

Permissive action of protein kinase C- ζ in insulin-induced CD36- and GLUT4 translocation in cardiac myocytes

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Abstract

Insulin stimulates cardiac long-chain fatty acid (LCFA) and glucose uptake via translocation of human homolog of rat fatty acid translocase (CD36) and GLUT4 respectively, from intracellular membrane compartments to the sarcolemma, a process dependent on the activation of phosphatidylinositol-3 kinase. To identify downstream kinases of insulin signaling involved in translocation of CD36 and GLUT4 in the heart, we tested i) which cardiac protein kinase C (PKC) isoforms (α , δ , ϵ or ζ) are activated by insulin, and ii) whether PKC isoform-specific inhibition affects insulin-stimulated substrate uptake in the heart. Insulin-stimulated LCFA and glucose uptake were completely blunted by inhibition of PKC- ζ , but not by inhibition of conventional or novel PKCs. Concomitantly, translocation of CD36 and GLUT4 to the sarcolemma was completely blunted upon inhibition of PKC- ζ . However, insulin, in contrast to the diacylglycerol-analog

phorbol-12-myristate-13-acetate (PMA), did not induce membrane-attachment of the conventional and novel PKCs- α , δ , and ϵ . PKC- ζ was already entirely membrane-bound in non-stimulated cells, and neither insulin nor PMA treatment had any effect on the subcellular localization of PKC- ζ . Furthermore, insulin treatment did not change phosphorylation of PKC- α , δ , and ζ or enzymatic activity of PKC- ζ towards a PKC- ζ substrate peptide. It is concluded that PKC- ζ , but not any other PKC isoform, is necessary for insulin-induced translocation of GLUT4 and CD36. However, PKC- ζ is already fully active under basal conditions and not further activated by insulin, indicating that its role in insulin-stimulated uptake of both glucose and LCFA is permissive rather than regulatory.

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Introduction

Insulin is a major effector of cardiac substrate utilization, regulating both glucose and long-chain fatty acid (LCFA) uptake into cardiomyocytes. Under basal conditions, more than 90% of the major glucose transporter isoform GLUT4 is stored intracellularly (Slot *et al.* 1991). Upon stimulation with insulin, around 50% of this intracellular GLUT4 is translocated to the plasma membrane (PM) to facilitate glucose uptake (Slot *et al.* 1991, Zorzano *et al.* 1997). The insulin-dependent intracellular signaling events has been unraveled in part, and are known to be involved in the activation of the insulin receptor, tyrosine phosphorylation of insulin receptor substrates (IRSs) followed by their binding to and activation of phosphatidylinositol-3 kinase (PI3K) and subsequent production of PI-3,4,5-(PO₄)₃ (PIP₃). In the heart, signaling events downstream of PI3K have been less extensively characterized, but for adipose

tissue and skeletal muscle, it is widely recognized that Akt/protein kinase B (Akt/PKB), and also atypical protein kinase C (PKC) isoforms are involved (Watson & Pessin 2001, Farese 2002).

Together, with the conventional and novel PKCs, the atypical PKCs constitute the PKCs, a family of serine/threonine kinases (Newton 1995). These three subfamilies can be distinguished based on their protein structure and ligand-binding domains. All PKC isoforms share a highly conserved catalytic domain and a pseudosubstrate region. In comparison to conventional and novel PKCs, the C1 domain (diacylglycerol (DAG)-binding domain) of atypical PKCs is modified and does not bind DAG. Moreover, atypical PKCs lack the C2 domain (Ca²⁺-binding domain) present in conventional PKCs, and therefore this subfamily lacks the ability to be activated by DAG or Ca²⁺. Instead of DAG, other lipid metabolites, such as PIP₃, have been proven suitable PKC- ζ activators *in vitro* (Farese 2002).

Isoform-specific expression of PKCs varies during cardiac ventricular development, however, the major atypical PKC isoform in adult rat cardiac myocytes is PKC- ζ (Puceat *et al.* 1994, Clerk *et al.* 1995, Shizukuda & Buttrick 2002). In addition, members of the other subclasses have been found to be present, including conventional (PKC- α) and novel PKCs (PKC- δ and PKC- ϵ ; Goldberg *et al.* 1997, Albert & Ford 1999).

Convincing evidence firmly establishes the involvement of PKC- ζ in GLUT4 translocation in a variety of adipose and muscle cell lines. Notably, transfection of constitutive PKC- ζ stimulates GLUT4 translocation, whereas dominant-negative PKC- ζ partially inhibited this process (Standaert *et al.* 1997). The potential of PIP₃ to activate PKC- ζ positions PKC- ζ directly downstream of PI3K. In line with this notion is the observation that the activation of PKC- ζ appears to be sensitive to inhibition by two structurally unrelated PI3K inhibitors wortmannin and LY294002 (Bandyopadhyay *et al.* 1997). However, in contrast to adipocytes, evidence about the role of PKC- ζ in insulin-stimulated GLUT4 translocation in the heart is lacking.

In addition to glucose uptake, cardiac LCFA uptake is a protein-mediated process regulated by insulin (Luiken *et al.* 2002). CD36 is the major LCFA transporter in the heart, and accounts for >75% of the cardiac LCFA flux (Luiken *et al.* 1997). CD36 is not only present at the sarcolemma, but also in an intracellular membrane compartment, presumably endosomes, where it, at least partially, colocalizes with GLUT4 (Luiken *et al.* 2004). Insulin has been found to stimulate LCFA uptake by 1.5- to 1.6-fold. The underlying mechanism has been pinpointed to a translocation of intracellularly stored CD36 to the sarcolemma (Luiken *et al.* 2002). Insulin-stimulated uptake of LCFA into myocytes is sensitive to wortmannin and LY294002, implicating the activation of PI3K as a key event in CD36 translocation (Luiken *et al.* 2002). Based on these data, the regulation of LCFA uptake by insulin is strikingly similar to that of glucose in that it includes a PI3K-dependent translocation of a transporter. However, beyond the involvement of PI3K, it has been hardly investigated whether this similarity extends to members of the PKC family. Only one recent study has reported the involvement of PKC- ζ in insulin-stimulated LCFA uptake in a cell line, but this study did not attempt to reveal mechanistical causes for this PKC- ζ action, such as translocation of CD36 (Kelly *et al.* 2008).

In this study, we examined whether PKC- ζ is involved in insulin-stimulated substrate uptake into cardiac myocytes. More specifically, we investigated whether PKC- ζ , and possibly other PKCs ($-\alpha$, $-\delta$, and $-\epsilon$), are involved in the stimulatory effect of insulin on LCFA uptake and CD36 translocation. Our results point towards an involvement of PKC- ζ , but not of $-\alpha$, $-\delta$, and $-\epsilon$, in insulin-stimulated uptake of both LCFA and glucose into cardiac myocytes. Moreover, the role of PKC- ζ appeared to be permissive rather than regulatory.

