Cytochrome *c* oxidase subunit IV as a marker of protein kinase C ϵ function in neonatal cardiac myocytes: implications for cytochrome *c* oxidase activity

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We have previously demonstrated that low concentrations of phorbol esters stimulate the selective translocation of protein kinase C (PKC) α and ϵ from the cell soluble to the particulate fraction in NCMs (neonatal rat cardiac myocytes). We therefore determined if the *in vitro* phosphorylation of substrates in these fractions could be used as assays of PKC α or ϵ activation. Intact cell phorbol ester treatment caused a decline in the *in vitro* ³²P-incorporation into several proteins in the cell-soluble fraction. These declines occurred in the presence or absence of *in vitro* Ca²⁺ and probably reflected the exit of PKC isoenzymes from the soluble fraction. In contrast, an approx. 18 kDa protein incorporated ³²P in particulate fractions isolated from 4 β -PMA-treated cells in a Ca²⁺-independent manner. Proteomic and immunoprecipitation analyses indicated that the protein is subunit IV of the cytochrome *c* oxidase complex (COIV). *In vitro* phosphorylation of COIV was attenuated by PKC pseudosubstrate peptides.

Introduction of an PKC ϵ -selective translocation inhibitor [Johnson, Gray, Chen and Mochly-Rosen (1996) J. Biol. Chem. 271, 24962–24966] into NCMs before 4 β -PMA treatments also attenuated the *in vitro* phosphorylation of COIV. In mitochondrial extracts from 4 β -PMA-treated NCMs, the PKC ϵ isoenzyme co-immunoprecipitated with COIV, and cytochrome *c* oxidase activity was enhanced 2-fold. The *in vitro* phosphorylation of COIV reflects a novel approach for monitoring PKC ϵ function in NCMs. Furthermore, PKC ϵ probably interacts with COIV in NCM mitochondria to enhance electron-transport chain complex IV activity.

Key words: cardiac myocyte, cytochrome *c* oxidase, immunoprecipitation, mitochondria, phosphorylation, protein kinase C ϵ (PKC ϵ).

INTRODUCTION

PKC (protein kinase C) has three subfamilies: the classical PKCs (α , β_1 , β_2 and γ), the novel PKCs (δ , ϵ , θ and η) and the atypical PKCs (ζ , μ and λ/ι) [1]. Individual PKC isoenzymes can have unique roles which are mediated in part by their selective targeting to different cell loci [2]. These localization signals involve protein–protein interactions between PKC isoenzymes and PKC-anchoring proteins such as RACKs (receptors for activated C-kinase) [2–6], AKAPs (A-kinase-anchoring proteins) [7,8], PKC-inhibitory proteins, PKC substrates for phosphorylation and others [2]. Lipids [9] and Ca²⁺ [10] are also thought to be important in the localization of PKC isoenzymes. Once positioned and activated, PKC isoenzymes regulate cell functions by phosphorylating proteins on serine or threonine residues [1]. All PKC isoenzymes require phospholipids such as phosphatidylserine for activation, and the classical PKC isoenzymes require Ca²⁺ for full activation [1]. In addition, the classical and novel PKC isoenzymes are activated to phosphorylate substrates by DAG (*sn*-1,2-diacylglycerol), which is produced following hormonal activation of PLC (phospholipase C) or PLD (phospholipase D). The cell-permeant drug 4 β -PMA can replace DAG as an activator of these PKC isoenzymes, and has been used extensively to investigate PKC functions in intact cells.

PKC is a key regulatory enzyme family in cardiac muscle. Treatment of heart cells with phorbol esters or hormones which

activate DAG accumulation promotes the translocation of PKC from the cytosol to distinct cell loci [12] and the cell particulate fraction [12–14]. Furthermore, phosphorylation of substrates [15,16], inotropic and chronotropic effects [12,17–20], gene expression [21–23], modulation of calcium [16–18,24,25], cardiac ischaemic pre-conditioning [26,27] and hypertrophy [14,28,29] have all been identified in cardiac cells following PKC activation. In some instances, the α and ϵ isoenzymes of PKC have been shown to translocate to the particulate cell fraction of NCMs (neonatal cardiac myocytes) before other PKC isoenzymes [20,30]. For example, we demonstrated previously selective translocation of the α and ϵ isoenzymes of PKC following 3 nM 4 β -PMA treatment, and translocation of the α , β , δ and ϵ isoenzymes of PKC following short 100 nM 4 β -PMA treatments [20]. A 1 h 100 nM 4 β -PMA treatment led to the down-regulation of the α and β isoenzymes of PKC without loss of the PKC ϵ isoenzyme [20]. In addition, Steinberg and co-workers have demonstrated selective translocation of the α and ϵ isoenzymes of PKC to the particulate cell fraction of NCMs subjected to hypoxia [30].

The mammalian cytochrome *c* oxidase enzyme complex has 13 subunits as previously described by Capaldi et al. [31]. It catalyses the last enzymic step in the ETC (electron-transport chain), resulting in the transfer of electrons from cytochrome *c* to molecular oxygen [31]. These reactions are tightly coupled to energy production and the maintenance of the mitochondrial proton

Abbreviations used: CAMK, Ca²⁺-calmodulin-dependent kinase; COIV, subunit IV of the cytochrome *c* oxidase complex; DAG, *sn*-1,2-diacylglycerol; ETC, electron-transport chain; IB, inhibitory buffer; IP, immunoprecipitation; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; MLC, myosin light chain; NCM, neonatal rat cardiac myocyte; PKA-NH, amide inhibitor of protein kinase A; PKC, protein kinase C; PKD, protein kinase D; RACK, receptor for activated C-kinase; ROS, reactive oxygen species; Ψ , pseudosubstrate.

