Novel mechanisms of the regulation of Protein kinase B in adipocytes; implications for Protein kinase A, Epac, Phosphodiesterases 3 and 4

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Abstract

Crosstalk between insulin and cAMP signalling pathways has a great impact on adipocyte metabolism. Whilst Protein kinase B (PKB) is a pivotal mediator of insulin action, in some cells regulation of PKB by cAMP has also been demonstrated. Here we provide evidence that, in a phosphatidyl inositol 3-kinase dependent manner, β3-adrenergic stimulation (using CL316243) in adipocytes induces PKB phosphorylation in the absence of insulin and also potentiates insulin-induced phosphorylation of PKB. Interestingly, insulin- and CL316243-induced PKB phosphorylation was found to be inhibited by pools of cAMP controlled by PDE3B and PDE4 (mainly in the context of insulin), whereas a cAMP pool controlling protein kinase A appeared to mediate stimulation of PKB phosphorylation (mainly in the context of CL316243). Furthermore, an Epac (exchange protein directly activated by cAMP) agonist (8-pCPT-2'-O-Me-cAMP) mimicked the effect of the PDE inhibitors, giving evidence that Epac has an inhibitory effect on PKB phosphorylation in adipocytes. Further, we put the results obtained at the level of PKB in the context of possible downstream signalling components in the regulation of adipocyte metabolism. Thus, we found that overexpression of PKB induced lipogenesis in a PDE3B-dependent manner. Furthermore, overexpression or inhibition of PDE3B was associated with reduced or increased phosphorylation of the key lipogenic enzyme acetyl-CoA carboxylase (ACC), respectively. These PDE3B-dependent effects on ACC correlated with changes in lipogenesis. The Epac agonist, 8-pCPT-2'-O-Me-cAMP, mimicked the effect of PDE3B inhibition on ACC phosphorylation and lipogenesis.

1. Introduction

In adipocytes, cAMP and insulin signalling pathways frequently interact to regulate metabolic functions such as glucose uptake, lipogenesis and lipolysis [1, 2]. cAMP signalling can be protein kinase A (PKA)-dependent or PKA-independent. There are two isoforms of PKA, PKA I and II, which are ubiquitously expressed and play a crucial role in cell metabolism, growth and other functions, through phosphorylation of numerous target proteins [3]. PKAs were thought to be the major effectors of cAMP until the discovery of Epacs, guanine nucleotide exchange factors directly activated by cAMP, which have been shown to activate the small G proteins Rap1 and Rap2 [3]. Epac1 has one and Epac2 has two binding sites for cAMP. Epac1 is ubiquitously expressed whereas Epac2 is predominant in the neuroendocrine and endocrine tissues. [4]. Epacs have been shown to affect diverse cellular functions including hormone/ transmitter secretion and cell adhesion [3, 4].

Cellular gradients of cAMP are generated by the combined actions of adenylate cyclases and cyclic nucleotide phosphodiesterases (PDEs) [5]. Highly conserved PDEs are tissue specific and differentially distributed in cells and cellular compartments. They form a large group of enzymes consisting of eleven families (PDE1-11), characterized by different substrate affinities, biochemical and physical properties, different mechanisms whereby they are regulated, and different sensitivities to inhibitors [6, 7]. Adipocytes express mainly PDE3B and PDE4s. PDE3B, one of two members of the PDE3 family,

has been shown to participate in a complex interplay between insulin and cAMP signalling networks, important for the regulation of insulin-induced glucose uptake and lipogenesis as well as insulin-induced inhibition of lipolysis in adipocytes [2, 8]. On the other hand, little is known about the role for different PDE4 isoforms in adipocytes. In general, PDE4 consists of four subfamilies which are widely expressed and involved in the regulation various cellular functions [9]. Via interactions with different signalling scaffold proteins like β-arrestin, PDE4 has been shown to generate localized gradients of cAMP and thus to control the activity of cAMP effectors [9].

One major pathway utilized by insulin involves activation of phosphatidylinositol 3-kinase (PI3K), which through phosphoinositide-dependent kinase (PDK)1 and 2 leads to serine and threonine phosphorylation, and thereby activation of three highly homologous protein kinase B (PKB) isoforms (PKBα, β, γ) [10, 11]. Phosphorylated (at Ser 473/4 and Thr 308/9 in PKBα/β), and thereby activated, PKB in turn phosphorylates proteins involved in many vital processes [11-13]. In adipocytes, PKB plays a crucial role in insulin-induced GLUT-4 translocation to the plasma membrane [14] and protein synthesis [15]. It also appears to be involved in the antilipolytic action of insulin through phosphorylation and activation of PDE3B [16, 17].