Materials and Methods

Materials

[1-¹⁴C]palmitic acid was obtained from Amersham Life Science Ltd, BSA (fraction V, essentially LCFA free) phloretin, insulin, staurosporine, bisindolylmaleimide-I (BIM-I), hexadecyl-methylglycerol (HMG), and wortmannin were all obtained from Sigma. HMG and myristoylated PKC- ζ pseudosubstrate were purchased from Biomol (Plymouth Meeting, PA, USA). Collagenase type 2 was purchased from Worthington (Lakewood, NJ, USA). Antibodies directed against PKC- α , PKC- δ , PKC- ϵ , phospho-Akt/PKB (Ser473), phospho-extracellular signal-regulated kinase (ERK; Thr202/Tyr204), phospho-PKC- α (Thr638), phospho-PKC- δ (Ser643), phospho-PKC- ζ (Thr410/403), and glyceraldehyde-3-phosphate dehydrogenase were obtained from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies against PKC- λ , PKC- ζ (C-20), and caveolin-3 were purchased from Transduction Laboratories (Sparks, MD, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively. Anti-phospho-AS160 (Thr642) was from Invitrogen. Antibodies directed against GLUT4 were obtained from Sanver Tech (Heerhugowaard, The Netherlands). Antibody MO25 was a gift from Dr Tandon, Thrombosis and Vascular Biology Laboratory, Otsuka America Pharmaceutical Inc. (Rockville, MD, USA). The monocarboxylate transporter-1 antibody was a gift from Dr Hatta, University of Tokyo (Tokyo, Japan). BCA protein assay reagent kit was from Pierce (Rockford, IL, USA).

Isolation of cardiac myocytes

Cardiac myocytes were isolated from male Lewis rats (200–250 g) using a Langendorff perfusion system and a Krebs–Henseleit bicarbonate medium supplemented with 11 mM glucose, and equilibrated with a 95% O₂ and 5% CO₂ gas phase (medium A) at 37 °C as previously described (Luiken *et al.* 1997). After isolation, the cells were washed twice with medium A supplemented with 1.0 mM CaCl₂ and 2% (w/v) BSA (medium B) and then suspended in 15 ml medium B. The isolated cells were allowed to recover for ~ 2 h at room temperature. At the end of the recovery period, cells were washed and suspended in medium B. Only when >80% of the cells had a rod-shaped appearance and excluded trypan blue, were they used for subsequent tracer uptake studies.

Substrate utilization by cardiac myocytes

Cells (2.0 ml; 5–8 mg wet mass per ml), suspended in medium B without glucose, were preincubated in capped 20 ml incubation vials for 15 min at 37 °C under continuous shaking. For palmitate and deoxyglucose uptake, 0.5 ml of a mixture of [1-¹⁴C]palmitate/BSA complex and [1-³H]deoxyglucose was added to pre-incubated cell

suspensions (final concentration: palmitate, 100 $\mu\text{mol/l}$; deoxyglucose, 100 $\mu\text{mol/l}$; palmitate/BSA ratio 0.3; Luiken *et al.* 1997). Uptake of ^{14}C -palmitate and ^3H -deoxyglucose (3 min incubation) was determined upon washing the cells twice for 2 min at 100 g in an ice-cold stop solution containing 0.2 mmol/l phloretin by scintillation counting.

Cardiac myocyte suspensions were pre-incubated with the PKC inhibitors staurosporine ($\leq 1 \mu\text{M}$; 15 min), BIM-I ($\leq 1 \mu\text{M}$; 15 min), HMG ($\leq 125 \mu\text{M}$; 15 min), or myristoylated PKC- ζ pseudosubstrate (50 μM ; 60 min) prior to stimulation by insulin (10 nM) or phorbol-myristate-acetate (1 μM), and subsequent substrate uptake assays. Stock solutions of staurosporine, BIM-I, HMG, and phorbol-myristate-acetate were prepared in DMSO, which in no case exceeded a final concentration of 0.5% in the cell suspensions. At this concentration, DMSO did not affect cellular substrate utilization (data not shown). All agents were added at the minimal concentration at which they exerted the maximal effect. None of these agents were found to affect the percentage of cells that i) was rod-shaped and ii) excluded trypan blue, as parameters of cellular integrity.

Subcellular fractionation of cardiac myocytes for assessment of translocation of GLUT4 and CD36

Cardiac myocytes (2.25 ml; 20–25 mg wet mass per ml) were incubated for 15 min in medium B in the absence and presence of additions. At the end of the incubation, the total volume of cell incubations was quickly transferred to a tightly fitting 5 ml Potter–Elvehjem 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. Immediately hereafter cell suspensions were homogenized with 10 strokes. Subsequently, fractionation was carried out as described previously (Fischer *et al.* 1995, Luiken *et al.* 2002). For determination of the GLUT4 and FAT/CD36 content in the PM and in light-density microsomes (LDM), aliquots of the membrane fractions were separated with SDS-PAGE and western blotting (Luiken *et al.* 2002). To detect FAT/CD36, we used a monoclonal antibody (MO25) directed against human CD36, and for detection of GLUT4, a polyclonal IgG antiserum was applied. Signals obtained by western blotting were quantified by densitometry.

The purity of the fractions obtained by this fractionation procedure was previously checked (Fischer *et al.* 1995). Specifically, the PM fraction is 13.5-fold enriched with ouabain-sensitive *p*-nitrophenyl-phosphatase, whereas the specific activity of the sarcoplasmic EGTA-sensitive Ca^{2+} -ATPase was 3.6-fold decreased. In addition, no activity of *p*-nitrophenyl-phosphatase or of Ca^{2+} -ATPase could be detected in the LDM fraction, indicating that this fraction was devoid of PM and of sarcoplasmic reticulum.

Subcellular fractionation of cardiac myocytes for assessment of PKC translocation

Cardiac myocytes (2.25 ml, 20–25 mg wet weight per ml) were incubated for 15 min in medium B in the absence and presence of additions, where after the suspension was split into two portions: 0.5 ml for obtaining the total tissue protein fraction, and 1.5 ml for fractionation into particulate and cytosolic fractions. Then, both aliquots were immediately centrifuged at 1000 g 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 phenylmethylsulfonyl fluoride, and 0.005% aprotinin), 0.3 ml for obtaining the total protein fraction (which was then immediately frozen in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$), 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 $-80 \text{ }^\circ\text{C}$.

