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RESEARCH PAPER

β-Adrenoceptor stimulation potentiates insulin-stimulated PKB phosphorylation in rat cardiomyocytes via cAMP and PKA

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Background and purpose: Genetic approaches have documented protein kinase B (PKB) as a pivotal regulator of heart function. Insulin strongly activates PKB, whereas adrenaline is not considered a major physiological regulator of PKB in heart. In skeletal muscles, however, adrenaline potentiates insulin-stimulated PKB activation without having effect in the absence of insulin. The purpose of the present study was to investigate the interaction between insulin and β-adrenergic stimulation in regulation of PKB phosphorylation.
Experimental approach: Cardiomyocytes were isolated from adult rats by collagenase, and incubated with insulin, isoprenaline, and other compounds. Protein phosphorylation was evaluated by Western blot and phospho-specific antibodies.
Key results: Isoprenaline increased insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation more than threefold in cardiomyocytes. Isoprenaline alone did not increase PKB phosphorylation. Isoprenaline also increased insulin-stimulated GSK-3β Ser^{9} phosphorylation approximately twofold, supporting that PKB phosphorylation increased kinase activity. Dobutamine (β_1-agonist) increased insulin-stimulated PKB phosphorylation as effectively as isoprenaline (more than threefold), whereas salbutamol (β_2-agonist) only potentiated insulin-stimulated PKB phosphorylation by approximately 80%. Dobutamine, but not salbutamol, increased phospholamban Ser^{16} phosphorylation and glycogen phosphorylase activation (PKA-mediated effects). Furthermore, the cAMP analogue that activates PKA (dibutyryl-cAMP and N^6-benzoyl-cAMP) increased insulin-stimulated PKB phosphorylation by more than threefold without effect alone. The Epac-specific activator 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (007) increased insulin-stimulated PKB phosphorylation by approximately 50%. Db-cAMP and N^6-benzoyl-cAMP, but not 007, increased phospholamban Ser^{16} phosphorylation.
Conclusions and implications: β-adrenoceptors are strong regulators of PKB phosphorylation via cAMP and PKA when insulin is present. We hypothesize that PKB mediates important signalling in the heart during β-adrenergic receptors stimulation.
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Abbreviations: DNA-PK, DNA-dependent protein kinase; Epac, exchange protein directly activated by cAMP; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase-3; IGF-1, insulin like growth factor-1; mAKAP, muscle-specific A-kinase anchoring protein; MEK, mitogen-activated protein kinase kinase; mTORC2, mammalian target of rapamyosin (mTOR) complex-2; PDK1, phosphoinositide–dependent kinase-1; PTEN, phosphatase and tensin homolog deleted on chromosome 10

Introduction

Genetic approaches have provided evidence that protein kinase B (PKB or Akt) is an important regulator of normal heart functions and dys-regulation causes cardiac disease (Debosch *et al.*, 2006). Deletion of PKBα decreases heart size (Debosch *et al.*, 2006) whereas overexpression of constitutively activated PKB increases heart size and causes dilated myopathy (Condorelli *et al.*, 2002; Matsui *et al.*, 2002). However, PKB has beneficial effects and protects the hearts during ischaemia–reperfusion (Matsui *et al.*, 2002). Growth factors like insulin and insulin-like growth factor-1 (IGF-1) activates PKB in the heart (Chesley *et al.*, 2000; Beauloye *et al.*, 2001). Insulin activates PKB via PI 3-kinase (Shepherd *et al.*, 1998; Shepherd,

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2005) and phosphorylation of PKB at Thr[308] and at Ser[473] (Vanhaesebroeck and Alessi, 2000). PDK1 phosphorylates PKB Thr[308] (Vanhaesebroeck and Alessi, 2000). PKB Ser[473] is phosphorylated by mTORC2 complex in insulin-sensitive tissues (Sarbassov *et al.*, 2005), but DNA-PK, PKCβII and integrin-linked kinase have been reported to phosphorylate PKB Ser[473], at least in some cell types (Persad *et al.*, 2001; Kawakami *et al.*, 2004; Huston *et al.*, 2008). Activated PKB phosphorylates glycogen synthase kinase (GSK)-3 (GSK-3α Ser[21] and GSK-3β Ser[9]) and GSK-3 is, like PKB, a point of integration of hypertrophic signalling in the heart (Sugden *et al.*, 2008). Genetic manipulations of regulators of PKB activity, like PDK1, PTEN and PI 3-kinase, have provided further evidence that regulation of PKB signalling is essential for normal heart growth and function (Shioi *et al.*, 2000; Schwartzbauer and Robbins, 2001; Crackower *et al.*, 2002; Mora *et al.*, 2003; Bayascas *et al.*, 2006; Heineke and Molkentin, 2006).

β-Adrenoceptors regulate central physiological functions in the heart (Ruehr *et al.*, 2004; Zheng *et al.*, 2005) and defective cAMP regulation promotes heart failure (Lehnart *et al.*, 2005). The signalling pathways for β_1- and β_2-adrenoceptors differ (Steinberg, 1999; Pavoine and Defer, 2005; Zheng *et al.*, 2005). Stimulation of β_1-adrenergic receptors increases concentration of cAMP that activates PKA leading to phosphorylation of phospholamban (PLB), ryanodine receptor, phosphorylase kinase (which activates glycogen phosphorylase) and numerous other proteins (Drago and Colyer, 1994; Steinberg, 1999). In contrast, stimulation of β_2-adrenoceptors is not thought to increase PLB Ser[16] phosphorylation or to activate glycogen phosphorylase (Kuschel *et al.*, 1999; Jo *et al.*, 2002), although a small increase in PLB Ser[16] phosphorylation has been observed (Bartel *et al.*, 2003). Although β_2-adrenergic receptors activates adenylyl cyclase, the produced cAMP is rapidly broken down (Xiang *et al.*, 2005) and β_2-adrenoceptors mediate additional signalling via PI 3-kinase, extracellular signal-regulated kinase (ERK) and phospholipase A_2 (Steinberg, 2004; Pavoine and Defer, 2005). Interestingly, prolonged stimulation of β_1-adrenoceptors causes apoptosis in cardiomyocytes whereas β_2-adrenoceptors may improve cell survival after hypoxia (Zhu *et al.*, 2001; Zhu *et al.*, 2003).

The γ-isoform of class 1 PI 3-kinase (PI3Kγ) is activated by G protein-coupled receptors (Wymann and Marone, 2005) and β-adrenoceptors have been reported to mediate effects via PI 3-kinase and PKB in heart cells (Chesley *et al.*, 2000; Oudit *et al.*, 2003; Tseng *et al.*, 2005). β_2-Adrenoceptors activate PI3Kγ via $G_{\alpha i}$-coupled $G_{\beta\gamma}$ but PKB activation is much less than during IGF-1 stimulation (Chesley *et al.*, 2000). Recently, cAMP has also been reported to mediate effect via exchange protein directly activated by cAMP (Epac) (De rooij *et al.*, 1998; Jensen, 2007) and it has been reported that Epac mediated hypertrophy in neonatal cardiomyocytes (Morel *et al.*, 2005; Métrich *et al.*, 2008).

Stimulation of β_2-adrenoceptors does not increase cAMP concentration throughout the cell and compartmentalized signalling allows activation of specific pools of PKA (Steinberg, 2004). A huge number of phosphodiesterases limit the spread of cAMP and compartmentalize β_2-adrenergic signalling (Fischmeister *et al.*, 2006). The PDE4 family is coded by four genes comprising ~20 members (Houslay *et al.*, 2005), and the PDE4 subfamily is involved in establishing compartmentalized β_2-adrenergic signalling in cardiomyocytes (Xiang *et al.*, 2005). ERK phosphorylates most PDE4 isoforms, and ERK-mediated phosphorylation decreases phosphodiesterase activity of the long isoforms whereas activity of most of the short PDE4 isoforms increases (Mackenzie *et al.*, 2000; Houslay *et al.*, 2005). The roles of ERK and PDE4 for β_2-adrenergic signalling have not achieved much attention in cardiomyocytes from adult rats.

Recently, we reported complex interaction between β-adrenoceptors and insulin signalling in skeletal muscles. While β-adrenoceptor stimulation did not activate PKB in the absence of insulin, β-adrenoceptor stimulation strongly potentiated insulin-stimulated PKB phosphorylation and activity, and cAMP mimicked the effect of β-adrenoceptor stimulation (Brennesvik *et al.*, 2005; Jensen *et al.*, 2008). The purpose of the present study was to test the hypothesis that stimulation of β-adrenoceptors potentiates insulin-stimulated PKB phosphorylation in cardiomyocytes via cAMP and PKA.