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gradient. Inhibition of cytochrome *c* oxidase activity also leads to increased production of mitochondrial ROS (reactive oxygen species) via indirect actions on complexes I and III of the ETC [32]. Therefore identification of cytochrome *c* oxidase regulators may improve our knowledge of cardiac energetics and mitochondrial ROS generation. The present study has identified for the first time: (i) a novel catalytic-activity-based marker of PKC ϵ function in NCMs and (ii) a mitochondrial PKC ϵ –COIV (cytochrome *c* oxidase subunit IV) interaction which is associated with a 2-fold enhancement of cytochrome *c* oxidase activity in NCMs. We hypothesize that activation of mitochondrial PKC ϵ increases cytochrome *c* oxidase activity to improve the efficiency of myocardial energetics and aerobic respiration, and to modulate mitochondrial ROS production. The present study may suggest novel mechanisms involved in mitochondrial disease and cardiac protection in neonatal heart cells.

EXPERIMENTAL

Primary NCMs

Cells were isolated from 1-day-old neonatal Sprague–Dawley rats as described previously [20,33]. All work with animals was conducted in compliance with institutional, state and federal guidelines for the humane care and use of laboratory animals. Animals experienced no unnecessary pain or distress in these experiments.

Transient permeabilization of NCMs

Introduction of PKC translocation inhibitors (β C2-4, ϵ V1 and δ V1) was carried out using our extensively characterized transient permeabilization methodology as described previously [33].

Cell lysis and isolation of particulate cell fraction

Culture medium was removed, and the cells were washed twice in chilled $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. Unless otherwise indicated, all other operations were conducted with chilled reagents on ice or at 4 °C. Care was taken to siphon off all excess PBS. Cells were scraped from the dish in 350 μl of IB (inhibitory buffer) (50 mM KH_2PO_4 , 5 mM EDTA, 0.5 mM EGTA, 10 nM calyculin A, 5 mM $\text{Na}_4\text{P}_2\text{O}_7$, and 25 $\mu\text{g}/\text{ml}$ each of PMSF, leupeptin, aprotinin and soybean trypsin inhibitor). Cells were triturated three times in IB using a tuberculin syringe and a 22-gauge needle. Next, cells were centrifuged at 100 000 g at 4 °C for 30 min. Supernatants were then assayed by using 40 μl of fraction in an *in vitro* phosphorylation assay (see below). Particulate fractions were then resuspended in IB plus 0.2% (v/v) Triton X-100 using a tuberculin syringe and a 22-gauge needle, and 40 μl of the particulate cell fraction was used for the *in vitro* ^{32}P -incorporation (phosphorylation) assay.

In vitro phosphorylation assays

Cell fractions isolated from NCMs were subjected to an *in vitro* assay for the times indicated in the Figures. The final phosphorylation assay buffer consisted of 50 mM Tris/HCl, pH 7.4, 5 mM KH_2PO_4 , 1 mM EDTA, 0.1 mM EGTA, 2 nM calyculin A, 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 5 $\mu\text{g}/\text{ml}$ each of PMSF, leupeptin, aprotinin and soybean trypsin inhibitor, 0.4 mM free Mg^{2+} (calculated as described previously [34]), 10 μM [γ - ^{32}P]ATP (3000 c.p.m./pmol), 1–3 μg of DAG and 5 μg of phosphatidylserine, with or without 0.5 mM CaCl_2 . *In vitro* phosphorylation assays were conducted at room temperature (22 °C) for 3 min. Reactions

were terminated by the addition of 90 °C SDS Laemmli sample buffer. Samples were then heated at 85 °C for 5 min and subjected to SDS/PAGE.

Electrophoresis, gel drying and autoradiography

SDS/PAGE on 1 mm thick, 13.5% polyacrylamide gels was performed according to the procedures of Laemmli. Gel solutions were degassed for 45 min before polymerizing gels. Gels were dried in a Bio-Rad gel-drying apparatus at 80 °C for 1 h, and subjected to autoradiography and densitometry.

Peptide mass spectrometric analyses

A total of 25 100-mm-diameter dishes of cells plated at 800 cells/ mm^2 were treated for 30 min with 3 nM 4 β -PMA. Particulate fractions were isolated and then subjected to the *in vitro* ^{32}P -labelling assay. One ^{32}P -labelling reaction was used for each dish of cells. Each ^{32}P -labelling reaction (0.8 ml total volume) was stopped by addition of two volumes of chilled stop buffer (50 mM Tris/HCl, pH 2.8). The pH of the ^{32}P -labelling reaction was pH 7.4; however, after addition of the pH 2.8 stop solution, the final pH of our 'stopped' reactions was pH 4.0. We next pre-equilibrated a 35 ml DE52 anion-exchange column with this same pH 4.0 buffer. The stopped reactions were incubated batch-style with the DE52 resin for 3 h at 4 °C with gentle rocking. The DE52 anion-exchange resin was harvested by centrifugation for 2 min at 200 g . The supernatant (which contained the majority of the ~ 18 kDa protein) was retrieved and saved. The DE52 resin was washed three times using the pH 4.0 buffer, and each wash was saved. Finally, proteins bound to the DE52 resin were eluted in three steps using 0.2, 0.5 and 1.0 M concentrations of NaCl prepared in the pH 4.0 buffer. Proteins from the supernatant after DE52 incubation, each of the three DE52 column washes and the 0.2, 0.5 and 1.0 M NaCl column elutions were subjected to acetone precipitation (sample/acetone ratio 1:3). Precipitated proteins were resuspended in Laemmli sample buffer and subjected to one-dimensional SDS/PAGE. We ran several aliquots of the final dissolved protein on a total of six gels with ten lanes each. Following electrophoresis, gels were stained with Coomassie Blue, dried and subjected to autoradiography. Most of the ~ 18 kDa protein was found to be in the supernatant after the 3 h incubation with the DE52 resin (i.e. the unbound fraction). Therefore gel pieces from all six SDS/PAGE gels containing the ^{32}P -labelled, ~ 18 kDa protein were excised and placed inside three large wells on a 13.5% polyacrylamide mini-gel. Following electrophoresis, the mini-gel was stained with Coomassie Blue, dried and subjected to autoradiography. The 18 kDa band was found to be the only radioactive band in the gel by autoradiography and Cerenkov counting of gel pieces. After localization of the ^{32}P radioactivity, gel pieces were excised from this region of the gel and were submitted to the Emory University Microchemical Facility. Samples were then digested in-gel overnight with trypsin at 37 °C. The resulting peptides were extracted, desalted using C18 ZipTip (50%) and analysed by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS using the Bruker Daltonics (Billerica, MA, U.S.A.) model Reflex III instrument operated in the positive reflector mode [35]. An additional tandem MS (MS/MS) analysis of selected peptide molecular ions was performed using the Applied Biosystems (Foster City, CA, U.S.A.) model 4700 Proteomics Analyzer MALDI-TOF/TOF [36]. The acquired MS and MS/MS data were searched against the NCBI nr all taxa and rodentia proteomes using GPS Explorer (Applied Biosystems) and Mascot (Matrix Science) algorithms.