Measurement of PKC- ζ -activation

Cardiac myocytes (2.25 ml; 20–25 mg wet mass per ml) were incubated for 15 min in medium B in the absence and presence of additions. Cells were lysed in an NP-40 based lysis buffer (see above) in the presence of 1 μM microcystin LR and immunoprecipitated with 5 μg mouse monoclonal PKC- λ for 1.5 h at 4 °C. Subsequently, Prot-G beads were added and complexes were harvested after another 1.5 h. The precipitate was washed three times with lysis buffer and two times with kinase assay buffer (100 mM HEPES pH 7.4, 10 mM MgCl_2 , 1 mM dithiothreitol). PtdSer (4 μg per sample) was dried under a N_2 atmosphere and dissolved in 25 μl kinase buffer per sample. Subsequently, PtdSer was waterbath-sonicated three times for 5 min and 25 μl sample kinase buffer, ATP (40 μM , final concentration), 5 μCi γ - ^{32}P -labelled ATP per reaction, dithiothreitol (1.5 mM), protein kinase-A inhibitor (PKI; 1 mM), and PKC- ϵ -substrate (40 μM) were added. Kinase reactions were allowed to proceed for 10 min at 37 °C under gentle agitation. Twenty microlitres of each reaction was spotted on p81 paper and washed three times for 5 min with 0.85% (v/v) phosphoric acid, and once for 5 min with acetone. P81 papers were air dried and analyzed in a scintillation counter.

Isolation of giant membrane vesicles for measurement of membrane-attachment of PKC- ζ in skeletal muscle

Rats were injected intravenously with insulin (2 U/kg body mass) or an equal volume of saline, and 15 min later killed for removal of hindlimb muscle for the preparation of giant

membrane vesicles as described previously (Koonen *et al.* 2002). These membranes were frozen upon isolation, and used for the assessment of PKC- ζ protein content.

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.

Other procedures

Cellular wet mass was obtained from cell samples taken during the incubation period and determined after centrifugation for 2–3 s at maximal speed in a microcentrifuge and subsequent removal of the supernatant. Protein was quantified with the Bicinchoninic acid protein assay (Pierce) according to the manufacturer's instructions.

Data presentation and statistics

All data are presented as means \pm S.E.M. for the indicated number of myocyte preparations. Statistical difference between groups of observations was tested with a paired Student's *t*-test. *P* values equal to or <0.05 were considered significant.

Results

Effects of PKC inhibitors on established PKC-mediated responses in cardiac myocytes

In order to investigate the involvement of isoforms of the PKC family in insulin-stimulated uptake of glucose and of LCFA, we have used several general and iso-form specific pharmacological PKC inhibitors, i.e. staurosporine, BIM-I, 1-*O*-HMG, and myristoylated PKC- ζ pseudosubstrate (ζ -PS). Staurosporine is among the most potent PKC inhibitors (IC_{50} 3 nM), acting on the catalytic domain shared by all PKC isoforms (Tamaoki *et al.* 1986). BIM-I is a staurosporine-derivative (Gschwendt *et al.* 1996) that has been reported to be specific for conventional PKCs at low concentrations (Khayat *et al.* 2002). HMG is a specific inhibitor of conventional and novel PKC and competes with DAG or phorbol esters for binding to the regulatory C1-domain (van Blitterswijk *et al.* 1987). ζ -PS is cell-permeable and irreversibly binds to atypical PKCs (House & Kemp 1987). N-linked myristoylation of these peptides allows their uptake by intact cells (Eichholtz *et al.* 1993). A myristoylated tridecapeptide corresponding to the pseudosubstrate region of PKC- ζ (ζ -PS) has been successfully used to specifically inhibit PKC- ζ in adipocytes (Standaert *et al.* 1997) and subsequently in cardiac myocytes (Shizukuda & Buttrick

2002). Because the pseudosubstrate region is well-conserved between the atypical PKC isoforms, ζ -PS is also inhibitory to other atypical PKCs besides PKC- ζ , such as PKC- ι and PKC- λ (Standaert *et al.* 1997). However, in rat heart the relative expression level of PKC- ι and λ is relatively low (Puceat *et al.* 1994, Shizukuda & Buttrick 2002), and therefore these atypical PKC isoforms should play minor roles in cardiac signaling. Nonetheless, PKC- ι and λ cannot be entirely excluded from mediating pivotal cellular processes, for example due to compartmentalization.

In order to test whether these inhibitors exert their desired effects in cardiac myocytes, we assessed whether they were able to block responses in which DAG-inducible PKC isoforms have been firmly established to be involved in. Phorbol esters are known to induce the phosphorylation of

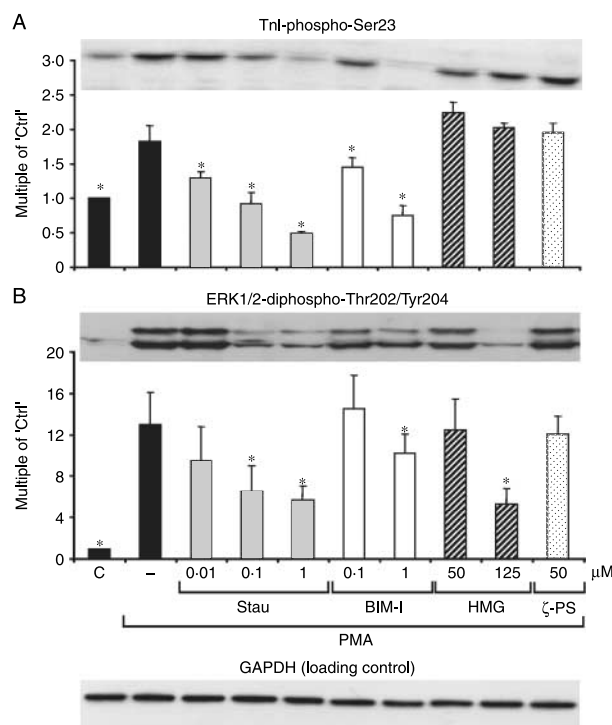


Figure 1 Verification of the isoform-specific potential of selected PKC inhibitors in cardiac myocytes by assessing their effect on phosphorylation of cTnI and of ERK1/2. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions (Ctrl) or with staurosporine (Stau), BIM-I, HMG, and ζ -PS at the concentrations indicated. Thereafter, cardiac myocytes were treated without additions or with 1 μ M PMA for 15 min at 37 °C. For assessment of phosphorylation of cTnI and ERK1/2, cardiac myocytes were pelleted, dissolved in sample buffer, after which westerns were performed with phosphospecific antibodies against phospho-cTnI (Ser23) and phospho-ERK1/2 (Thr202/Tyr204). Gels were loaded with equal quantities of protein for each cell lysate (20 μ g/lane). Values are expressed as multiple of non-stimulated control. Representative western blots are displayed. Western detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Data are presented as means \pm S.E.M., *n*=3. *Significantly different from PMA-stimulated control cardiac myocytes (*P*<0.05).

troponin-I, involving conventional PKCs (Liu *et al.* 1989, Venema & Kuo 1993), and phosphorylation of ERK1/2 via both conventional and novel PKCs (Zou *et al.* 1996, Montessuit & Thorburn 1999).