Methods

Chemicals and antibodies

Collagenase 2 and DNAse were from Worthington (Lakewood, NJ, USA) and Natural Mouse Laminin from Invitrogen (Carlsbad, CA, USA). Isoprenaline, dobutamine, salbutamol, PD98,059, dibutyryl-cAMP (db-cAMP; #D0627), rolipram, adrenaline, noradrenaline and wortmannin were from Sigma (St. Louis, MO, USA). N^6-Benzoyl-cAMP (N^6-cAMP; B009), 8-(4-chlorophenylthio)-2′-O-methyl-cAMP (007; C041) and 8-Bromoadenosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer (Rp-8-Br-cAMPS; B001) were from BIOLOG Life Science Institute (Bremen, Germany). Insulin (Actrapid) was from Novo Nordisk (Bagsværd, Denmark). Anti-anti-GSK-3 (#05-412) and anti-mouse HRP-conjugate antibodies (#12-349) were from Upstate (Lake Placid, NY, USA). Anti-phospho-Akt Ser[473] (#9271), anti-phospho-Akt Thr[308] (#9275), anti-phospho-GSK-3α/β Ser[21]/Ser[9] (#9331) and anti-rabbit HRP-linked antibodies (#7074) were from Cell Signaling Technology (Danvers, MA, USA). Anti-PLB (#A010-14) and anti-phospho-PLB Ser[16] (A010-12) were from Badrilla (Leeds, UK). ECL (RPN2106) was from Amersham Pharmacia (Buckinghamshire, UK) and ECL (WBKLS0500) from Millipore (Bedford, MA, USA). Other chemicals were standard analytical grades from Merck (Darmstadt, Germany), Sigma (St. Louis, MO, USA) and Bio-Rad (Hercules, CA, USA).

Animals

Male Wistar rats were obtained from B & K Universal (Nittedal, Norway) and acclimatized in our laboratory animal facilities for 2 weeks with free access to food and tap water before the experiment. Experiments were approved and conducted in conformity with laws and regulations controlling experiments and procedures for animal research in Norway and the European Convention for the Protection of Vertebrate Animals used in Experimental and Other Scientific Purposes.

Heart cell isolation and incubation

Hearts from adult male rats (450 g) were quickly removed under pentobarbitone anaesthesia (0.8 mL pentobarbitone

50 mg·mL^{-1} i.p.) and placed in ice-cold saline. The hearts were, still immersed in saline, connected to the Langendorff perfusion apparatus through a cannula inserted into the aorta and cardiomyocytes were isolated by a procedure modified after Stokke *et al*. (Stokke *et al*. 1996). The hearts were initially perfused for ~10 min with a Ca^{2+}-free Joklik S-MEM medium (Invitrogen, 22300-016) added 24 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1 mM DL-carnitine, pH 7.4 (buffer A). Perfusion was continued for 25 min at 37°C with buffer A added 200 U·mL^{-1} collagenase 2 and 0.1% BSA at a flow rate of 6–7 mL·min^{-1} with recirculation. Perfusion buffers were gassed with 95% O_2/5% CO_2. In some of the initial experiments trypsin (63 U·mL^{-1}, Sigma) was added. The effect of isoprenaline on insulin-stimulated PKB phosphorylation was similar in cardiomyocytes isolated with and without trypsin and data are pooled. The hearts were removed from the cannulae and the ventricular tissue cut and torn into small fragments in 30 mL buffer A added 0.5 mM $CaCl_2$ and 1% BSA. Coagulated blood and visible connective tissue were removed, and the suspension was incubated for 10 min, shaking (100 stroke per minute), at 37°C and gently gassed. The heart tissue was transferred to a glass tube and centrifuged (20× *g*, ~20 s). The pellet was resuspended in 30 mL with buffer A added 200 U·mL^{-1} collagenase 2, 0.1% BSA and DNase I (0.06 U·mL^{-1}), and heart cells were allowed to dissociate for 15–20 min (shaking: 100 stroke per minute, 37°C and gently gassed). Centrifugation was repeated and the cardiomyocyte pellet resuspended in ~30 mL buffer A added 0.5 mM $CaCl_2$ and 1% BSA, and allowed to rest at 37°C for 5 min without agitation. The cell suspension was filtered through a nylon mesh (size ~250 μm), centrifuged as above. The final pellet containing the cardiomyocytes was resuspended in culture medium (Medium 199 with 0.2% BSA, 2 mM DL-carnitine, 5 mM creatine, 5 mM taurine, 100 μU·mL^{-1} insulin, 1 × 10^{-10} M 5-triiodo-D-thyronine, 100 IU·mL^{-1} penicillin and 100 IU·mL^{-1} streptomycin; ~15 mL per heart). After 10–30 min at 37°C equilibrated with 95% O_2/5% CO_2, cardiomyocytes were plated in 9.5 cm^2 TC dishes (Corning, NY, USA) coated with laminin. After 2 h in incubator (37°C, 5% CO_2) in 2 mL culture medium the culture medium was changed to remove non-attached cells and cell debris. After change of medium, nearly all cells attached to laminin were rod-shaped cardiomyocytes. The cardiomyocytes were incubated overnight for experiments the following day. Protein content within experiments was rather similar in each well. In different experiment, mean protein content per well varied between 400 and 800 μg. For laminin coating, dishes were treated with 500 μL 10 μg·mL^{-1} laminin dissolved in Medium 199 for 1 h.

Prior to experiments, cardiomyocytes were preincubated for 2 h in 2 mL buffer containing 120 mM NaCl, 3.3 mM KCl, 1.2 mM KH_2PO_4, 24 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% BSA, 5.5 mM D-glucose, pH 7.4 (PB). In experiments, cardiomyocytes were incubated for 15 min in buffer as above with or without insulin (10 000 μU·mL^{-1}), isoprenaline (10^{-6} M), dobutamine (10^{-6} M) or salbutamol (10^{-6} M). In experiments with cAMP analogues and MEK inhibitor (PD98,059), substances were added after 90 min of preincubation. Cardiomyocytes were therefore incubated for 30 min with PD98,059 (50 μM) or 0.5 mM of the cAMP analogues (db-cAMP, N^6-benzoyl cAMP or 007) prior to the 15 min incubation with insulin (10 000 μU·mL^{-1}) unless otherwise stated in legends. In experiments with the PDE4 inhibitor rolipram (1 μM) the inhibitor was added after 105 min of preincubation. Cardiomyocytes were therefore incubated with rolipram for 15 min before insulin (10 000 μU·mL^{-1}) and salbutamol (10^{-6} M) were added for 15 min. PD98,059 and rolipram were dissolved in DMSO; in these experiments, 0.1% DMSO was included in wells.

Western blot analysis

Cardiomyocytes were scraped off the wells in 250 or 350 μL·well^{-1} of ice-cold lysis buffer containing 10 mM $NaPO_4$ buffer pH 7.2, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS (w/v), 0.2 mM Na_3VO_4 and 2 μL·mL^{-1} of protease inhibitor cocktail (P8340; Sigma). The lysates were transferred to microtubes and rotated for 45 min at 4°C. After centrifugation (11 600× *g*; 15 min; 4°C) protein concentration was determined in the supernatant (DC Protein Assay, Bio-Rad, Hercules, CA, USA) with protein standard from Sigma (P8119). Lysates were diluted to equal concentration on experimental days (1 μg·μL^{-1}) and stored at –70°C.

For Western blot, lysates were prepared with Laemmli buffer and proteins (~15 μg) were separated by electrophoresis (Mini-PROTEAN 3 #165-3315 from Bio-Rad) in 10% SDS-PAGE. A 15% SDS-PAGE was run for anti-PLB and anti-PLB Ser^{16} probing. Proteins were transferred from gel into PVDF membrane (Immobilon-P 0.45 μM, #IPVH00010 from Millipore) for 1 h at 0.25 A with ice-container in transfer buffer (25 mM Tris, 192 mM glycine and 10% methanol). Membranes were washed 3 × 10 min in PBS-T (80 mM NA_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl and 0.1% Tween-20, pH 7.4) and incubated (blocked) in 5% dried non-fat skim milk solved in PBS-T for 2 h at room temperature to minimize non-specific binding. All washing and incubation were done with gentle shaking. Membranes were washed 2 × 30 s in PBS-T before incubation overnight at 4°C with primary antibodies diluted in PBS-T with 3% BSA (w/v). Dilutions of primary antibodies varied from 1:500 to 1:40 000. After 6 × 10 min wash, membranes were incubated at room temperature for 1 h with secondary antibody diluted in PBS-T with 1% BSA (w/v). Dilutions of secondary antibodies varied from 1:20 000 to 1:40 000. After 6 × 10 min wash, membranes were incubated in ECL. Signals were detected from enhanced chemiluminescence after exposed to film (Kodak X-OMAT UV Plus Film or FUJI RX Cat. nr. 90101104, Tokyo, Japan). The films were scanned, and by using a densitometry the signals were quantified (Scion Image, Scion Corporation).

Glycogen phosphorylase activity

Cardiomyocytes were scraped off the wells in 350 μL homogenizing buffer [50 mM MES, 100 mM NaF, 5 mM EDTA and 1 mM 2-mercaptoethanol (pH 6.1)]. The cell suspension was minced in a MixerMill (Retsch, Haan, Germany) for 2 × 30 s and centrifuged (3000× *g*; 10 min; 4°C) and the supernatant frozen at –70°C. Glycogen phosphorylase activity was measured in reverse direction with 48 mM glucose 1-phosphate

and 0.5 μCi·mL^{-1} [^{14}C(U)]-glucose 1-phosphate (PerkinElmer, Shelton, CT, USA) by the filter paper method as described previously (Gilboe *et al.*, 1972; Franch *et al.*, 1999). Total phosphorylase activity was determined in the presence of 3 mM 5′-AMP in the assay buffer, phosphorylase *a* activity in the absence of AMP, and percentage of phosphorylase in the *a*-form was calculated. Total protein concentration in the supernatant was determined (DC Protein Assay) using Protein Standard (P8119, Sigma) as reference.

Statistics

Data are presented as mean ± SE. Analysis of variance was performed to investigate differences and Fishers least significant difference was used as *post hoc* test to compare different treatments. $P < 0.05$ was considered as significant.