In addition to its role in insulin-induced signalling pathways, PKB in adipocytes has also been shown to be activated in response to isoproterenol, a cAMP-increasing β-adrenergic receptor agonist [18]. However, mechanisms whereby this activation occurs are not yet understood. When it comes to other cell types, both PKA and Epac have been suggested to mediate cAMP-induced activation of PKB; PKA in COS cells [19], Epac in HEK cells [20], and Epac in the presence of insulin, in skeletal muscle [21]. In skeletal muscle, PKB

has also been shown to be negatively regulated by cAMP and PKA signalling [21]. In hepatocytes, the ability of insulin and leptin to activate PKB was potentiated by glucagon via increases in cAMP and PKA [22]. Thus, PKB seems to be a target not only for insulin action, but also for multiple cAMP signalling pathways, resulting in an increase or decrease of its kinase activity. The aim of this study was to elucidate the mechanisms whereby cAMP regulates PKB phosphorylation and its downstream effects in adipocytes, with particular focus on possible interactions between the insulin and cAMP signalling networks.

2. Materials and methods

2.1. Materials:

Adipocytes were treated with OPC 3911 (10 μM), N-cyclohexyl-n-(2-hydroxyethyl)-4-((1,2,3,4-tetrahydro-2-oxo-6-quinolinyl)oxy)butyramide, a selective and potent (IC_{50}=0.06 μM) PDE3 inhibitor [23]; RO 20-1724 (10 μM), 4-(3-Butoxy-4-methoxybenzyl)-2-imidazolidinone, a selective PDE4 inhibitor (IC_{50} values: 6 μM for PDE4A, B, D and 22 μM for PDE4C) [24]; 8-pCPT-2'-O-Me-cAMP (10 μM), 8-(4-Chlorophentylthio)-2'-O-methyl-cAMP, a high affinity Epac1 agonist (EC_{50}=2.2 μM, whereas for cAMP EC_{50}=30 μM) and a very weak activator of PKA [25]; H89 (25 μM), N-(2-(4-bromocinnamylamino)ethyl)-5-isoquinolinesulfonamide, a potent PKA inhibitor (inhibition constant of 0.048 +/- 0.008 μM) [26]; CL 316243 (30 nM): disodium(R,R)-5-

(2-((2-(3-chlorophenyl)-2-hydroxyethyl)-amino)propyl)-1,3-benzodioxole-2,3-dicarboxylate, a potent agonist specific for β3 adrenergic receptors (dissociation constant of 1 μM) [27].

2.2. Methods:

2.2.1. Preparation of adipocytes

Adipocytes were isolated from epididymal fat pads of 36-38 day-old male Sprague-Dawley rats (B&K Universal, Stockholm) and prepared as described previously [28, 29]. Lund University Ethic Committee approved the study (permission number: M170-03).

2.2.2. Preparation of adipocyte homogenates

One milliliter aliquots of 10% (v/v) adipocytes in KRH were incubated as indicated in "Results" for 10 min at 37°C. Cells were then homogenized in 1 ml of homogenization buffer (50 mM TES, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 40 mM phenylphosphate, 5 mM NaF, 1 mM dithioerythriol, 1 mM phenylmethylsulphonylfluoride, 0.05 mM sodium orthovanadate, 10 μg/ml antipain, 10 μg/ml leupeptin and 1 μg/ml pepstatin A, pH 7.4) and centrifuged at 15500 x g for 5 min at 4°C. The solidified fat was removed and the infranatant was collected.