Treatment of cardiac myocytes with phorbol-12-myristate-13-acetate (PMA) induced a moderate (twofold) increase in troponin-I (Ser23) phosphorylation and a large (13-fold) increase in ERK1/2 (Thr202/Tyr204) phosphorylation (Fig. 1). Staurosporine dose-dependently inhibited PMA-induced phosphorylation of both troponin-I and ERK1/2 to a maximally observed inhibition of > 100 and 61% respectively, at 1 µM concentration. BIM-I exerted a dose-dependent inhibition of maximally > 100 and 23% on PMA-induced phosphorylation of troponin-I and ERK1/2 respectively, at 1 µM concentration. HMG did not inhibit either one of the PMA-induced protein phosphorylation events at 50 µM, but specifically inhibited PMA-induced ERK phosphorylation by 65% at 125 µM. ζ-PS inhibited neither PMA-induced troponin-I phosphorylation nor PMA-induced ERK phosphorylation at 50 µM (Fig. 1).

We also examined whether the foregoing inhibitors were able to inhibit the DAG-insensitive atypical PKC-isoform PKC-ζ in cardiac myocytes by measuring phosphorylation of the PKC-ζ target peptide upon lysis of the cells followed by PKC-ζ immunoprecipitation and *in vitro* kinase activity of the precipitated PKC-ζ against this peptide. Staurosporine and ζ-PS markedly inhibited PKC-ζ activity by 43 and 70% respectively, whereas BIM-I and HMG had no effect (Fig. 2).

Next, we examined whether the used PKC inhibitors would affect insulin signaling through the IRS – PI3K – Akt/PKB axis. This appeared not to be the case, because insulin-induced Akt/PKB (Ser473) phosphorylation was not reduced by pre-treatment of cardiac myocytes with these inhibitors (Fig. 3).

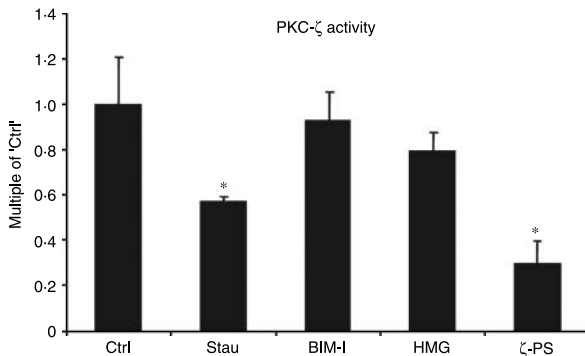


Figure 2 Influence of selected PKC inhibitors on activity of PKC-ζ in cardiac myocytes. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions (Ctrl) or with 1 µM staurosporine (Stau), 1 µM BIM-I, 125 µM HMG or 50 µM ζ-PS. For assessment of PKC-ζ activity, cardiac myocytes were lysed and used for immunoprecipitation with a PKC-λ antibody (see Materials and Methods). PKC-ζ activity is measured by the incorporation of [γ-32P]-ATP into a PKC-target peptide. Data are presented as means ± S.E.M., n=4. *Significantly different from non-treated (Ctrl) cardiomyocytes (P<0.05).

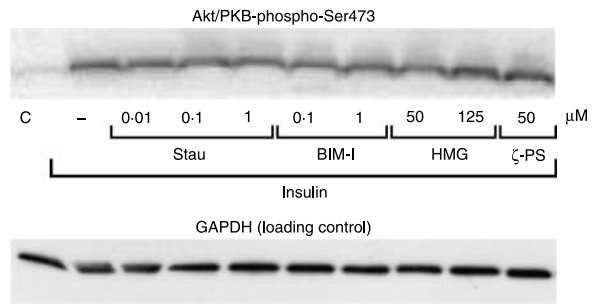


Figure 3 Influence of selected PKC inhibitors on Akt/PKB phosphorylation. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions (Ctrl) or with the various PKC inhibitors, as described in the legend of Fig. 1. Thereafter, cardiac myocytes were treated without or with 100 nM insulin for 15 min at 37 °C. For assessment of phosphorylation of Akt/PKB, cardiac myocytes were pelleted, dissolved in sample buffer, after which westerns were performed with phosphospecific antibodies against phospho-Akt (Ser473). Gels were loaded with equal quantities of protein for each cell lysate (20 µg/lane). A representative western blot out of three independent experiments is displayed. Western detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Taken together, 1 µM staurosporine efficiently blocks conventional, novel PKC as well as atypical PKCs in cardiac myocytes. In addition, 1 µM BIM-I largely blocked conventional PKCs in cardiac myocytes based on its ability to markedly inhibit troponin-I (Ser23) phosphorylation but had only minor effects on PMA-induced ERK1/2 (Thr202/Tyr204) phosphorylation. Furthermore, HMG only appeared to affect novel PKCs in cardiac myocytes, because PMA-induced troponin-I phosphorylation was not affected while ERK1/2 phosphorylation was markedly inhibited. Finally, ζ-PS specifically inhibited PKC-ζ, without any confounding effects on conventional or novel PKCs. Hence, these selected PKC inhibitors are suitable tools to investigate the general and isoform-specific involvement of PKCs in insulin-induced substrate utilization in cardiac myocytes, without non-specific side actions on other key enzymes involved in insulin signaling, as far as investigated.

Effects of PKC inhibitors on insulin-stimulated substrate uptake and transporter translocation in cardiac myocytes

Prior to measuring insulin-stimulated substrate uptake, we assessed the effects of the PKC inhibitors on basal substrate uptake without observing any differences (data not shown). Upon treatment with 10 nM insulin, deoxyglucose, and palmitate uptake were 4.3- and 1.6-fold respectively, increased (Fig. 4). Insulin-stimulated glucose uptake was largely inhibited by pre-treatment of cardiac myocytes with staurosporine (–56% at 10 nM; –76% at ≥ 100 nM) and with ζ-PS (–73%), while BIM-I and HMG were not effective (Fig. 4). Insulin-stimulated LCFA uptake was completely inhibited by staurosporine (at all tested concentrations) and by ζ-PS, but not affected by BIM-I and HMG (Fig. 4).