Results

Protein kinase B Ser^{473} and Thr^{308} phosphorylation was not detectable in cardiomyocytes incubated in buffer without hormones. As expected, insulin increased phosphorylation of PKB at both Ser^{473} and Thr^{308} (Figure 1). Isoprenaline did not stimulate PKB Ser^{473} or Thr^{308} phosphorylation when present alone. Interestingly, isoprenaline increased insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation by more than threefold (Figure 1A and B). Insulin increased phosphorylation of GSK-3β at Ser^{9} (PKB phosphorylation site) and combination of insulin and isoprenaline increased GSK-3β Ser^{9} phosphorylation further supporting that PKB activity was increased (Figure 1C). Dose–response curve for insulin-stimulated PKB Thr^{308} phosphorylation in the presence and absence of isoprenaline showed that isoprenaline also had a strong effect at physiological concentrations of insulin (Figure 1F). Dose–response curve for isoprenaline-mediated PKB Ser^{473} phosphorylation showed that high physiological concentrations of isoprenaline were required to see effect on insulin-stimulated PKB phosphorylation (Figure 1G). PLB Ser^{16} phosphorylation (PKA phosphorylation site) was not detectable in basal condition and during insulin stimulation. Isoprenaline increased PLB Ser^{16} phosphorylation and insulin did not influence isoprenaline-stimulated PLB Ser^{16} phosphorylation (Figure 1). About 20% of glycogen phosphorylase was in *a*-form in basal condition, and isoprenaline increased glycogen phosphorylase activation to about 55% (Table 1). Insulin decreased isoprenaline-mediated glycogen phosphorylase activation (Table 1) and tended to decrease basal glycogen phosphorylase activation ($P = 0.091$).

Wortmannin completely blocked PKB phosphorylation in cardiomyocytes stimulated with insulin alone and combination of insulin and isoprenaline (Figure 2). In parallel with the inhibited PKB phosphorylation, wortmannin also completely blocked GSK-3β Ser^{9} phosphorylation stimulated by insulin and isoprenaline (Figure 2).

The β_1-agonist dobutamine and the β_2-agonist salbutamol were used to get indications of whether isoprenaline mediated its effect via β_1- or β_2-adrenergic receptors. Dobutamine (β_1-agonist) increased insulin-stimulated PKB phosphorylation at both Thr^{308} and Ser^{473} to similar level as isoprenaline did (Figure 3A and B). Salbutamol (β_2-agonist) also increased insulin-stimulated PKB phosphorylation but was far less potent than isoprenaline and dobutamine (Figure 3). Neither dobutamine nor salbutamol increased PKB phosphorylation in the absence of insulin. GSK-3β Ser^{9} phosphorylation was high in cardiomyocytes incubated with insulin and dobutamine supporting that PKB phosphorylation increased activity (Figure 3C). Salbutamol did not significantly activate glycogen phosphorylase or phosphorylate PLB at Ser^{16} as expected (Table 1; Figure 3D). Dobutamine, on the other hand, increased glycogen phosphorylase activation and PLB Ser^{16} phosphorylation (Table 1; Figure 3D). Adrenaline and noradrenaline increased insulin-stimulated PKB Ser^{473}, PKB Thr^{308} and GSK-3β Ser^{9} phosphorylation in a similar manner as isoprenaline (Figure 3E). Moreover, adrenaline and noradrenaline increased PLB Ser^{16} phosphorylation (Figure 3E). In the absence of insulin, adrenaline and noradrenaline did not influence PKB or GSK-3β phosphorylation (Figure 3E).

The cAMP analogue db-cAMP mimicked the effect of isoprenaline on PKB phosphorylation. Alone, db-cAMP did not increase PKB phosphorylation, but db-cAMP potentiated insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation (Figure 4A and B). GSK-3β Ser^{9} phosphorylation was also higher in cardiomyocytes incubated with db-cAMP and insulin compared with cardiomyocytes incubated with insulin alone (Figure 4C). N^{6}-cAMP, a PKA-selective cAMP analogue, also increased insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation approximately fourfold without having effect alone (Figure 4A and B). N^{6}-cAMP (0.5 mM) increased glycogen phosphorylase activation (Table 1) and caused PLB Ser^{16} phosphorylation (Figure 4D). The Epac-specific cAMP analogue 007 increased insulin-stimulated PKB phosphorylation by about 80% (Figure 4). The Epac activator (0.5 mM) did not activate glycogen phosphorylase (Table 1) or stimulate PLB Ser^{16} phosphorylation (Figure 4D) supporting that PKA was not activated.

Inhibition of PKA with Rp-8-Br-cAMPS (0.5 mM) reduced glycogen phosphorylase activation in cardiomyocytes incubated with 3×10^{-9} M isoprenaline from 32.1 ± 1.4% to 27.4 ± 1.6% ($P < 0.05$; $n = 4$ in both groups). Rp-8-Br-cAMPS also reduced PKB Ser^{473} phosphorylation in cardiomyocytes incubated with insulin and isoprenaline (Figure 5) whereas Rp-8-Br-cAMPS did not influence insulin-stimulated PKB Ser^{473} phosphorylation (Figure 5).

PDE4 is required for β_2-adrenoceptor subtype-specific signalling in cardiomyocytes (Xiang *et al.*, 2005). Furthermore, a large complex consisting of PDE4, ERK, MEK, mAKAP, Epac and PKA has been reported to exist in neonatal cardiomyocytes (Dodge-kafka *et al.*, 2005). In the present study, we used rolipram (PDE4 inhibitor) and PD98,059 (MEK inhibitor) to test the hypothesis that ERK-mediated PDE4 phosphorylation reduced the ability of β_2-adrenoceptor stimulation to increase insulin-stimulated PKB phosphorylation. Rolipram increased PKB phosphorylation when cardiomyocytes were incubated with both insulin and salbutamol (Figure 6A and B). Rolipram also increased GSK-3β Ser^{9} phosphorylation in heart cells incubated with both salbutamol and insulin (Figure 6C). Inhibition of PDE4 by rolipram did not increase PLB Ser^{16} phosphorylation in basal condition but increased PLB Ser^{16} phosphorylation when salbutamol was present