Isolation of mitochondria from NCMs

Four 100-mm-diameter dishes of NCMs were used per treatment group. Cells washed twice in chilled Ca²⁺/Mg²⁺-free PBS containing 1 mM Na₄P₂O₇ and 10 nM calyculin A were then gently scraped from each dish and collected in a total volume of 1 ml of lysis buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM Na₄P₂O₇, 10 nM calyculin A, and 20 μ g/ml each of PMSF, soybean trypsin inhibitor, aprotinin and leupeptin) followed by Dounce homogenization. The homogenate was sequentially centrifuged twice at 600 *g* for 10 min. The resulting pellets were discarded and the 600 *g* supernatant was subjected to a 10 000 *g* spin for 10 min to pellet intact mitochondria. Mitochondria were resuspended in lysis buffer and the supernatant from the 10 000 *g* spin was saved. Each of these fractions was used for the co-IP (co-immunoprecipitation) (see Figure 10) and cytochrome *c* oxidase assays.

Cytochrome *c* oxidase assays

Intact mitochondria and the 10 000 *g* supernatants were subjected to a spectrophotometric cytochrome *c* oxidase assay according to the manufacturer's instructions (Sigma Chemical Co.). Aliquots from the 10 000 *g* supernatant and intact mitochondria fractions were incubated in assay buffer (10 mM Tris/HCl, pH 7.0, and 120 mM KCl) in the absence (results not shown) and presence of 1 mM *n*-dodecyl- β -D-maltoside. For the 1 mM *n*-dodecyl- β -D-maltoside solubilization, mitochondria were triturated on ice using a tuberculin syringe and a 22-gauge needle. As described in the manufacturer's instructions, the cytochrome *c* oxidase activity before solubilization divided by the total activity [(before solubilization + after solubilization) $\times$ 100 %] gives an estimate of outer-mitochondrial membrane integrity. We observed no measurable activity in the 10 000 *g* supernatant fractions in any of our assays. Activity was maximal during the first 1 min of the assay and only minor sustained activity remained after that time. We therefore reported our results using the initial reaction rates (1 min). Approx. 90–95 % of the cytochrome *c* oxidase activity was found in the 1 mM *n*-dodecyl- β -D-maltoside solubilized mitochondrial pellet. The average activities observed were approx. 600 m-units/mg of protein per min where 1 unit is the amount of enzyme required to oxidize 1 μ M of ferrocytochrome *c* per min at pH 7.0 at 25 °C. All assays were conducted in duplicate within the linear range of the assay. We consistently found approx. 5–10 % of the total cytochrome *c* oxidase activity in non-mitochondrial fractions and this percentage did not vary between treatment groups (results not shown). Equivalent amounts of protein for each mitochondrial extract was assayed and we observed no differences in COIV immunoreactivity in mitochondria isolated from control and phorbol ester treatment groups.

IPs

Following *in vitro* ³²P-labelling protocols, reactions were stopped using IP stop buffer [10 mM Tris/HCl, pH 7.4, 0.125 % (w/v) BSA, 5 mM EDTA, 5 mM EGTA, 5 mM Na₄P₂O₇, 10 nM calyculin A, and 20 μ g/ml each of PMSF, leupeptin, aprotinin and soybean trypsin inhibitor]. Samples were heated at 60 °C for 3 min and then subjected to standard IP procedures [37]. All subsequent steps were conducted at or below 4 °C. Briefly, stopped reactions were placed on ice for three 5 min incubations, with vortex-mixing between each incubation. They were then subjected to a 600 *g* centrifugation for 5 min to precipitate detergent-insoluble material. Next, lysates were pre-cleared using Protein A–agarose that had been pre-incubated with rabbit anti-mouse antisera (Jackson ImmunoResearch Laboratories). After the 1 h

pre-clearing step, the Protein A–agarose was removed by centrifugation at 400 *g* for 5 min, and pre-cleared supernatants were then incubated with primary antisera [anti-COIV (Molecular Probes) or anti-PKC ϵ (BD Transduction Labs)] for 4 h. A second batch of Protein A–agarose was incubated with rabbit anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories; 1:100 dilution) for 2 h before incubation with the supernatant containing the primary antisera for an additional 1 h. Immune complexes were collected by centrifugation at 400 *g* for 5 min, and the resulting Protein A–agarose pellets were washed three times with wash buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 5 mM Na₄P₂O₇, 10 nM calyculin A, 20 μ g/ml each of PMSF, leupeptin, aprotinin and soybean trypsin inhibitor, and 1 % Triton X-100). Proteins were liberated from Protein A–agarose by heating in Laemmli sample buffer at 85 °C for 5 min. Samples were then subjected to SDS/PAGE and transferred on to nitrocellulose paper using standard Western blot techniques. The resulting blots were probed for COIV or PKC ϵ using ¹²⁵I-labelled Protein A detection as described previously [20].