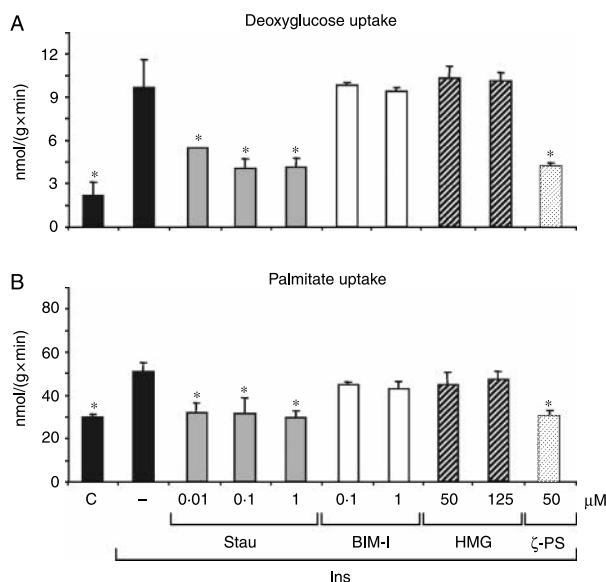


Figure 4 Influence of selected PKC inhibitors on insulin-induced glucose- and LCFA uptake into cardiac myocytes. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions (Ctrl) or with the various PKC inhibitors, as described in the legend of Fig. 1. Thereafter, cardiac myocytes were treated without or with 100 nM insulin for 15 min at 37 °C. Finally, initial rates of (A) [³H]deoxyglucose uptake and (B) [¹⁴C]palmitate uptake were determined and expressed as nmol/(g cellular wet mass × min). Data are presented as means ± S.E.M., *n* = 5. *Significantly different from insulin-treated control cardiomyocytes (*P* < 0.05).

Concomitant with stimulation of glucose and LCFA uptake, insulin treatment increases the PM content of GLUT4 (2.0-fold) and CD36 (1.5-fold) at the expense of their contents at the LDM (GLUT4: -55%; CD36: -66%; Fig. 5). This insulin-stimulated translocation of GLUT4 and CD36 was completely inhibited by 1 μM staurosporine (Fig. 5).

These results demonstrate that inhibition of total PKC activity or of atypical PKCs, but not of novel and conventional PKCs, results in a large inhibition of insulin-stimulated glucose uptake and a complete inhibition of insulin-stimulated LCFA uptake, likely mediated by inhibition of GLUT4- and CD36 translocation.

Effects of insulin and PMA on activation of PKCs in cardiac myocytes

PKC- α , - δ , - ϵ , and - ζ are the major PKC isoforms in the heart (Eichholtz *et al.* 1993, Puceat *et al.* 1994, Clerk *et al.* 1995, Goldberg *et al.* 1997, Shizukuda & Buttrick 2002). Their activation results in the translocation to subcellular membranes possibly in combination with phosphorylation of Ser/Thr residues in the activation loop (Keranen *et al.* 1995, Newton 1995). In order to test their responsiveness to insulin, we investigated whether the cardiac PKC isoforms are subjected to membrane translocation in response to insulin.

In these cardiac myocyte incubations, PMA was used as a positive control for PKC activation. During the incubation period of 15 min, the total protein content of PKC- α , - δ , and - ϵ in cardiac myocytes was unaltered upon treatment with either insulin or PMA compared with untreated cardiac myocytes (Fig. 6A). In non-stimulated myocytes, PKC- α and - δ were mostly present in the soluble form, whereas PKC- ϵ was evenly distributed between soluble and membrane compartments. By contrast, PKC- ζ was undetectable in the cytosolic fraction and completely resided in the membrane fraction. In agreement with its ability to activate all conventional and novel PKCs, PMA treatment caused a complete shift in the contents of PKC- α , - δ , and - ϵ from the cytosolic to the particulate fraction, and had no effect on PKC- ζ distribution (Fig. 6A). By contrast, insulin treatment did not affect the subcellular distribution of either of the tested PKC isoforms (Fig. 6A).

We also tested whether commercially available phospho-specific antibodies against the major cardiac PKCs could provide an indication for insulin-induced PKC activation. Thus, we examined phosphorylation of PKC- α at Thr638, phosphorylation of PKC- δ at Ser643, and phosphorylation of PKC- ζ at Thr410/403. Thr410/403 is within the activation loop of PKC- ζ , and, hence, is directly involved in its activation. However, the Thr638 and Ser643 sites seem not to

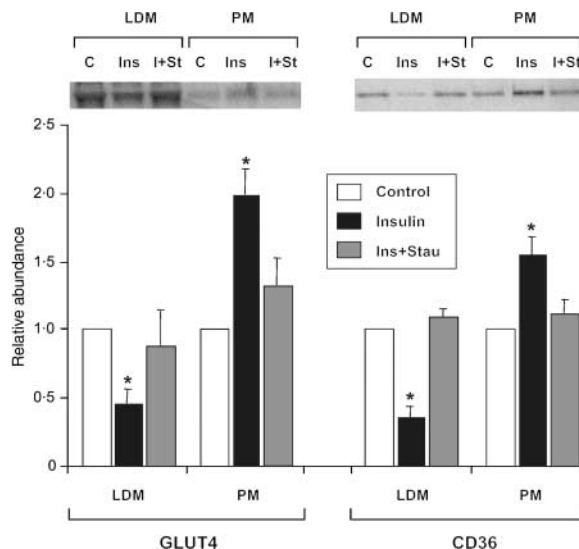


Figure 5 Effect of staurosporine on insulin-induced GLUT4- and CD36 translocation. Isolated rat cardiac myocytes were pre-incubated for 15 min at 37 °C without additions or with 1 μM staurosporine (Stau). Thereafter, cardiac myocytes were treated without or with 100 nM insulin for 15 min at 37 °C. Then, cardiac myocytes were separated into a plasma membrane (PM) fraction and a light-density microsomal (LDM) fraction. In these fractions, the protein contents of GLUT4 and CD36 was determined by western blotting. Gels were loaded with equal quantities of protein for each fraction (10 μg/lane). Values are expressed as multiple of control (C) for each fraction. Representative western blots are displayed. Data are presented as means ± S.E.M., *n* = 4. *Significantly different from non-treated (control) cardiomyocytes (*P* < 0.05).