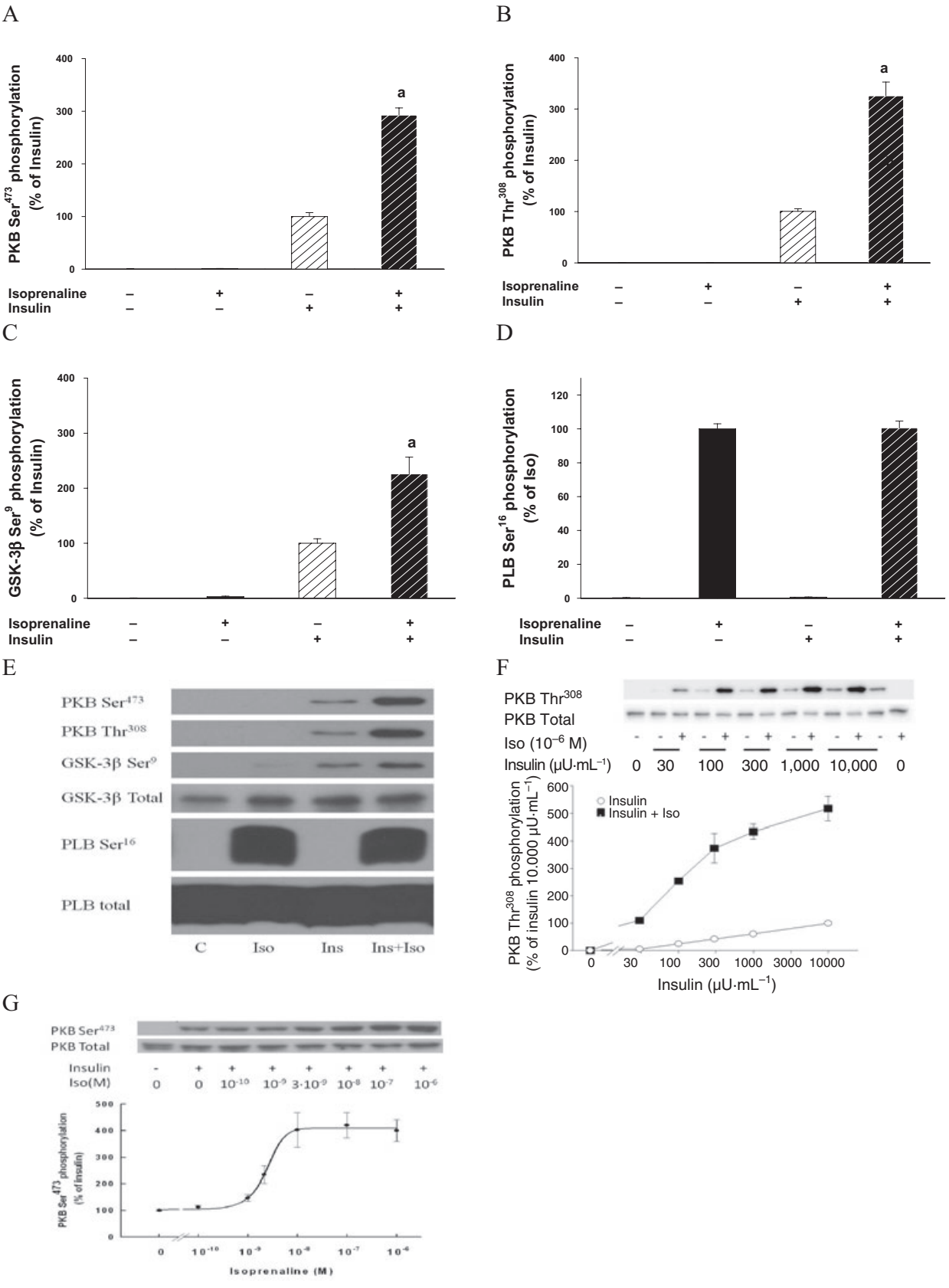

A
PKB Ser473 phosphorylation (% of Insulin)
400
300
200
100
0
a
Isoprenaline – + – +
Insulin – – + +
B
PKB Thr308 phosphorylation (% of Insulin)
400
300
200
100
0
a
Isoprenaline – + – +
Insulin – – + +
C
GSK-3β Ser9 phosphorylation (% of Insulin)
400
300
200
100
0
a
Isoprenaline – + – +
Insulin – – + +
D
PLB Ser16 phosphorylation (% of Iso)
120
100
80
60
40
20
0
Isoprenaline – + – +
Insulin – – + +
E
PKB Ser473
PKB Thr308
GSK-3β Ser9
GSK-3β Total
PLB Ser16
PLB total
C Iso Ins Ins+Iso
F
PKB Thr308
PKB Total
Iso (10−6 M) - - + - + - + - + - +
Insulin (μU·mL−1) 0 30 100 300 1,000 10,000 0
PKB Thr308 phosphorylation (% of insulin 10.000 μU·mL−1)
600
500
400
300
200
100
0
Insulin
Insulin + Iso
0 30 100 300 1000 3000 10000
Insulin (μU·mL−1)
G
PKB Ser473
PKB Total
Insulin - + + + + + + +
Iso(M) 0 0 10-10 10-9 3·10-9 10-8 10-7 10-6
PKB Ser473 phosphorylation (% of Insulin)
500
400
300
200
100
0
0 10-10 10-9 10-8 10-7 10-6
Isoprenaline (M)

Figure 1 Effect of isoprenaline and insulin on phosphorylation of protein kinase B (PKB), glycogen synthase kinase (GSK)-3β and phospholamban (PLB) in isolated cardiomyocytes. After an overnight incubation in medium, cardiomyocytes were preincubated in buffer without any hormones for 2 h prior to 15 min incubation with or without isoprenaline (10^{-6} M) in the absence or presence of insulin (10 000 μU·mL^{-1}). Cardiomyocytes were prepared for Western blot as described in *Methods*. (A) Effect of isoprenaline and insulin on PKB Ser^{473} phosphorylation. Graph shows means of quantified blots with insulin as 100%; data are from three different experiments; $n = 6–9$ in each group; representative blot is shown in (E). (B) Effect of isoprenaline and insulin on PKB Thr^{308} phosphorylation. Graph shows means of quantified blots with insulin as 100%; data are from three different experiments; $n = 6–7$ in each group. (C) Effect of isoprenaline and insulin on phosphorylation GSK-3β Ser^{9} phosphorylation. Graph shows means of quantified blots with insulin as 100%; data are from three different experiments; $n = 6–7$ in each group. (D) Effect of isoprenaline and insulin on PLB Ser^{16} phosphorylation. Graph shows means of quantified blots with isoprenaline as 100%; data are from four different experiments; $n = 8–9$ in each group. (E) Representative blots showing PKB Ser^{473}, PKB Thr^{308}, GSK-3β Ser^{9}, PLB Ser^{16} phosphorylation and total GSK-3β and total PLB in different treatments groups. (F) Dose–response curve for insulin-stimulated PKB Ser^{308} phosphorylation in the absence (open circles) and presence of 10^{-6} M isoprenaline (filled squares); symbols are means of quantified blots (with 10 000 μU·mL^{-1} of insulin as 100%) from three different experiments; $n = 9$ for insulin 10 000 μU·mL^{-1} and $n = 6$ for other symbols. (G) Dose–response curve for isoprenaline-mediated PKB Ser^{473} phosphorylation in the presence of 10 000 μU·mL^{-1} insulin; symbols are means of quantified blots (with insulin as 100%) from four different experiments; $n = 19$ for insulin and $n = 9–12$ for other symbols. [a]Significantly higher than insulin.

Table 1 Glycogen phosphorylase activation in cardiomyocytes after exposure to β-adrenergic receptor agonists or cAMP analogues

	Glycogen phosphorylase (% a-form)	
	(–) Insulin	*(+) Insulin*
Control	20.9 ± 0.9 (22)	18.1 ± 0.8 (12)
Isoprenaline	55.5 ± 1.4[a] (21)	42.3 ± 0.4[b] (6)
Dobutamine	39.6 ± 1.9[a,b] (12)	
Salbutamol	23.9 ± 1.2 (6)	
N^6-cAMP	45.6 ± 3.2[a] (8)	
007	22.8 ± 2.2 (6)	

Cardiomyocytes were incubated overnight in medium and preincubated for 2 h in buffer without any hormones prior to 15 min exposure to isoprenaline (10^{-6} M), dobutamine (10^{-6} M) salbutamol (10^{-6} M) and insulin (10 000 μU·mL^{-1}). Cardiomyocytes were exposed to 0.5 mM of the cAMP analogues for 45 min. Adrenaline and noradrenaline increased glycogen phosphorylase %*a* to 46.2 ± 2.4 and 49.4 ± 1.1 respectively ($n = 4$ in both groups; $P < 0.05$ compared with control).
Data are mean ± SEM. Number of samples in parentheses.
[a]Significantly higher than control and salbutamol.
[b]Significantly lower than isoprenaline without insulin.
007, 8-(4-chlorophenylthio)-2′-O-methyl-Camp; N^6-cAMP, N^6-benzoyl-cAMP.

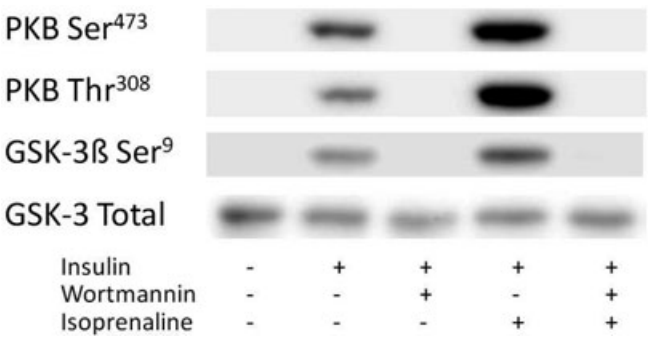


Figure 2 Wortmannin blocks phosphorylation of protein kinase B (PKB) and glycogen synthase kinase (GSK)-3β stimulated by insulin alone and in combination with isoprenaline. Representative blots for PKB Ser^{473}, PKB Thr^{308} and GSK-3β Ser^{9} phosphorylation in cardiomyocytes incubated with insulin (10 000 μU·mL^{-1}), isoprenaline (10^{-6} M) and wortmannin as indicated. After an overnight incubation in medium, cardiomyocytes were preincubated in buffer without any hormones for 2 h with 1 μM wortmannin added after 105 min. After preincubation (and 15 min incubation with wortmannin) insulin and isoprenaline was added for 15 min.

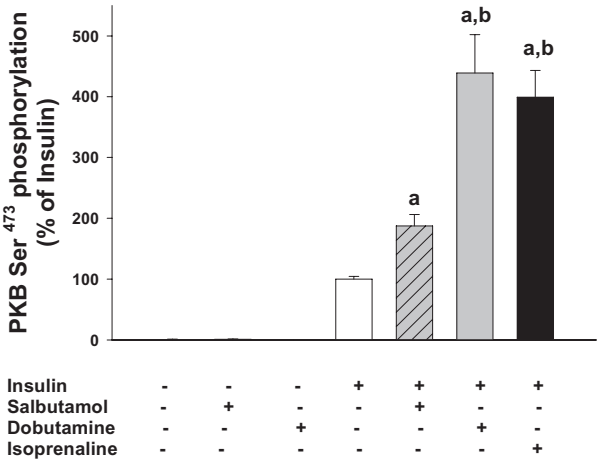
(Figure 6D). Rolipram did not influence basal or insulin-stimulated PKB phosphorylation. Inhibition of MEK by PD98,059 increased PKB Ser^{473}, PKB Thr^{308} and GSK-3 Ser^{9} phosphorylation in cardiomyocytes when both insulin and salbutamol were present (Figure 7A–C). PD98,059 also increased salbutamol-mediated PLB Ser^{16} phosphorylation (Figure 7D) supporting that PKA became activated. Basal and insulin-stimulated PKB Ser^{473} phosphorylation was not influenced by PD98,059.