RESULTS AND DISCUSSION

Treatment of cells with 3 nM 4 β -PMA induces changes in *in vitro* ³²P-incorporation into cardiac myocyte proteins

In the experiment shown in Figure 1, cells were treated with 3 nM 4 α - or 4 β -PMA for 0–60 min at 37 °C. Cell-soluble and particulate fractions were then subjected to an *in vitro* phosphorylation assay as described in the Experimental section. Figure 1(A) shows little change in ³²P-incorporation into cell-soluble-fraction proteins during the first 5 min of intact cell 3 nM 4 β -PMA treatment. Following 10–60 min of 3 nM 4 β -PMA treatment, however, there was a decline in ³²P-incorporation into a ~25 kDa protein (Figures 1A and 2A). When 0.5 mM CaCl₂ was included in the *in vitro* assays, we observed a modest increase in ³²P-labelling of the ~25 kDa protein (1 min), followed by a decrease in ³²P-labelling (Figures 1B and 2B). When compared with soluble fractions from 4 α -PMA treated cells, the ~25 kDa protein (delineated by an arrow in Figure 1B) showed 38.4 $\pm$ 13.1, 50.9 $\pm$ 7.7 and 55.8 $\pm$ 8.4 % (means $\pm$ S.E.M.; *n* = 4) losses from the cell-soluble fraction following 10, 30 and 60 min of 3 nM 4 β -PMA treatment respectively. The enhanced ³²P-incorporation into cell-soluble-fraction proteins isolated from control (4 α -PMA)-treated cells when Ca²⁺ was included in the *in vitro* assays (compare Figure 1A with 1B) may reflect contributions from classical PKC isoenzymes, suggesting that both Ca²⁺-dependent and Ca²⁺-independent PKC isoenzymes may contribute to the ³²P-incorporation into the ~25 kDa protein. Densitometric analysis for these data are summarized in Figures 2(A) and 2(B), which, in general, show a decline in ³²P-incorporation into the ~25 kDa protein that was dependent on the duration of intact cell 4 β -PMA treatment.

However, the most striking change detected using this assay was found in the particulate fractions isolated from 3 nM 4 β -PMA treated cells (Figures 1C and 1D). We observed a time-dependent increase in ³²P-incorporation into an ~18 kDa protein in particulate fractions isolated from 4 β -PMA-treated cells (see arrow, Figures 1C and 1D). This correlated closely with 3 nM 4 β -PMA induced translocation of the α and ϵ isoenzymes of PKC to the particulate cell fraction and the PKC ϵ -induced slowing of the contraction rate characterized previously [3,20]. We therefore hypothesized early on that this might be a selective marker of PKC ϵ function. The percentage of maximum ³²P-incorporation into the ~18 kDa protein in particulate fractions isolated from cells treated for 0, 10, 30 and 60 min with 3 nM 4 β -PMA and then

Figure 1 Treatment with 3 nM 4 β -PMA induces changes in the *in vitro* ^{32} P-incorporation into NCM proteins

Cells were treated with 3 nM concentrations of 4 α -PMA for 60 min (0 min lane) or 4 β -PMA for the times shown. Following cell lysis and fractionation, soluble (A, B) and particulate (C, D) fractions were assayed for 3 min in an *in vitro* phosphorylation assay (see the Experimental section). *In vitro* assays conducted in the absence (A, C) or presence (B, D) of 0.5 mM CaCl₂ were terminated with SDS/PAGE sample buffer. Samples were subjected to SDS/PAGE and autoradiography. The arrows on the right-hand side of the Figure indicate the positions of the ~25 kDa (soluble fraction) and the ~18 kDa (particulate fraction) proteins. Shown are autoradiographs taken from a single experiment typical of four independent experiments.

assayed in the absence of Ca²⁺ was 3.1 ± 1.6 , 52.5 ± 10.8 , 89.5 ± 7.6 and 85.4 ± 12.6 % respectively ($n = 4$). Similarly, when 0.5 mM Ca²⁺ was included in the *in vitro* assays, these percentages of maximum values were 3.0 ± 1.7 , 39.1 ± 8.5 , 84.6 ± 7.8 and 87.4 ± 10.0 % respectively ($n = 4$).

Treatment of NMCs with 100 nM 4 β -PMA enhances *in vitro* phosphorylation responses

We observed accelerated and more dramatic losses of ^{32} P-incorporation into cell-soluble-fraction proteins following 100 nM 4 β -PMA treatment (Figures 3 and 4). In *in vitro* assays without added Ca²⁺, the average percentage decreases in ^{32} P-incorporation into the ~25 kDa cell-soluble-fraction protein following 3, 10, 30 and 60 min intact cell exposures to 100 nM 4 β -PMA were 30.1 ± 10.9 , 70.5 ± 3.3 , 87.9 ± 3.1 and 84.6 ± 4.1 respectively ($n = 4$). Similarly, when these soluble fractions were assayed in the presence of 0.5 mM Ca²⁺, the percentage decreases in ^{32} P-incorporation into the ~25 kDa protein were 42.1 ± 12.3 ,

Figure 2 Quantification of 3 nM 4 β -PMA intact cell treatment-induced changes in *in vitro* cell-soluble and particulate fraction ^{32} P-incorporation

Autoradiographs from the experiments summarized in Figure 1 were subjected to densitometry. Results were normalized to the percentage of the maximal 3 nM 4 β -PMA response. Analyses were conducted on one protein from the cell soluble (~25 kDa) and one from the cell particulate fraction (~18 kDa). Shown are the mean $\pm$ S.E.M. responses from four independent experiments each from a separate myocyte preparation.

Figure 3 Treatment with 100 nM 4 β -PMA induces changes in the *in vitro* ^{32} P-incorporation into NCM proteins

All conditions were similar to those in Figure 1, except that 100 nM 4 β -PMA concentrations were used.

79.5 ± 9.4 , 97.4 ± 1.3 and 97.6 ± 1.4 respectively ($n = 4$). In experiments conducted similarly to those in Figures 3(A) and 3(B), except that exogenous purified rat brain PKC was added to

Figure 4 Quantification of 100 nM 4 β -PMA-induced changes in *in vitro* ^{32} P-incorporations into NCM proteins

Densitometric analyses were conducted on autoradiographs from the four independent experiments represented in Figure 3. Other details are as in Figure 2.

cell-soluble fractions, we observed similar ^{32} P-labelling in control and 0–60 min 100 nM 4 β -PMA (intact cell) treatment groups (results not shown). This indicated that the loss of ^{32} P-labelling following 4 β -PMA treatments (Figures 1A, 1B, 3A and 3B) was not due to back phosphorylation. We therefore propose that 4 β -PMA triggers the translocation of PKC isoenzymes out of the cell-soluble fraction, resulting in a decline in ^{32} P-incorporation in these fractions.