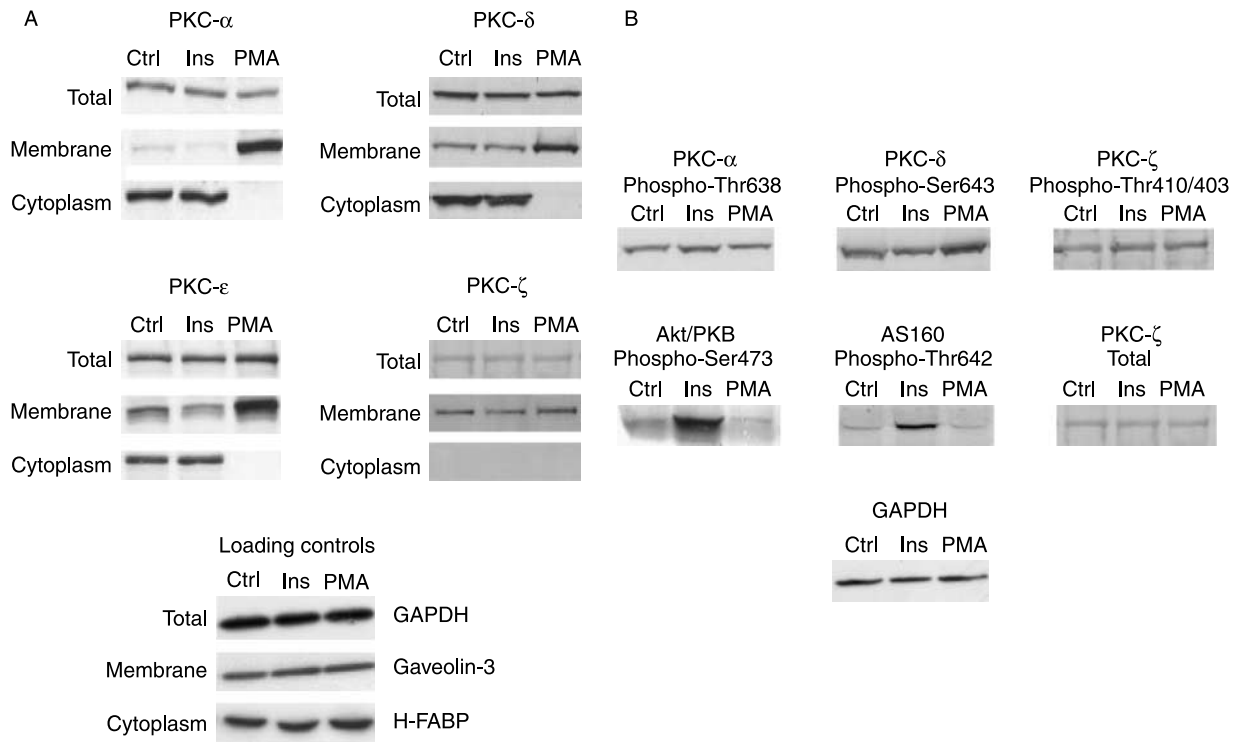


Figure 6 Effects of insulin and PMA on activation of major cardiac PKC isoforms. (A) Effects of insulin and PMA on membrane-attachment of PKC- α , - δ , - ϵ , and - ζ . Isolated adult rat cardiac myocytes were incubated for 15 min at 37 °C without additions (Ctrl), with 100 nM insulin (Ins) or with 1 μ M 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 μ g for each fraction was subjected to western blotting using antibodies against PKC- α , - δ , - ϵ , and - ζ . Representative western blots out of three independent experiments are presented. Loading controls for westerns of each of the PKC isoforms have been performed, in which GAPDH was used as loading control for the total cellular fraction, caveolin-3 as loading control for the particulate fraction, and heart-type fatty acid-binding protein (H-FABP) for the cytosolic fraction. A representative loading control (belonging to the PKC- ζ westerns) is shown, but also the loading controls performed in parallel to the westerns of the other PKC isoforms did not show any significant changes between the various treatments. (B) Effects of insulin and PMA on phosphorylation of PKC- α , - δ , and - ζ and for comparison of Akt/PKB and AS160. Isolated adult rat cardiac myocytes were incubated for 15 min at 37 °C without additions or with 100 nM insulin (Ins) or 1 μ M PMA. For assessment of phosphorylation of PKC- α , PKC- δ , PKC- ζ , Akt/PKB, and AS160, cardiac myocytes were pelleted, dissolved in sample buffer, after which westerns were performed with phosphospecific antibodies against PKC- α (Thr638), PKC- δ (Ser643), PKC- ζ (Thr410/403), Akt/PKB (Ser473), and AS160 (Thr642). Gels were loaded with equal quantities of protein (20 μ g/lane) for each cell lysate. Representative western blots out of three independent experiments are presented. A western against the total PKC- ζ protein was performed as loading control for the phospho-PKC- ζ western. For westerns of each of the other PKC isoforms a GAPDH western was performed as loading control, which did not show any significant changes between the various treatments. One of these GAPDH loading controls is displayed.

be directly involved in activation of PKC- α and - δ respectively (Bornancin & Parker 1996, Li *et al.* 1997). Nonetheless, phosphorylation of these sites might still reflect activation due to subsequent poorly understood autophosphorylation events. PMA treatment increased Ser643 phosphorylation of PKC- δ (1.7-fold \pm 0.3, $n=3$), but not Thr638 phosphorylation of PKC- α . Insulin treatment had no effect on phosphorylation at either of these sites (Fig. 6B).

Hence, PMA was able to induce the membrane attachment of PKC- α , - δ , and - ϵ and phosphorylation of PKC- δ in cardiac myocytes, showing that in these cells these isoforms possess the intrinsic ability to be activated, but not by insulin (Fig. 6A and B). However, such positive control on activation is lacking in case of PKC- ζ , since this isoform is not

responsive to PMA (see Introduction). Therefore, we have additionally measured the enzymatic activity of PKC- ζ in cardiac myocytes, and the effects of insulin or PMA treatment hereon. Neither insulin nor PMA resulted in increased [32 P]ATP incorporation into the PKC target peptide, indicating that neither stimulus altered the enzymatic activity of PKC- ζ (Fig. 7).

Effects of insulin on activation of PKC- ζ in skeletal muscle

Because of the surprising finding that insulin-treatment of cardiac myocytes did not alter the activity state of PKC- ζ , we sought to confirm the general consensus that at least in peripheral insulin-sensitive tissues insulin would be indeed

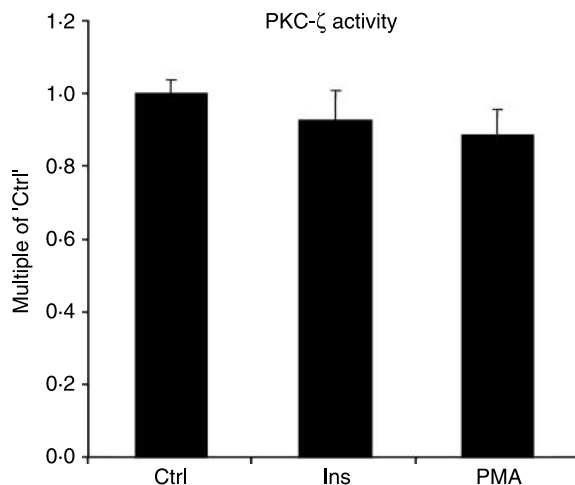


Figure 7 Effect of insulin on activity of PKC- ζ in cardiac myocytes. Isolated adult rat cardiac myocytes were incubated for 15 min at 37 °C without additions or with 100 nM insulin (Ins) or 1 μ M PMA. For assessment of PKC- ζ activity in cardiac myocytes, see legends of Fig. 2. Data are presented as means \pm s.e.m., $n=4$. *Significantly different from non-treated (control) cardiomyocytes ($P<0.05$).

capable of activating PKC- ζ (Standaert *et al.* 1999, Braiman *et al.* 2001, Liu *et al.* 2006). For this purpose, we studied the effects of 15 min insulin-administration to rats on PKC- ζ membrane attachment in skeletal muscle using the procedure of preparation of giant membrane vesicles. Insulin-treatment indeed resulted in an increased content of PKC- ζ (>3-fold, $n=4$, $P<0.05$, see Fig. 8) in giant membrane vesicles from hindlimb muscle. This indicates that insulin indeed activates PKC- ζ in skeletal muscle.