Discussion

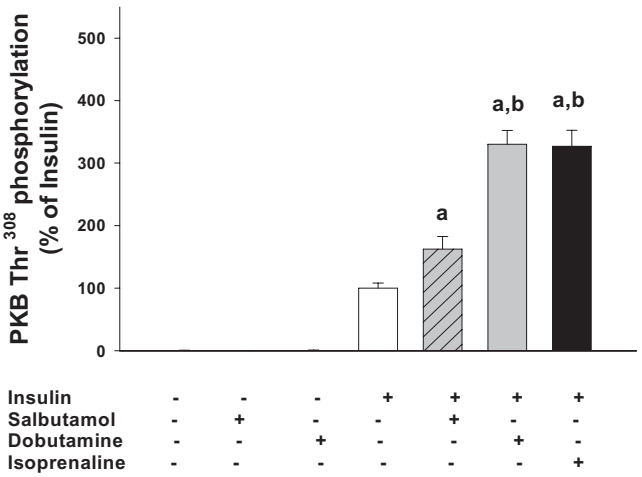
A novel and important finding in the present study was that stimulation of β-adrenergic receptors increased insulin-stimulated PKB phosphorylation in cardiomyocytes. Interestingly, stimulation of β_1-adrenergic receptors increased insulin-stimulated PKB phosphorylation (>300%) much more than stimulation of β_2-adrenergic receptor (~80%). Furthermore, the cAMP analogues activating PKA (db-cAMP and N^6-cAMP) increased insulin-stimulated PKB as much as isoprenaline. In addition, the Epac-specific cAMP analogue 007 increased insulin-stimulated PKB phosphorylation although to lesser extent. Our findings couples for the first time β-adrenoceptor-cAMP-PKA signalling to stimulation of PKB phosphorylation in cardiomyocytes and indicates the need to reconsider the role of β-adrenergic receptor in the regulation of PKB. In particular, studies of β-adrenoceptor signalling in heart need to take into account the potential synergistic crosstalk with other hormones.

Insulin is considered a strong activator of PKB (Bertrand *et al.*, 2008) and it is therefore notable that stimulation of β-adrenoceptors increased insulin-stimulated PKB Ser^{473} and Thr^{308} phosphorylation by three- to fourfold. Isoprenaline also increased insulin-stimulated GSK-3β Ser^{9} phosphorylation supporting that PKB activity was increased, and our data suggest that β-adrenoceptors are powerful regulators of PKB when insulin is present. PKB has a central role for regulation of cardiac function and it has been shown that overexpression of constitutively activated PKB increases heart size and causes dilated myopathy (Condorelli *et al.*, 2002; Matsui *et al.*, 2002) whereas deletion of PKBα decreases heart size (Debosch *et al.*, 2006). Furthermore, PKB protects the hearts during ischaemia–reperfusion (Matsui *et al.*, 2002) and regulates

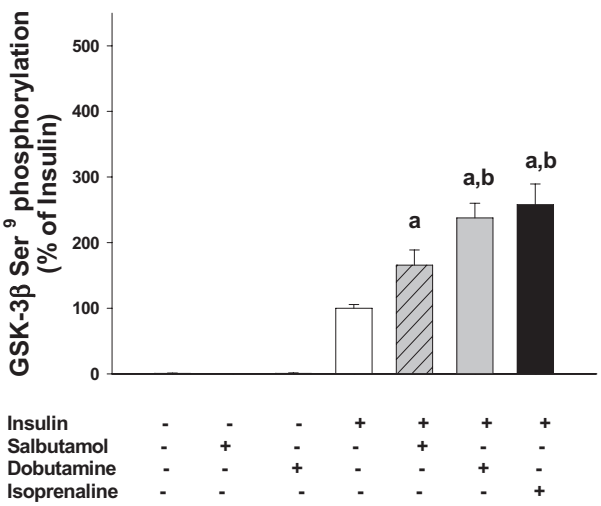
A


PKB Ser 473 phosphorylation (% of Insulin)
500
400
300
200
100
0
a
a,b
a,b
Insulin - - - + + + +
Salbutamol - + - - + - -
Dobutamine - - + - - + -
Isoprenaline - - - - - - +

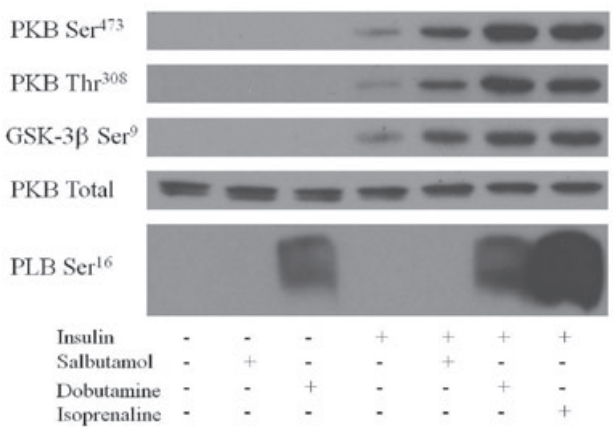
B


PKB Thr 308 phosphorylation (% of Insulin)
500
400
300
200
100
0
a
a,b
a,b
Insulin - - - + + + +
Salbutamol - + - - + - -
Dobutamine - - + - - + -
Isoprenaline - - - - - - +

C


GSK-3β Ser 9 phosphorylation (% of Insulin)
500
400
300
200
100
0
a
a,b
a,b
Insulin - - - + + + +
Salbutamol - + - - + - -
Dobutamine - - + - - + -
Isoprenaline - - - - - - +

D


PKB Ser473
PKB Thr308
GSK-3β Ser9
PKB Total
PLB Ser16
Insulin - - - + + + +
Salbutamol - + - - + - -
Dobutamine - - + - - + -
Isoprenaline - - - - - - +

E

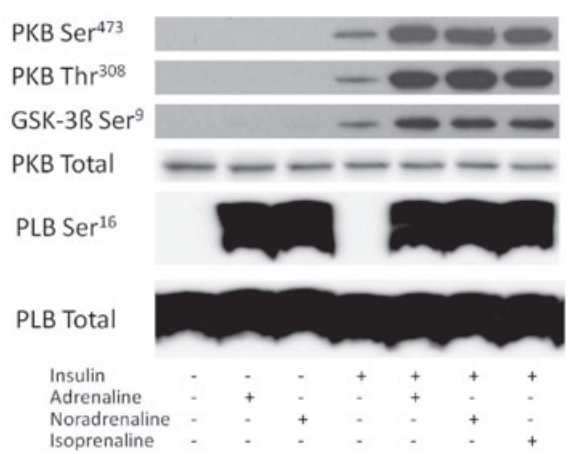

PKB Ser473
PKB Thr308
GSK-3ß Ser9
PKB Total
PLB Ser16
PLB Total
Insulin - - - + + + +
Adrenaline - + - - + - -
Noradrenaline - - + - - + -
Isoprenaline - - - - - - +

Figure 3 Effect of dobutamine (β_1-agonist), salbutamol (β_2-agonist), isoprenaline, adrenaline and noradrenaline on insulin-stimulated protein kinase B (PKB), glycogen synthase kinase (GSK)-3β and phospholamban (PLB) phosphorylation. After an overnight incubation in medium, cardiomyocytes were preincubated in buffer without any hormones for 2 h prior to 15 min incubation with salbutamol (10^{-6} M), dobutamine (10^{-6} M), isoprenaline (10^{-6} M), adrenaline (10^{-6} M) and noradrenaline (10^{-6} M) in the absence or presence of insulin (10 000 μU·mL^{-1}). Graphs show means of quantified blots with insulin as 100%; representative blots are shown in (D). (A) Effect of dobutamine, salbutamol and isoprenaline on insulin-stimulated PKB Ser^{473} phosphorylation. Data are from five different experiments; $n = 20$ for insulin and $n = 10$–14 for other groups. (B) Effect of dobutamine, salbutamol and isoprenaline on insulin-stimulated PKB Thr^{308} phosphorylation. Data are from five different experiments; $n = 18$ for insulin and $n = 8$–11 for other groups. (C) Effect of dobutamine, salbutamol and isoprenaline on insulin-stimulated GSK-3β Ser^{9} phosphorylation. Data are from five different experiments; $n = 17$ for insulin and $n = 8$–11 for other groups. (D) Representative blots showing PKB Ser^{473}, PKB Thr^{308}, GSK-3β Ser^{9} and PLB Ser^{16} phosphorylation and total PKB in different treatments groups. (E) Representative blots showing PKB Ser^{473}, PKB Thr^{308}, GSK-3β Ser^{9} and PLB Ser^{16} phosphorylation in cardiomyocytes incubated with adrenaline (10^{-6} M) and noradrenaline (10^{-6} M) alone or in combination with insulin. [a]Significantly higher than insulin; [b]Significantly higher than insulin + salbutamol.