When particulate fractions were assayed from cells treated with 100 nM 4 β -PMA for 3, 10, 30 and 60 min, the percentages of maximal ^{32} P-incorporation observed in Ca^{2+} -free assays were 55.5 ± 5 , 85.5 ± 6.7 , 100 ± 0 and 72.1 ± 13.0 respectively ($n = 4$). In assays with 0.5 mM Ca^{2+} present, these values were found to be 54.2 ± 10.3 , 97.9 ± 2.1 , 87.9 ± 12.1 and 63.3 ± 11.0 respectively ($n = 4$). Therefore 100 nM 4 β -PMA intact cell treatments promoted a more rapid *in vitro* phosphorylation of the ~18 kDa protein than was observed with 3 nM 4 β -PMA treatments, and, for the most part, the response was sustained for up to 60 min (Figures 1 and 2 compared with Figures 3 and 4). Our results therefore demonstrated a Ca^{2+} -independent *in vitro* phosphorylation of the ~18 kDa protein even after a 1 h 100 nM 4 β -PMA treatment, a condition which we have shown previously down-regulates the α and β isoenzymes of PKC but not the ϵ or δ isoenzymes in these cells [20]. These patterns of down-regulation were also confirmed in the present study (results not shown). Collectively, our data are consistent with the PKC ϵ isoenzyme mediating the *in vitro* phosphorylation of the ~18 kDa protein.

In vitro ^{32} P-incorporation into the ~18 kDa protein is mediated by PKC isoenzymes

We next isolated particulate fractions from NCMs treated for 30 min with 3 nM 4 β -PMA and then conducted *in vitro* ^{32} P-labelling reactions in the presence of 0–100 μM concentrations of the inhibitory PKC pseudosubstrate (Ψ) peptide (Figures 5, 6A and 6E), the amide inhibitor of protein kinase A (PKA-NH) or the CAMK (Ca^{2+} -calmodulin-dependent kinase) II Ψ peptide (Figures 5, 6C, 6D, 6G and 6H). In the absence of Ca^{2+} , we found dose-dependent attenuations of ^{32} P-incorporation into the

Figure 5 Incubation of particulate cell fractions with the PKC Ψ peptide attenuates *in vitro* ^{32} P-incorporation into the ~18 kDa protein

Cells were treated with 3 nM 4 β -PMA for 30 min. Particulate cell fractions were isolated and subjected to an *in vitro* phosphorylation assay as in Figure 1. Included in these incubations were 0–100 μM concentrations of the PKC Ψ peptide $\text{NH}_2\text{-RFARKGALRQKNVHEVK-COOH}$, scrambled-sequence (inactive) version of the PKC Ψ peptide (Scram Ψ), CAMK II Ψ peptide $\text{NH}_2\text{-LKKFNARRKLKGAILTTMLA-COOH}$ or PKA-NH $\text{NH}_2\text{-TYADFIASGRTGRRNANNI-COOH}$. Results shown are from a single experiment typical of five experiments.

Figure 6 Densitometric analysis of PKC Ψ -mediated attenuation of *in vitro* cardiac myocyte protein phosphorylation

Autoradiographs from the experiments described in Figure 5 were subjected to quantification by densitometry. Raw densitometric scores were converted into percentages of the 0 peptide concentration (control) group. Results are means $\pm$ S.E.M. from the five experiments each from a separate myocyte preparation.

Figure 7 The recombinant ϵ V1 domain inhibits intact cell 100 nM 4 β -PMA-induced *in vitro* ^{32} P-incorporation into the ~18 kDa protein

NCMs were permeabilized in the absence (sham) or presence of PKC-isoenzyme-selective translocation inhibitors. The inhibitors used were 100 μ M β C2-4 (NH₂-SLNPEWNET-COOH) [4], 200 μ g/ml δ V1 (amino acids 2–144 of PKC δ) [3] and 200 μ g/ml ϵ V1 (amino acids 2–144 of PKC ϵ) [3]. Next, cells were treated with 100 nM 4 α - (inactive) or 4 β -PMA for 10 min. Particulate fractions were isolated and subjected to *in vitro* ^{32} P-incorporation reactions in the presence of 0.5 mM CaCl₂, followed by SDS/PAGE and autoradiography. The autoradiograph (**A**) is from a single experiment typical of three identical experiments. Densitometry (**B**) was conducted on the experiments represented in (**A**).

~18 kDa protein by the PKC Ψ (Figures 5 and 6A). The PKC Ψ was slightly more efficacious in the presence of 0.5 mM *in vitro* Ca²⁺ (Figure 5 and 6A compared with Figure 6E). We found no effects of a scrambled-sequence (inactive) version of the PKC Ψ peptide, the PKA-NH inhibitor or the CAMK Ψ peptide on this response (Figures 5, 6B–6D and 6F–6H). Finally, we found no effects of the tyrosine kinase inhibitor genistein, the phosphoinositide 3-kinase inhibitor wortmannin or the MEK (mitogen-activated protein kinase/extracellular-signal-related kinase kinase) inhibitor PD98059 (results not shown). Our results are therefore, consistent with a PKC-mediated *in vitro* phosphorylation of the ~18 kDa protein.