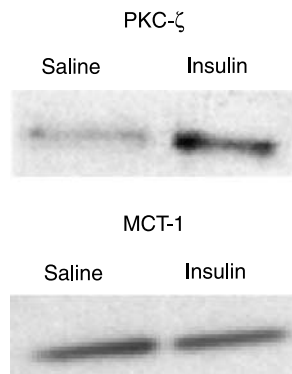


Figure 8 Effect of insulin on PKC- ζ translocation to the plasma membrane in skeletal muscle. Rats were injected with saline or insulin 15 min prior to removal of hindlimb muscle for the preparation of giant membrane vesicles. These giant vesicles were pelleted, dissolved in sample buffer, after which westerns were performed against PKC- ζ . Gels were loaded with equal quantities of protein (5 μ g/lane) for each giant vesicle preparation. A representative western blot out of four independent experiments is presented. Western detection of the resident sarcolemmal protein MCT-1 was used as a loading control.

Discussion

Glucose uptake and LCFA uptake appear to be similarly regulated by insulin, i.e. via PI3K-dependent translocation of GLUT4 and CD36 respectively. The main purpose of this study was to assess whether this similarity also extends to the level of PKCs, possibly acting downstream of PI3K. The role of PKC- ζ and also of other PKCs in GLUT4 translocation has been extensively investigated in adipocytes and to a lesser extent in skeletal muscle, but not in the heart. Moreover, PKCs have never been associated with insulin-stimulated CD36 translocation and LCFA uptake.

The major findings of this study were that i) inhibition of the atypical PKC- ζ , but not inhibition of conventional or novel PKCs, inhibited insulin-stimulated glucose and insulin-stimulated LCFA uptake via blocking of the translocation of GLUT4 and CD36 respectively, and ii) insulin did not alter a) the distribution of PKC- α , - δ , - ϵ or - ζ between soluble and particulate cardiac myocyte fractions or b) the phosphorylation of PKC- α , - δ or - ζ , or c) the catalytic activity of PKC- ζ . It is concluded that PKC- ζ , but not any other PKC isoform, is involved in insulin-induced glucose- and LCFA uptake, but its role is permissive rather than regulatory.

PKC- ζ is the only cardiac PKC isoform involved in insulin-stimulated glucose- and LCFA uptake

Although genetic approaches to silence PKC isoforms in cardiac myocytes could unequivocally proof their role in insulin-stimulated glucose- and LCFA uptake, we chose to use pharmacological compounds to inhibit PKC classes for the following reasons: i) adult cardiac myocytes are very difficult to transfect and will lose their characteristic features within a few days of culturing; ii) it has already been reported that the culture of cardiac myocytes results in a down-regulation of several PKCs (Cuello *et al.* 2007), making a siRNA transfection to silence PKC- ζ redundant. Alternatively, PKC inhibitors are commonly used to test the involvement of PKCs in a given cellular process. We have used a number of PKC inhibitors and verified the specificity of their actions on the different isoforms, with staurosporine being a general PKC inhibitor, whereas the actions of BIM-I, HMG, and ζ -PS were restricted to inhibition of conventional PKCs, novel PKCs and PKC- ζ respectively.

Previously, insulin-induced glucose uptake was reported to be inhibited by staurosporine and ζ -PS in adipocytes and skeletal muscle (Farese *et al.* 1993, Standaert *et al.* 1997), indicating the involvement of PKC- ζ and leaving open the possibility of the involvement of other PKC isoforms. In case of PKC- ζ , not only pharmacological evidence, but also genetic evidence in cell lines in which it was overexpressed (Goldberg *et al.* 1997, Braiman *et al.* 2001) or silenced (Sajan *et al.* 2006) pointed towards an undisputed role of this atypical PKC in insulin-stimulated glucose uptake. In the present study, we confirmed that staurosporine and ζ -PS were able to

inhibit insulin-stimulated glucose uptake and made the novel observation that these same inhibitors completely inhibited insulin-stimulated LCFA uptake into cardiac myocytes. Recently, inhibition of insulin-stimulated LCFA uptake by ζ -PS was also reported in the L6 muscle cell line (Kelly *et al.* 2008). This inhibitory action of both PKC inhibitors cannot be attributed to inhibition of insulin-induced Akt/PKB activity because both compounds did not affect Ser473 phosphorylation (Fig. 3). By contrast, BIM-I and HMG did not affect insulin-stimulated glucose- and LCFA uptake. Together, these findings point out that the PKC family is involved in insulin-stimulated glucose- and LCFA uptake, but that this involvement only applies for the atypical isoform PKC- ζ , but not for conventional or novel PKC isoforms. Moreover, the inhibitory potential of ζ -PS on both insulin-stimulated glucose- and insulin-stimulated LCFA uptake is similar to that of staurosporine, indicating that PKC- ζ fully accounts for the overall PKC involvement in these processes. The ability of staurosporine to completely inhibit insulin-induced GLUT4- and CD36 translocation clearly indicates that the mechanism by which PKC- ζ mediates insulin-induced glucose- and LCFA uptake involves translocation of GLUT4 and CD36.

Insulin treatment of cardiac myocytes does neither alter PKC- ζ activity nor that of other PKC's