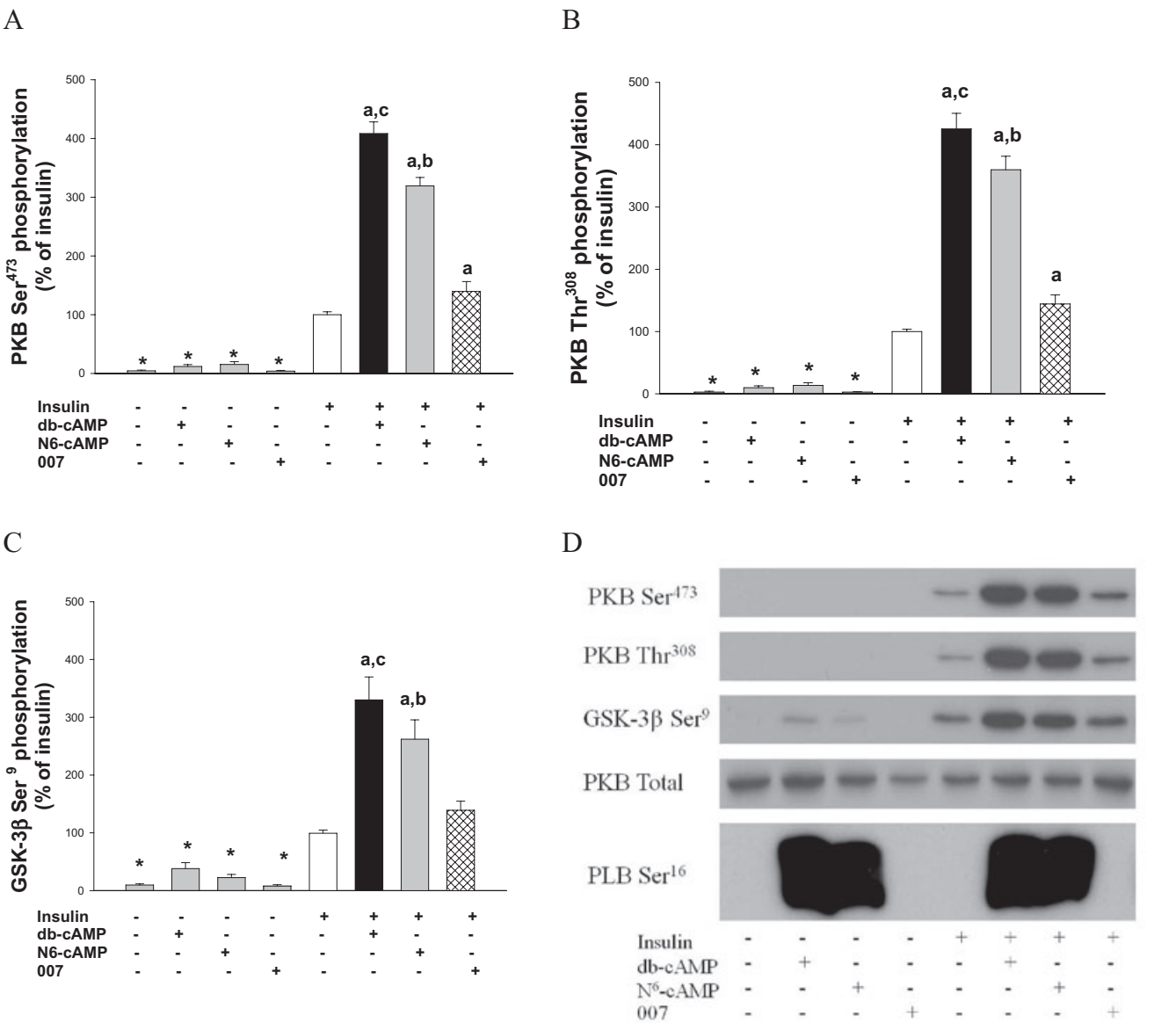


Figure 4 Effect of cAMP analogues on phosphorylation of protein kinase B (PKB), glycogen synthase kinase (GSK)-3β and phospholamban (PLB). After an overnight incubation in medium, cardiomyocytes were preincubated in buffer without any hormones for 2 h with cAMP analogues (0.5 mM) added after 90 min. After preincubation (and 30 min incubation with cAMP analogues) insulin was added for 15 min. Graphs show means of quantified blots with insulin as 100%; representative blots are shown in (D). (A) Effect of cAMP analogues on PKB Ser^{473} phosphorylation in the absence or presence of 10 000 μU·mL^{-1} insulin. Data are from five different experiments; $n = 19$ for insulin and $n = 10$ for other groups. (B) Effect of cAMP analogues on PKB Thr^{308} phosphorylation in the absence or presence of 10 000 μU·mL^{-1} insulin. Data are from five different experiments; $n = 19$ for insulin and $n = 10$ for other groups. (C) Effect of cAMP analogues on GSK-3β Ser^{9} phosphorylation in the absence or presence of 10 000 μU·mL^{-1} insulin. Data are from five different experiments; $n = 27$ for insulin and $n = 10$ for other groups. (D) Representative blots showing PKB Ser^{473}, PKB Thr^{308}, GSK-3β Ser^{9} and PLB Ser^{16} phosphorylation and total PKB in different treatments groups. [a]Significantly higher than insulin; [b]Significantly higher than insulin + 007; [c]Significantly higher than insulin + N^6-cAMP. *Significantly lower than insulin. 007, 8-(4-chlorophenylthio)-2′-O-methyl-cAMP; db-cAMP, dibutyryl-cAMP; N^6-cAMP, N^6-benzoyl-cAMP.

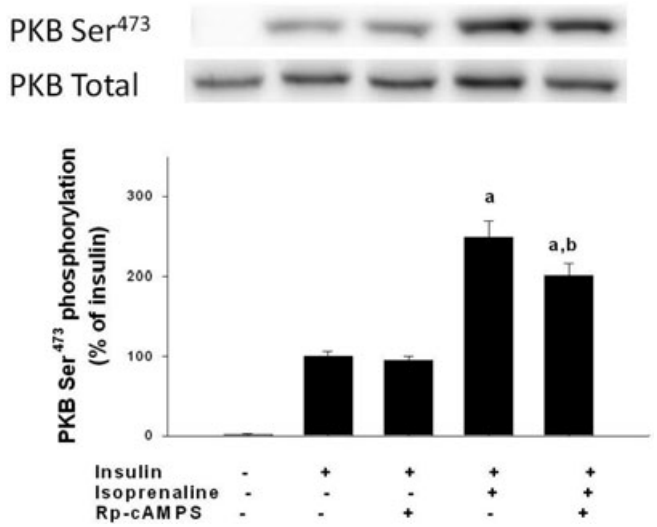


Figure 5 The protein kinase A (PKA) inhibitor Rp-8-Br-cAMPS reduces PKB Ser^{473} phosphorylation in cardiomyocytes incubated with insulin and isoprenaline. After an overnight incubation in medium, cardiomyocytes were preincubated in buffer for 3 h with Rp-8-Br-cAMPS (0.5 mM) and without any hormones. After preincubation insulin (10 000 μU·mL^{-1}) and isoprenaline (3×10^{-9} M) was added for 15 min. Data are from three different experiments; $n = 6–7$ for all groups. Representative blots for PKB Ser^{473} and total PKB are shown above the graph. [a]Significantly higher than insulin; [b]Significantly lower than insulin + isoprenaline.

other functions in the heart (Shiojima and Walsh, 2006). Although we mainly used GSK-3 Ser^{9} phosphorylation as a readout of PKB activity, it is worth to note that GSK-3 *per se* is considered as a key hypertrophic signalling molecule in the heart (Sugden *et al.*, 2008). Taken together, our data show that stimulation of β-adrenoceptors, in the presence of insulin, strongly increases phosphorylation of the two hypertrophic signalling molecules PKB and GSK-3.

Isoprenaline alone did not significantly increase PKB phosphorylation in cardiomyocytes from adult rats. Previously, β-adrenergic stimulation has been reported to increase PKB Ser^{473} phosphorylation in neonatal cardiomyocytes (Chesley *et al.*, 2000; Morisco *et al.*, 2005) and in H9c2 cardiomyocytes (Yano *et al.*, 2007). However, β-adrenoceptor-mediated PKB activation is low compared with IGF (Chesley *et al.*, 2000) and β-adrenoceptors are normally not considered as an important regulator of PKB in the heart (Shiojima and Walsh, 2006). Indeed, *in vivo* injection of isoprenaline has also shown to increase PKB phosphorylation in adult rats (Tseng *et al.*, 2005) and mice (Oudit *et al.*, 2003); *in vivo* insulin will always be present and it is possible that the effect of isoprenaline represents a potentiation of insulin action. Leblais *et al.* (Leblais *et al.* 2004) have also reported that stimulation of β_1-adrenoceptors increase PI 3-kinase activity, but data on PKB were not reported. Even though these data seem to contradict the finding that β-adrenoceptor stimulation did not increase PKB phosphorylation in the absence of insulin, we have previously reported similar effect of β-adrenergic stimulation on PKB phosphorylation in skeletal muscles: adrenaline had no effect in the absence of insulin but potentiated insulin-stimulated PKB phosphorylation and activity (Brennesvik *et al.*, 2005; Jensen *et al.*, 2008). In the present study, adrenaline and noradrenaline increased insulin-stimulated PKB phosphorylation as efficiently as isoprenaline, supporting a physiological role of the sympathoadrenal system in the regulation of PKB in the heart.

Stimulation of β_1-adrenoceptors increased insulin-stimulated PKB phosphorylation much more than stimulation of β_2-adrenoceptors. This was not expected since β_2-adrenoceptor stimulation, previously has been coupled to PKB activation (Chesley *et al.*, 2000; Oudit *et al.*, 2003; Tseng *et al.*, 2005), but not β_1-adrenoceptor stimulation. It is well documented that β_1- and β_2-adrenoceptors have different signalling mechanism in cardiomyocytes from adult rats (Steinberg, 1999; Pavoine and Defer, 2005; Zheng *et al.*, 2005). In agreement with these studies, we found that stimulation of β_1-adrenergic receptors agonist dobutamine increased glycogen phosphorylase activation and PLB Ser^{16} phosphorylation whereas salbutamol did not significantly activate glycogen phosphorylase and phosphorylates PLB Ser^{16} (Kuschel *et al.*, 1999; Jo *et al.*, 2002). The physiological role of β_1- and β_2-adrenoceptors also differs and it has been reported that stimulation of β_1-adrenoceptors causes apoptosis whereas stimulation of β_2-adrenoceptors prevents apoptosis (Communal *et al.*, 1999; Zhu *et al.*, 2001; Zhu *et al.*, 2003). Because activation of PKB prevents apoptosis, it was exciting that addition of insulin made β_1-adrenoceptor stimulation to a powerful activator of PKB phosphorylation. Furthermore, β_1-adrenoceptors stimulates hypertrophy much more than β_2-adrenoceptors, and our results raise the possibility that β_1-adrenoceptors mediate hypertrophic signalling via PKB and GSK-3.