The PKC ϵ -selective translocation inhibitor ϵ V1 and the chemical PKC inhibitor GF109203X attenuate 4 β -PMA-induced ^{32} P-incorporation into the ~18 kDa protein

Figure 7(A) shows an experiment in which NCMs were transiently permeabilized in the presence or absence of PKC-isoenzyme-selective inhibitors [2–6]. Cells were then treated for 10 min with 100 nM 4 α -PMA (inactive isomer) or 4 β -PMA (active isomer). Particulate fractions were then subjected to the *in vitro* phosphorylation assay in the presence of 0.5 mM CaCl₂. The recombinant ϵ V1 domain produced a 40% attenuation of the intact cell 4 β -PMA-treatment-induced response (Figures 7A and 7B). We found this impressive in the face of such a strong intact cell PKC stimulus (100 nM 4 β -PMA for 10 min). Since the ϵ V1–PKC ϵ RACK interaction is thought to be competitive

Figure 8 GF109203X and the recombinant ϵ V1 domain inhibit intact cell 3 nM 4 β -PMA-treatment-induced *in vitro* ^{32} P-incorporation into the ~18 kDa protein

In the two left-hand lanes of the Figure, NCMs were treated with 3 nM 4 β -PMA for 1 h. Next, particulate cell fractions were isolated and incubated in the *in vitro* ^{32} P-labelling assay in the presence of 1 or 10 μ M GF109203X. The remaining treatment groups were conducted in parallel with the GF109203X treatments and involved introduction of the recombinant ϵ V1 and δ V1 PKC-isoenzyme-selective inhibitors (250 μ g/ml in the permeabilization step) into cells before PMA treatments. Unless otherwise indicated, the intact cell PMA treatments were 3 nM 4 β -PMA for 1 h. The top of the Figure shows a representative autoradiograph. Below, the histogram represents corresponding densitometry scores. Histogram results are mean $\pm$ S.E.M. percentages of maximum ^{32} P-incorporation into the ~18 kDa protein from three independent experiments, each conducted on a separate myocyte preparation.

with endogenous PKC ϵ [2] and since 100 nM 4 β -PMA would 'drive' endogenous PKC ϵ to its RACK with great intensity, we would not expect ϵ V1 to completely block the 100 nM 4 β -PMA-induced response. In support of this, we reported previously a greater ϵ V1-induced antagonism of PKC ϵ translocation using 3 nM 4 β -PMA stimulation than was observed following 100 nM 4 β -PMA stimulation [3]. To test this more directly, separate experiments were performed. In these experiments, the inhibition by the recombinant ϵ V1 fragment of the 3 nM 4 β -PMA-induced increase in ~18 kDa ^{32} P-labelling was $92 \pm 8\%$ ($n=3$) (Figure 8). In comparison, the ϵ V1 fragment inhibited 100 nM 4 β -PMA-induced increases in ^{32} P-incorporation into the ~18 kDa protein by $71 \pm 9\%$ ($n=3$). In contrast, the δ V1 PKC δ -selective inhibitor had no effect on the 3 nM (Figure 8) or 100 nM (Figures 7 and 8) 4 β -PMA-induced ^{32} P-labelling. Therefore the ϵ V1 inhibitor attenuated the 3 nM 4 β -PMA-induced response more effectively than it inhibited the 100 nM 4 β -PMA-induced labelling of the ~18 kDa protein.

We also found no inhibitory effects of 100 μ M concentrations of the classical PKC inhibitor β C2-4 [3–6] (Figure 7). Furthermore, the response requires intact cell 4 β -PMA treatment, so we do not believe that the non-4 β -PMA-responsive atypical PKC isoforms are involved. Also, the PKC η isoenzyme is always found in the particulate fraction of our cells and does not show changes in localization following 4 β -PMA treatments (results not shown). In isolated experiments, we also added 0–100 μ g/ml concentrations of the ϵ V1 recombinant fragment directly to the *in vitro* phosphorylation assays, and found no effect on these responses (results not shown).

To investigate the involvement of other PMA-responsive kinases such as PKD (protein kinase D) in the *in vitro*

phosphorylation of the ~ 18 kDa protein, we next conducted studies with the chemical PKC-inhibiting drug GF109203X. GF109203X inhibits PKC ϵ and other PKC isoenzymes, but it does not inhibit the PMA-responsive PKD enzyme [38]. NCMs were treated with 3 nM 4 β -PMA for 60 min, before particulate cell fractions were isolated and subjected to the *in vitro* phosphorylation reaction in the presence of 1 or 10 μ M GF109203X. Both concentrations of GF109203X virtually eliminated the labelling of the ~ 18 kDa protein (Figure 8). Our results suggest that PKD is not involved in the *in vitro* phosphorylation of the ~ 18 kDa protein.

Proteomic analyses suggest that the ~ 18 kDa protein is COIV

We next determined the identity of the ~ 18 kDa protein. Surprisingly, we found that $>90\%$ of the ^{32} P-labelled ~ 18 kDa protein did not bind a DE52 anion-exchange column when chromatography was conducted at pH 4. We therefore acetone-precipitated the ^{32} P-labelled ~ 18 kDa protein from the DE52 column supernatant (unbound material), ran these samples out on a series of SDS/polyacrylamide gels and performed autoradiography and Coomassie Blue staining as described in the Experimental section. Gel pieces containing the ~ 18 kDa protein were excised and were submitted to the Emory University Microchemical Facility for peptide MS. After digestion of the ~ 18 kDa band, C18 ZipTip desalting (50%) was performed and the mixture was analysed by MALDI-TOF MS. The monoisotopic peaks were searched in the NCBI database using Profound. A confident identification was made to COIV ($P < 0.0001$; Z score = 2.18) with protein sequence coverage of 45.6%. We also conducted additional TOF/TOF MS/MS analyses. The mass spectrometer first obtained MS spectra followed by MS/MS analysis of the 15 most abundant peptide molecular ions from the MS spectra. The combined MS and MS/MS data was then subjected to the NCBI database search using the Mascot server (all taxa and rodentia only). In the rodentia search, other than COIV, only one peptide was identified by MS/MS which contained a sequence found in cardiac MLC (myosin light chain) 1 and 3.

An antibody against COIV immunoprecipitates an ~ 18 kDa phosphoprotein from *in vitro* ^{32} P-labelled cardiac myocyte particulate fractions

Cells were treated with 3 nM 4 α - or 4 β -PMA for 60 min before lysis, and particulate cell fractions were isolated and subjected to the *in vitro* ^{32} P-labelling protocol as in Figure 1. Reactions were then stopped and subjected to IP protocols using a commercially available COIV antisera. COIV IPs revealed a 9.1 ± 1.2 -fold ($n = 4$) increase in ^{32} P-incorporation into an ~ 18 kDa protein when particulate fractions from 4 β -PMA-treated cells were used in our *in vitro* ^{32} P-labelling reactions (Figure 9). Finally, we did not observe ^{32} P-labelling of an ~ 18 kDa species when IPs were conducted using antisera selective for the MLC-1/3 proteins (results not shown). We therefore hypothesize based on our MS and IP analyses that the protein phosphorylated in the *in vitro* ^{32} P-labelling reaction is most likely to be COIV.