The ability of insulin to induce GLUT4- and CD36 translocation in a PKC- ζ -dependent manner suggests that activation of PKC- ζ by insulin is involved in these processes. In fact, it has been reported that in adipose tissue and skeletal muscle insulin is able to activate PKC- ζ , as measured by PKC- ζ membrane attachment, phosphorylation at Thr410/403, or by an increased enzymatic activity of immunoprecipitated PKC- ζ towards a synthetic substrate (Standaert *et al.* 1999, Braiman *et al.* 2001, Liu *et al.* 2006). We also confirmed that insulin-treatment of rats resulted in an increased attachment of PKC- ζ to sarcolemmal membranes from hindlimb muscle. However, in cardiac myocytes, as shown in the present study, insulin does not alter the subcellular distribution of PKC- ζ , its phosphorylation at Thr410/403 or its intrinsic activity. A possible cellular defect in signaling and/or membrane-anchoring events using these freshly isolated cardiac myocytes most likely can be excluded for a number of reasons. These are as follows: i) Insulin treatment results in full phosphorylation of Akt/PKB at Ser403 and AS160 at Thr642. In addition, ii) full membrane attachment of PKC- α , - δ , and - ϵ is achieved upon PMA treatment. Possible defects in the methods used to measure PKC- ζ activation can also be excluded for the following reasons: i) The western blot signals recognized by the total and phosphospecific antibodies against PKC- ζ used for measurement of PKC- ζ translocation and -phosphorylation respectively, are specific for PKC- ζ , because a) these signals are detected at 78 kDa, which corresponds exactly to the reported molecular mass of PKC- ζ , b) the signals from samples from brain tissue show a

much higher intensity than those from cardiac myocytes, which corresponds well with the relatively high expression of PKC- ζ in brain (data not shown), and c) the signals are largely absent in hearts from heart/muscle-specific atypical PKC null mice compared with wild-type hearts (data not shown). ii) The enzymatic activity in the *in vitro* PKC- ζ kinase assay is sensitive to inhibition by ζ -PS and staurosporine, indicating that indeed PKC- ζ activity is measured. Thus, it appears that our results are neither attributable to cellular defects in signaling and/or membrane-anchoring events nor to methodological detection problems. Therefore, our observations strongly suggest that unlike in adipocytes or skeletal muscle, in the heart insulin does not alter the activity state of PKC- ζ .

How does the inability of insulin to increase the activation state of PKC- ζ match with the reliance of insulin-induced GLUT4 translocation on PKC- ζ ? The inability of insulin to activate PKC- ζ does not imply that PKC- ζ is inactive during insulin stimulation. In fact, under basal conditions, the total PKC- ζ population within cardiac myocytes is membrane-bound, indicating that PKC- ζ is in the active state. This is in contrast with the subcellular localization of PKC- α , - δ or - ϵ ,

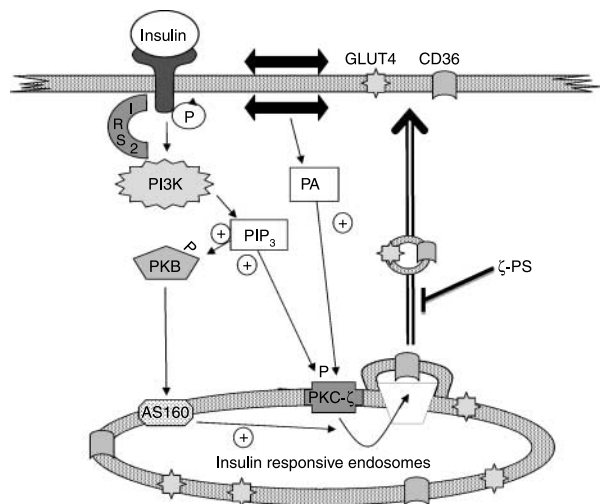


Figure 9 PKC- ζ plays a permissive role in insulin-induced GLUT4- and CD36 translocation in the heart. Upon binding to its receptor at the cell surface, insulin sequentially activates insulin receptor-substrate 2 (IRS2) and PI3K, resulting in production of PIP₃. Beyond PI3K, the insulin signal is split into two parallel branches. PIP₃ binds to both Akt/PKB and PKC- ζ . In case of Akt/PKB, this results in the activation and subsequent AS160 phosphorylation. In case of PKC- ζ , this does not result in further activation because the basally high levels of PA in the heart impose PKC- ζ to be already bound to endosomal membranes in the absence of insulin treatment. Hence, PKC- ζ maintains the endosomal membranes in a pre-active state, waiting for the insulin signal through activation of Akt/PKB and AS160 to complete the final stages in the budding of GLUT4- and CD36-containing vesicles for translocation to the sarcolemma. In agreement with the dependence of insulin-stimulated GLUT4- and CD36 translocation of PKC- ζ , these processes will be subjected to inhibition by ζ -PS.

all of which are merely present in the soluble cytosol, and likely inactive. Moreover, the membrane-bound state of PKC- ζ in non-stimulated cardiac myocytes is accompanied by a Thr410/403 phosphorylation level and an enzymatic activity that are not further increased by insulin. These findings indicate that in non-stimulated cardiac myocytes PKC- ζ is already maximally active, so that insulin fails to further activate it. A possible explanation for the difference in basal activity of PKC- ζ in heart versus skeletal muscle and adipose tissue could reside in basal intracellular concentrations of specific lipid second messengers. Besides, by PIP₃, produced by PI3K, PKC- ζ can be activated by a number of other lipid metabolites, such as phosphatidic acid (PA; Limatola *et al.* 1994, 1997). Notably, PA levels are increased in muscle by exercise, and remain elevated up to at least 60 min after termination of contraction (Cleland *et al.* 1989). The elevated PA levels are likely due to phospholipase-D activation upon a rise in intracellular Ca²⁺ (Eskildsen-Helmond *et al.* 1996). The heart being in a continuously contracting state will then contain permanently elevated PA levels, in contrast to skeletal muscle. These high PA levels will likely be maintained in cardiac myocytes upon their isolation from rat heart (see Materials and Methods), and will cause PKC- ζ to be maximally activated under basal conditions. Exposure of these cells to insulin, resulting in PIP₃ formation, will then be ineffective in further activating PKC- ζ .

Concluding remarks

The combined findings that PKC- ζ is necessary for both insulin-inducible GLUT4- and CD36 translocation and that in the heart the basal activity of PKC- ζ is not further stimulated by insulin, indicate that PKC- ζ plays a permissive rather than a regulatory role in insulin-induced glucose- and LCFA uptake. Based on this permissive role of PKC- ζ , we speculate that the following sequence of events is occurring: in non-stimulated cardiac myocytes, PKC- ζ is present in the endosomal compartment, fully bound to endosomal membranes, and is actively participating in the sorting of GLUT4 and CD36. Hence, PKC- ζ keeps the sorting machinery for both GLUT4 and CD36 in a pre-active state, so that an incoming insulin signal through PI3K and Akt/PKB, and possibly AS160, will allow completion of the final steps in budding and excision of GLUT4- and CD36-containing vesicles for their translocation to the sarcolemma (Fig. 9).

Clearly, insulin-induced GLUT4 translocation and insulin-induced CD36 translocation appear to rely on virtually the same signaling machinery. As already mentioned, we showed that PI3K, and likely also Akt/PKB, are involved in cardiac insulin-induced GLUT4- and insulin-induced CD36 translocation. The present data indicate that additionally PKC- ζ can be added to the list of shared signaling components between both translocation processes. It would be a challenge for future research to identify protein components that are

specifically dedicated to either insulin-induced GLUT4- or CD36 translocation, because these proteins would be attractive targets to manipulate the cardiac substrate balance.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of the scientific work.

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