We hypothesized that β-adrenoceptors potentiated insulin-stimulated PKB phosphorylation via cAMP; the hypothesis was supported by the fact that db-cAMP increased insulin-stimulated PKB phosphorylation. To further dissect the signalling pathway we used the PKA-specific cAMP analogues N^6-cAMP, which strongly increased insulin-stimulated PKB phosphorylation without having effect alone and therefore mimicked the effect of isoprenaline on PKB phosphorylation. Moreover, the cAMP analogue Rp-8-Br-cAMPS, which prevents PKA activation, reduced PKB Ser^{473} phosphorylation in cardiomyocytes incubated with insulin and isoprenaline. These data show for the first time that cAMP increases PKB phosphorylation via PKA in heart. Both PKB and cAMP signalling has previously been reported to mediate dilated cardiomyopathy (Lehnart *et al.*, 2005; Shiojima and Walsh, 2006). Our findings now couples cAMP and PKA signalling to phosphorylation of PKB, and links the two hypertrophic signalling pathways in heart that were previously regarded as independent.

The Epac-specific cAMP analogue 007 also increased insulin-stimulated PKB phosphorylation in heart, but less than db-cAMP and N^6-cAMP. The fact that 007 did not stimulate PLB Ser^{16} phosphorylation or activate glycogen phosphorylase supports that the effect was specific for Epac, and our data suggest that both PKA and Epac increase insulin-stimulated PKB phosphorylation. In skeletal muscles, we reported that 007 increased insulin-stimulated PKB phosphorylation (Brennesvik *et al.*, 2005), and suggested that the effect was mediated via Epac only because the PKA inhibitor H89 further increased the adrenaline-mediated potentiation of

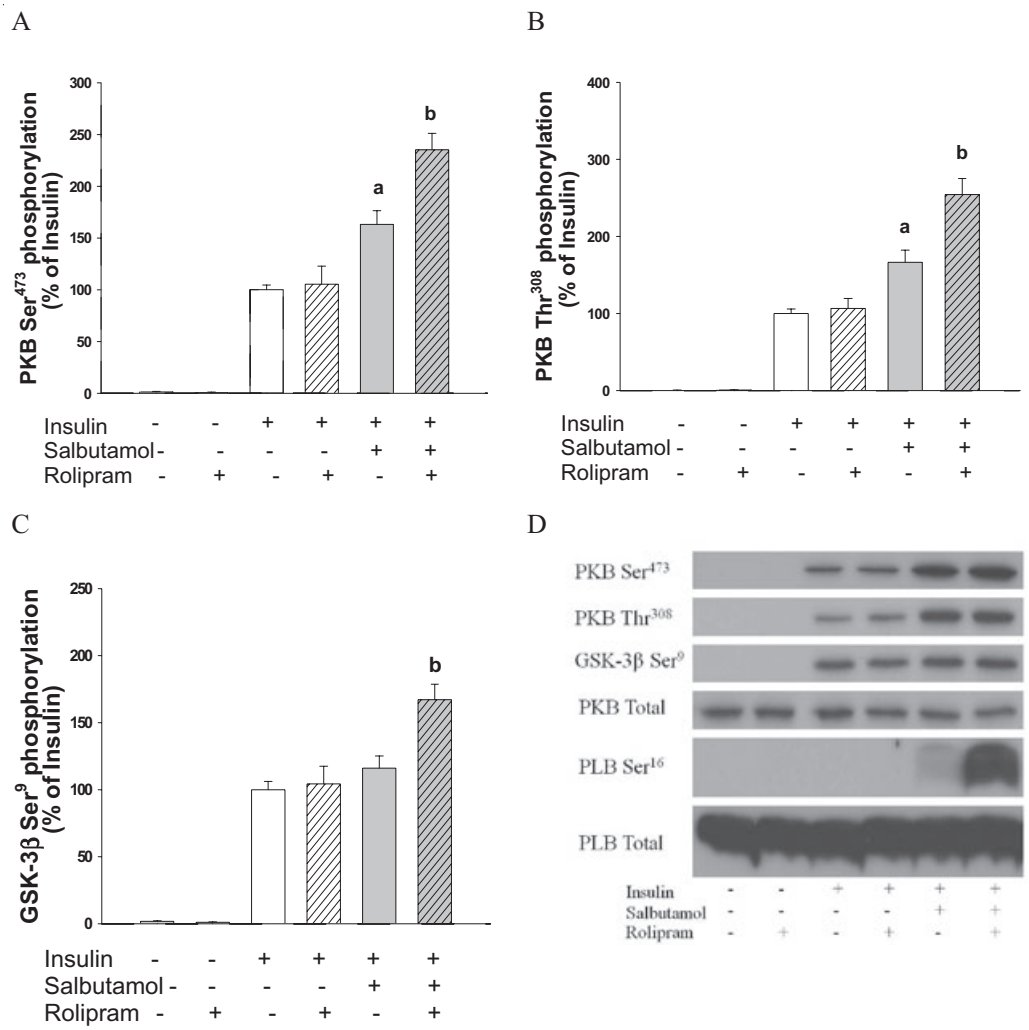


Figure 6 Rolipram increases phosphorylation of protein kinase B (PKB), glycogen synthase kinase (GSK)-3β and phospholamban (PLB) in cardiomyocytes incubated with salbutamol and insulin. After an overnight incubation in medium, cardiomyocytes were preincubated in buffer without any hormones for 2 h with 1 μM rolipram added after 105 min. After preincubation (and 15 min incubation with 1 μM rolipram) insulin (10 000 $\mu U \cdot mL^{-1}$) and salbutamol (10^{-6} M) were added for 15 min. Graph shows means of quantified blots with insulin as 100%; representative blot is shown in (D). (A) Effect of rolipram on insulin-stimulated PKB Ser^{473} phosphorylation in the absence or presence of salbutamol. Data are from four different experiments; n = 16 for insulin and n = 7–15 for other groups. (B) Effect of rolipram on insulin-stimulated PKB Thr^{308} phosphorylation in the absence or presence of salbutamol. Data are from six different experiments; n = 22 for insulin and n = 9–12 for other groups. (C) Effect of rolipram on insulin-stimulated GSK-3β Ser^{9} phosphorylation in the absence or presence of salbutamol. Data are from six different experiments; n = 24 for insulin and n = 9–12 for other groups. (D) Representative blots showing PKB Ser^{473}, PKB Thr^{308}, GSK-3β Ser^{9} and PLB Ser^{16} phosphorylation and total GSK-3β and PLB in different treatments groups. [a]Significantly higher than insulin; [b]Significantly higher than insulin + salbutamol.

insulin-stimulated PKB phosphorylation. However, H89 is a rather unspecific inhibitor for PKA (Lochner and Moolman, 2006), and we have later seen that N^6-cAMP increases insulin-stimulated PKB phosphorylation in soleus muscles (J. Jensen, unpublished). Therefore, we suspect that H89 influences PKB phosphorylation via mechanisms independent of PKA and there are no contradictions between the studies. Our data suggest that activation of both PKA and Epac can increase insulin-stimulated PKB phosphorylation in cardiomyocytes from adult rats. However, Epac seems much less effective than PKA.

The mechanism for PKA-mediated PKB phosphorylation is not obvious, but requires PI 3-kinase activation as wortmannin completely blocked PKB and GSK-3 phosphorylation in cardiomyocytes stimulated with insulin and isoprenaline. A possibility is that β-adrenoceptor stimulation increased insulin-stimulated PI 3-kinase activity, but this does not occur in skeletal muscles where stimulation of β-adrenoceptors also potentiated insulin-stimulated PKB and GSK-3 phosphorylation (Brennesvik *et al.*, 2005; Jensen *et al.*, 2007; Jensen *et al.*, 2008). Therefore, it is also likely that stimulation of β-adrenoceptors regulate other components in the PI 3-kinase signalling pathway such as PTEN, PDK1, mTORC2 or the phosphatase that dephosphorylates PKB; this idea is supported by a recent study showing that inhibition of PKA attenuated PDGF-mediated PIP_3 accumulation independent of PI 3-kinase activity in fibroblasts (Deming *et al.*, 2008). It is also possible that β-adrenoceptor