PKC ϵ and COIV co-immunoprecipitate from *in vitro* ^{32} P-labelling reactions conducted with particulate fractions isolated from 3 nM 4 β -PMA-treated NCMs

Following IPs with anti-COIV or anti-PKC ϵ antisera, samples were subjected to SDS/PAGE and electrotransferred on to nitrocellulose paper. The resulting blots were then probed with anti-COIV antisera. We observed that equal amounts of COIV

Figure 9 Antisera to COIV immunoprecipitates an ~ 18 kDa ^{32} P-labelled protein from *in vitro* reactions containing particulate fractions isolated from 4 β -PMA-treated NCMs

Cells were treated with 3 nM 4 α - or 4 β -PMA for 60 min. Particulate cell fractions were then subjected to the *in vitro* ^{32} P-labelling procedure and IP with COIV antisera. (A) Autoradiograph demonstrating the selective IP of an ~ 18 kDa phosphoprotein in reactions using particulate fractions from 4 β -PMA treated cells. (B) Densitometric analysis results (means $\pm$ S.E.M.) of the autoradiographs derived from four independent experiments each from separate cell preparations.

were immunoprecipitated from *in vitro* labelling reactions using particulate fractions isolated from either 4 α - or 4 β -PMA-treated cells. This indicated that the levels and immunocapture of COIV were the same in each treatment group, and could not explain the differences in ^{32} P-incorporation observed in Figure 9. Of interest, however, when IPs were conducted with the PKC ϵ antisera and then COIV was probed in Western blots, we observed a 3.1 ± 0.5 -fold increase in COIV detection in the 4 β -PMA treatment group ($n = 3$). Our previous studies [20], and experiments conducted in parallel with the current work, indicate that more PKC ϵ translocates to the particulate fraction following 3 nM 4 β -PMA treatment than exists under control conditions. We therefore believe that these increased particulate levels of PKC ϵ 'capture' more COIV in this assay. We also probed blots for MLC-1/3, and did not observe co-precipitation of these proteins when IPs were conducted with COIV antisera (results not shown). Collectively, our data are consistent with a PKC ϵ -mediated phosphorylation of COIV in our *in vitro* assays.

COIV and PKC ϵ co-immunoprecipitate from mitochondria isolated from 3 nM 4 β -PMA-treated NCMs

In vivo COIV exists on the inner-mitochondrial membrane [31,32], and a portion of the total PKC ϵ in heart cells has also been reported to exist inside mitochondria [39]. We determined whether or not activated PKC ϵ interacted with COIV inside NCM mitochondria (Figure 10). Cells were treated with 3 nM 4 α - or 4 β -PMA for 60 min, and mitochondria were isolated. Mitochondria were lysed, subjected to IP protocols with anti-PKC ϵ antisera and then Western blot analysis with anti-COIV antisera. Lane 1 of Figure 10 shows a lack of COIV immunoreactivity in mitochondria isolated from 4 α -PMA-treated cells. In contrast, mitochondria isolated from 4 β -PMA-treated cells showed a strong COIV immunoreactivity (arrow in Figure 10) indicating a PKC ϵ –COIV interaction (Figure 10, lane 2). Lanes 3 and 4 in Figure 10 represent control IPs with secondary antibody alone. Note that no COIV was immunoprecipitated in these control

Figure 10 Antisera to PKC ϵ co-immunoprecipitates COIV from mitochondria isolated from 4 β -PMA-treated NCMs

Cells were treated with 3 nM concentrations of 4 α - or 4 β -PMA for 1 h. Next, mitochondrial extracts were prepared and subjected to IP protocols with PKC ϵ -selective antisera. The resulting IPs were subjected to SDS/PAGE and Western blot analyses with COIV antisera (lanes 1 and 2). Lanes 3 and 4 were control IPs in which no primary antisera and only secondary antisera were used. Results shown are from a single experiment typical of three experiments each conducted using a different myocyte preparation.

groups. Our results suggest that 3 nM 4 β -PMA treatment of intact NCMs stimulates a PKC ϵ –COIV interaction inside mitochondria.

4 β -PMA enhances cytochrome *c* oxidase activity in NCMs

Since COIV is only one of 13 subunits of cytochrome *c* oxidase and all subunits are required to exist in a complex for full activity [31], we next determined if the PKC ϵ –COIV interaction had any significant effects on cytochrome *c* oxidase activity. NCMs were treated for 1 h with 100 nM 4 α -PMA, 3 nM 4 β -PMA or 100 nM 4 β -PMA. Intact mitochondria were then isolated, extracts were prepared and then assayed for cytochrome *c* oxidase activity. We found no significant cytochrome *c* oxidase activity in non-mitochondrial fractions, indicating that our procedures did not cause non-selective rupture of the outer-mitochondrial membrane. In three independent myocyte preparations, we observed a 2.1 ± 0.2 -fold enhancement of mitochondrial cytochrome *c* oxidase activity following 3–100 nM 4 β -PMA treatment ($n=3$). Of interest, the enhancements caused by either 3 or 100 nM 4 β -PMA were nearly identical in magnitude (results not shown). We therefore hypothesize that 4 β -PMA stimulates the activation of PKC ϵ inside mitochondria to enhance the activity of cytochrome *c* oxidase via an interaction with its IV subunit. Our previous back-phosphorylation analyses [40] suggest that PKC ϵ may phosphorylate an ~ 18 kDa protein in the particulate fraction of NCMs, but we did not confirm the identity of the ~ 18 kDa protein to be COIV in that study. Our present study does, however, demonstrate co-IP of COIV and PKC ϵ from mitochondria isolated from 4 β -PMA-treated cells, suggesting a stable phorbol-ester-induced binding interaction between the two proteins inside mitochondria.

