD, we suggest that it is only this membrane-bound PKD that is involved in stimulation of GLUT4 translocation.

With respect to the localization of the membrane-bound PKD pools, these pools have been found to be present within the sarcolemma, in mitochondria and at the *trans*-Golgi network in different cell types [55,56]. The presence of PKD within the *trans*-Golgi network is particularly interesting, because PKD activation at the Golgi complex has been shown to be involved in the formation and budding of vesicles bringing cargo to the plasma membrane [57]. Based on the notion that the *trans*-Golgi network is connected to the recycling endosomes via an extended network of membranes [58], we anticipate that the recycling endosomes might also harbour PKD. In fact, the continuous exchange of small membrane vesicles between Golgi and endosomes makes the morphological distinction of these intracellular compartments rather artificial. There is evidence that in the translocation of vesicles from the Golgi complex to the plasma membrane, Golgi-bound PKD forms a budding complex with other proteins such as the lipid kinase phosphatidylinositol-4 kinase-IIIβ, thereby initiating vesicle fission [57,59]. In this way, PKD is involved in the delivery of cargo from the Golgi complex to the plasma membrane [57,59]. By analogy, endosomal PKD could be involved in translocation of GLUT4-containing vesicles to the sarcolemma of cardiac myocytes. In this case, the endosomal compartment would represent the source of the membrane-bound PKD in non-stimulated cells. This population of PKD bound to endosomal membranes is then expected to become activated by contraction/oligomycin, resulting in fission of GLUT-4 containing vesicles. Subsequently, the excised GLUT4-vesicles translocate to the sarcolemma, leading to an increase in cellular glucose uptake. Furthermore, we speculate that PMA-induced phosphorylation of cytoplasmic PKD would cause a membrane attachment to non-endosomal compartments (such as the sarcolemma) explaining the non-involvement of this PKD subpopulation in GLUT4 translocation.

4.4. Concluding remarks

The primary finding in the present study is the apparent role of PKD in contraction-induced GLUT4-mediated glucose uptake. In the light of novel evidence from transgenic mouse models with blunted AMPK signaling but with a retained

response of skeletal muscle glucose uptake to contraction [60,61], PKD rather than AMPK could be the main protein kinase involved in contraction signaling to GLUT4 translocation. However, a more balanced view of integration of PKD in contraction-induced glucose uptake would include that contraction-induced GLUT4 translocation would require the input of two parallel pathways, i.e., AMPK signaling and PKD signaling, either of which is necessary to move GLUT4 to the sarcolemma in the contracting heart. This dual signaling input is not a novel concept in regulation of cellular glucose uptake, because also insulin-induced GLUT4 translocation has been established to depend on simultaneous and parallel activation of the wortmannin-sensitive phosphatidylinositol-3 kinase/protein kinase B (Akt) signaling axis and a wortmannin-insensitive pathway beginning with the phosphorylation of the adaptor protein Cbl by the insulin receptor and resulting in activation of a small GTP-binding protein TC10 [62].

Interestingly, in a search for myocardial substrates for PKD, a yeast 2-hybrid screen of a human cardiac library yielded a number of proteins involved in contraction mechanics. These proteins include troponin-I, myosin-binding protein-C and telethonin, and the notion that they serve as direct targets for PKD has been subsequently verified in in vitro kinase assays [37]. Especially, the ability of PKD to phosphorylate troponin-I at Ser23/24 implicates a role of PKD in the acceleration of relaxation through increased myofilament Ca^{2+} sensitivity and crossbridge cycling [37]. Overall, the PKD-mediated phosphorylation of myofilament proteins combined with GLUT4 translocation could implicate PKD in the broad responses of the heart to contraction. In this way PKD could connect the increased energy demand resulting from an acceleration of contraction mechanics with an increased uptake of energy-providing substrates.

Finally, the ability of PKD to induce GLUT4 translocation could make this protein kinase a novel therapeutic target for normalization of insulin sensitivity in heart and skeletal muscle in type-2 diabetic patients.

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