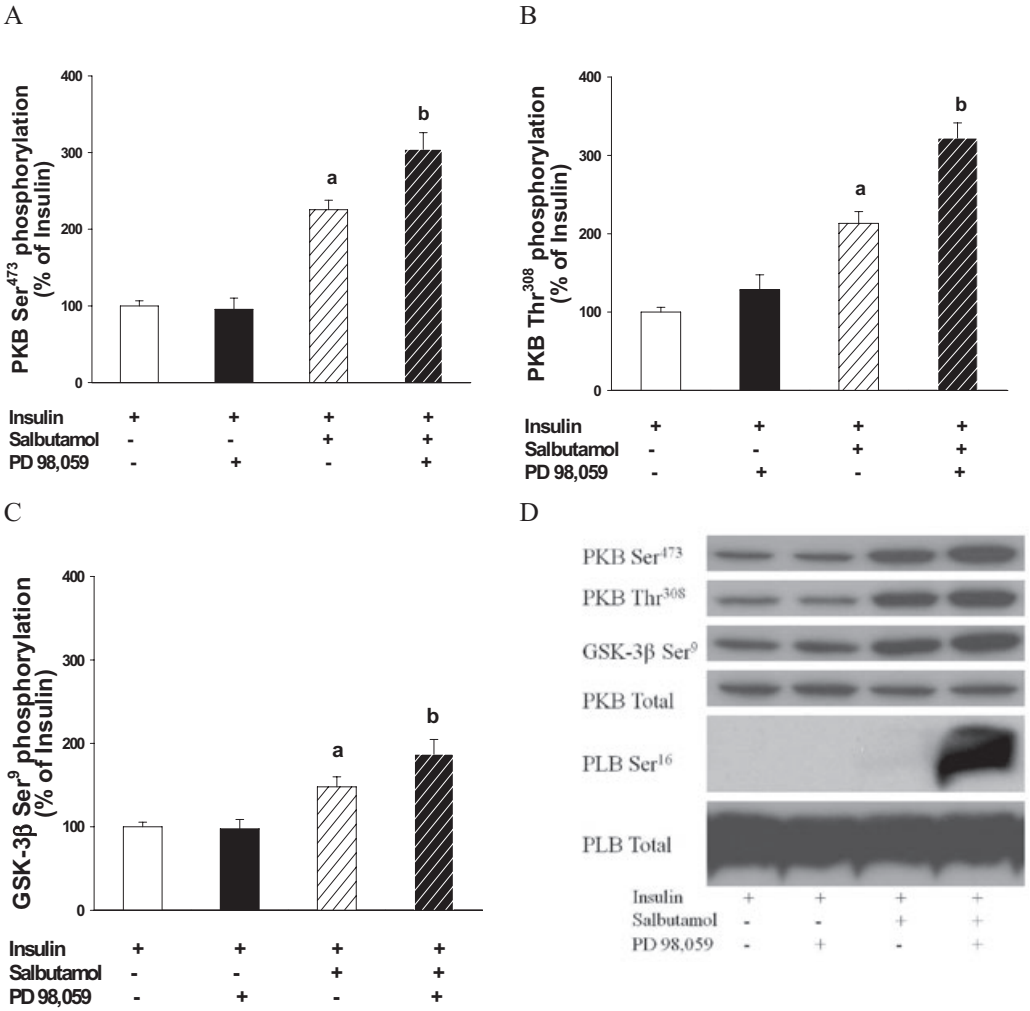


Figure 7 The MEK inhibitor PD98,059 increases phosphorylation of protein kinase B (PKB), glycogen synthase kinase (GSK)-3β and phospholamban (PLB) in cardiomyocytes incubated with salbutamol and insulin. After an overnight incubation in medium, cardiomyocytes were preincubated in buffer without any hormones for 2 h with 50 μM PD98,059 added after 90 min. After preincubation (and 30 min incubation with 50 μM PD98,059) insulin (10 000 $\mu U \cdot mL^{-1}$) and salbutamol (10^{-6} M) were added for 15 min. Graph shows means of quantified blots with insulin as 100%; representative blot is shown in (D). (A) Effect of PD98,059 on insulin-stimulated PKB Ser^{473} phosphorylation in the absence or presence of salbutamol. Data are from three different experiments; $n = 10$ for insulin and $n = 4–6$ for other groups. (B) Effect of PD98,059 on insulin-stimulated PKB Thr^{308} phosphorylation in the absence or presence of salbutamol. Data are from five different experiments; $n = 16$ for insulin and $n = 7–10$ for other groups. (C) Effect of PD98,059 on insulin-stimulated GSK-3β Ser^{9} phosphorylation in the absence or presence of salbutamol. Data are from four different experiments; $n = 14$ for insulin and $n = 6–8$ for other groups. (D) Representative blots showing PKB Ser^{473}, PKB Thr^{308}, GSK-3β Ser^{9} and PLB Ser^{16} phosphorylation and total PKB and PLB in different treatments groups. [a]Significantly higher than insulin; [b]Significantly higher than insulin + salbutamol.

stimulation activates other kinases, like DNA-PK, PKCβ2 or integrin-linked kinase, which have been reported to phosphorylate PKB Ser^{473} in some cell types (Persad *et al.*, 2001; Kawakami *et al.*, 2004; Huston *et al.*, 2008). Although the present study does clarify the mechanisms by which β-adrenoceptor stimulation increases insulin-stimulated PKB phosphorylation in cardiomyocytes, our data show that the PI 3-kinase activation is involved.

PDE4 is required for β_2-adrenoceptor subtype-specific signalling in cardiomyocytes (Xiang *et al.*, 2005). ERK phosphorylates most PDE4 isoforms and phosphorylation decreases phosphodiesterase activity of the long isoforms whereas activity of most of the short isoforms increases (Mackenzie *et al.*, 2000; Houslay *et al.*, 2005). In the present study, we used rolipram (PDE4 inhibitor) and PD98,059 (MEK inhibitor) to test the hypothesis ERK-mediated PDE4 phosphorylation reduced the ability of β_2-adrenoceptor stimulation to increase insulin-stimulated PKB phosphorylation. Interestingly, inhibition of PDE4 (rolipram) or MEK (PD98,059) increased the effect of salbutamol on insulin-stimulated PKB phosphorylation. Rolipram and PD98,059 also increased PLB Ser^{16} when salbutamol was present supporting that PKA became activated. These data also support that stimulation of β_2-adrenoceptors activate adenylyl cyclase in adult rat cardiomyocytes, but the produced cAMP is broken down immediately by PDE4 in a process that requires ERK activation. Unfortunately, the present study does not determine which isoforms of ERK and PDE4 that mediate this effect in adult cardiomyocytes. Compartmentalized β_2-adrenergic signalling involves PDE4 in large protein complexes (Baillie and

Houslay, 2005) and Dodge-Kafka *et al*. recently reported a large complex consisting of PDE4, ERK, MEK, mAKAP, Epac and PKA in neonatal cardiomyocytes (Dodge-kafka *et al*., 2005). The data in the present study support that ERK-mediated phosphorylation of PDE4 contribute to compartmentalization of β_2-adrenoceptor signalling in adult cardiomyocytes and reduce PKA-mediated potentation of insulin-stimulated PKB phosphorylation.

A clear limitation of the present study is that we do not describe a complete signalling pathway for the increase in insulin-stimulated PKB phosphorylation during stimulation of β-adrenoceptors. However, the present study for the first time report that cAMP-mediated PKA activation increases PKB phosphorylation in cardiomyocytes. It is also a limitation that we do not report a physiological role of β-adrenoceptor-mediated potentation of insulin-stimulated PKB phosphorylation. However, the fact that isoprenaline-mediated PKB phosphorylation depends on insulin in cardiomyocytes highlights that insulin should be included in studies with β-adrenoceptor agonist, and it would be particular interesting to investigate whether stimulation of β_1-adrenoceptors still causes apoptosis in cardiomyocytes when insulin is present.

The present study also opens new perspectives. Defective regulation of both PKB and cAMP signalling have been coupled to cardiac hypertrophy (Zheng *et al*., 2005), and the crosstalk between cAMP-PKA and PKB now couples these two pathways. cAMP signalling has, to the best of our knowledge, not been coupled to PI3Kγ activation, and it is therefore appealing to speculate that cAMP-mediated PKB phosphorylation may involves class I A PI 3-kinase. Crackower *et al*. (Crackower *et al*. 2002) reported that cardiac specific deletion of PTEN induced cardiac hypertrophy via PI3Kα and elevated PKB phosphorylation, and it is possible that combined activation of insulin and β-adrenoceptor signalling may induce cardiac hypertrophy via this signalling pathway. Metabolic syndrome is associated with increased sympathetic nervous activity and hyperinsulinemic and it may be attractive to hypothesize that this interaction between insulin and β-adrenoceptor signalling contributes to the increased risk for development of heart failure in type 2 diabetes (Ashrafian *et al*., 2007). Moreover, as PKB activation improves recovery from ischaemia–reperfusion (Matsui *et al*., 2001), it would also be relevant to investigate whether combination of insulin and β-adrenoceptor will improve recovery from ischaemia. The crosstalk mechanisms between insulin and β-adrenoceptors in the regulation of PKB should be fully characterized in the heart, as they may represent novel targets for treatment of cardiac diseases.

In conclusion, β-adrenoceptors are powerful regulators of PKB phosphorylation in the presence of insulin. The potentiation of insulin-stimulated PKB phosphorylation occurs via cAMP and PKA, and stimulation of β_1-adrenergic receptors increased insulin-stimulated PKB phosphorylation much more than β_2-adrenergic receptor stimulation. Stimulation of β_2-adrenoceptors activates adenylyl cyclase but co-activation of PDE4 prevents cAMP accumulation and PKA activation in adult rat cardiomyocytes. Moreover, the crosstalk between insulin and β-adrenergic receptors in cardiomyocytes show that it is important to study β-adrenergic signalling in the presence of insulin or other growth factors.

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Conflicts of interest

The authors declare no conflicts of interest.

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