We have shown previously that a 1 h 100 nM 4 β -PMA treatment down-regulates the α and β isoenzymes of PKC, but not the PKC ϵ isoenzyme in these cells [20]. Since there does not appear to be a larger increase in cytochrome *c* oxidase activity using 100 nM 4 β -PMA than there is with 3 nM 4 β -PMA treatment, our results suggest that down-regulation of the PKC α and β isoenzymes does not change this 4 β -PMA response. Our results therefore suggest that under conditions where the PKC ϵ iso-

enzyme is preferentially translocated from the cell-soluble to the particulate fraction ([20]; and results not shown), there is a 2.1-fold enhancement of cytochrome *c* oxidase activity and a sustained PKC ϵ –COIV interaction inside NCM mitochondria. It is possible that PKC ϵ achieves this effect by phosphorylation of COIV inside NCM mitochondria. Alternatively, COIV may serve as a PKC ϵ -localizing protein that allows PKC ϵ to phosphorylate other cytochrome *c* oxidase subunits or proteins which in turn lead to enhanced cytochrome *c* oxidase activity. Finally, recent studies have suggested that PKC isoenzymes can modulate the function of other enzymes via phosphorylation-independent binding interactions [41]. It is therefore possible that the binding of PKC ϵ to COIV causes conformational changes in the cytochrome *c* oxidase enzyme complex to enhance its activity. Further resolution of the mechanism(s) by which the PKC ϵ isoenzyme modulates cytochrome *c* oxidase activity are current focuses of our work.

Significance of the *in vitro* phosphorylation of COIV and mitochondrial PKC ϵ –COIV binding interactions

We first presented evidence that the *in vitro* phosphorylation of COIV can be used as an PKC ϵ -selective catalytic-activity-based assay in phorbol-ester-treated NCMs. To our knowledge, this is the first assay of its kind for monitoring PKC ϵ -selective catalytic activity. PKC-translocation assays are often used to estimate indirectly PKC isoenzyme activation in cells, but translocation assays do not monitor actual enzymic activity. Phospho-immunoreactivity studies have also been used to monitor PKC isoenzyme catalytic activation, but these studies focus on auto-phosphorylation of PKC isoenzymes and not on downstream substrates for phosphorylation as our assay does. Furthermore, this assay may conserve important cofactors not found in *in vitro* phosphorylation assays with purified kinases and purified or synthetic substrates.

We next demonstrated a stable PKC ϵ –COIV interaction in mitochondria isolated from phorbol-ester-treated cells which correlated with a 2-fold enhancement of cytochrome *c* oxidase activity. This is the first report of the modulation of cytochrome *c* oxidase activity by the PKC ϵ isoenzyme. Our work has potential significance for a number of reasons. First, PKC ϵ may regulate mitochondrial ETC complex IV. PKC ϵ -mediated enhancement of cytochrome *c* oxidase activities should improve the efficiency of electron flow from cytochrome *c* to molecular oxygen, which in turn would aid maintenance of the inner-mitochondrial-membrane proton gradient and improve myocardial aerobic respiration. Secondly, inhibition of cytochrome *c* oxidase shifts the component complexes of the ETC to a more reduced state which favours superoxide production from ETC complexes I and III [32]. Therefore enhanced cytochrome *c* oxidase activities should minimize excessive ROS production via improved electron flow through complexes I and III, which could prevent oxidative damage to heart cells. Thirdly, there are a number of diseases associated with deficiencies in cytochrome *c* oxidase including Leigh syndrome, Kearns–Sayre syndrome, fatal and benign infantile mitochondrial myopathy, and aging syndromes [31]. Since the PKC ϵ isoenzyme enhances cytochrome *c* oxidase activity, our findings may be therapeutically relevant to these and other disorders. Fourthly, cytochrome *c* oxidase activity declines during myocardial ischaemia or metabolic inhibition by cyanide or elevated levels of carbon monoxide [32]. PKC ϵ activation and modulation of cytochrome *c* oxidase in these states may play a partial role in protecting heart cells from damage associated with ischaemia/reperfusion injury or metabolic inhibition. It is well known that the PKC ϵ isoenzyme induces cardioprotection during cardiac hypoxic/ischaemic pre-conditioning [26,37,42,43], a

paradoxical response whereby brief periods of oxygen deprivation lead to protection against a subsequent prolonged oxygen deprivation. Many important signalling events inside mitochondria contribute to this protection, including the formation of signalling modules between PKC ϵ and mitogen-activated protein kinases [39], as well as the elevation of mitochondrial ROS [26,42]. Of interest, cardiac hypoxic/ischaemic pre-conditioning can be induced by low doses of nitric oxide (NO) donors via mechanisms that involve nitrosylation and activation of the PKC ϵ isoenzyme [43] and the elevation of mitochondrial ROS [26,42]. When experiments are conducted with *in vivo* cellular levels of oxygen, NO will bind to and inhibit cytochrome *c* oxidase [32]. In fact, under these conditions, the affinity of NO for cytochrome *c* oxidase is equivalent to the NO affinity for guanylate cyclase [32]. NO-induced inhibition of cytochrome *c* oxidase leads to an elevation of ROS production (via indirect effects on ETC complexes I and III) [32]. This mechanism may contribute to mitochondrial superoxide (and, as NO is present, peroxynitrite) production, which may play a role in NO-induced cardiac hypoxic/ischaemic pre-conditioning. Further studies will be required to test these hypotheses. In further studies, we have demonstrated that the *in vitro* phosphorylation of an 18 kDa protein (presumably COIV) correlates with NCM hypoxic pre-conditioning (M. Ogbi and J. A. Johnson, unpublished work). We also have preliminary evidence that the PKC ϵ –COIV binding interaction inside mitochondrial occurs in adult rat hearts exposed to ischaemic pre-conditioning protocols. We therefore propose a potential role for the PKC ϵ isoenzyme in cardiac mitochondrial energetics and ROS production, which may be of significance in myocardial ischaemia/reperfusion damage, metabolic inhibition by cyanide, carbon monoxide or NO, and potentially in protection induced by cardiac hypoxic/ischaemic pre-conditioning.

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