2.2.3. SDS-PAGE and western blot analysis

Samples were mixed with Laemmli sample buffer and subjected to polyacrylamide gel electrophoresis (PAGE) (9% acrylamide) and western blot analysis. After the electrotransfer of proteins onto a polyvinylidene difluoride membrane (Millipore), membranes were blocked for 30 min with 5% milk in TBS-T buffer (50 mM Tris pH 7.5, 150 mM NaCl and 0.1% (w/v) Tween-20), and incubated overnight with a 1:5000 dilution of anti-P-Ser473 PKB antibodies (Biosource), a 1:1000 dilution of anti-P-Ser473/474 PKB antibodies (kind gift from Lars Rönnstrand), a 1:2000 dilution of anti-PKB beta antibodies (raised in rabbits against the peptide RYDSLGSLELDQRTHC and affinity purified), a 1:1000 dilution of anti-PDE3B antibodies (raised in rabbits as described, see 30), a 1:2000 dilution of anti-P-ACC or anti-ACC antibodies (Cell Signaling). After washing in TBS-T buffer membranes were incubated for 1h with 1:5000 dilution of anti-rabbit antibodies linked with Horseradish peroxidase (Biosource), washed again and developed with the 1:1 solution of Peroxide and Luminol (Pierce). The chemoluminescent signal was analyzed and quantified with use of the Fuji LAS 1000 Plus system (Fuji Photo Film, Tokyo, Japan).

2.2.4. Determination of PDE3 activity

PDE3 activity was measured as described previously [31]. Assays were performed at 30°C for 45 min.

2.2.5. Adenovirus infection

High titer adenoviral stocks (approximately 10^{10} pfu/ml) were used to infect primary rat adipocytes (2-3 ml of 12 % cells) as indicated in the figure legend. Infection was performed for 5.5 hours (AdFlag-PDE3B) or overnight (AdVPKBmyr) in DMEM supplemented with 0.5% BSA and 5% FCS at 37°C, 5% CO_2. Control virus expressing β-galactosidase (AdVβ-gal) and adenoviral vectors were kindly provided by Drs. C.N. Newgard and H. Mulder. Flag-PDE3B-expressing virus was constructed as previously described [32]. PKB myr-expressing virus (kind gift from Dr. Michael Quon from the NIH)

2.2.6. Lipogenesis assay

Lipogenesis was measured as previously described [33]. One milliliter aliquots of 2% (v/v) adipocytes in Krebs-Ringer-HEPES (KRH) buffer with low glucose (0.55 mM) and 3.5% BSA were added to vials containing 0,4 μCi D-[6-^{3}H]-glucose (Amersham) and incubated as indicated in "Results" for 1 h at 37 °C. Reactions were stopped with a toluol-based scintillation liquid containing 0.3 g/l POPOP (1, 4-bis[5-phenyl-2-oxazolyl]benzene, 2,2'-p-phenylene-bis[5-phenyloxazole]) and 5 g/l PPO (2,5-diphenyl oxazole). Incorporation of D-[6-^{3}H]-glucose into cellular lipids was measured by scintillation counting.

Data analysis

Data are expressed as mean values +/- SEM. of the indicated number of experiments. Statistical significance (p<0.05) was evaluated with use of unpaired Student's *t* test and showed as stars in the figures.

3. Results

3.1. Serine phosphorylation of PKB is regulated by a complex interplay between insulin- and cAMP dependent signalling pathways in rat adipocytes: implications for Epac, PKA, PDE3B and PDE4

As shown in Fig 1A, inhibition of PDE3B (using OPC3911) and PDE4 (using RO20-1724) resulted in lowering of insulin-induced phosphorylation of PKB at Ser 473/4 (PKB α/β) by 45% and 35% respectively. The Epac agonist, 8-pCPT-2'-O-Me-cAMP, and a PKA inhibitor, H89, also caused a decrease in insulin-induced Ser 473/4 PKB phosphorylation, by 50% and 35% respectively. These results indicate that cAMP pools regulated by PDE3B and PDE4 lead to decreased PKB phosphorylation, possibly mediated by Epac. Insulin-induced Ser 473/4 phosphorylation of PKB was also tested in the presence of CL316243, a β3-adrenergic receptor agonist. As shown in Fig 1B, CL316243 efficiently potentiated insulin-induced phosphorylation of PKB. Insulin+CL316243-induced Ser 473/4 phosphorylation of PKB was lowered in the presence of OPC3911 and RO20-1724 by 40% in each case and in the presence of H89 by almost 60%. These results suggest that cAMP exerts stimulatory, as well as inhibitory effects on PKB phosphorylation. As shown in Fig.1C, CL316243 not only potentiates insulin-induced Ser 473/4 phosphorylation of PKB but also acts in the absence of insulin.

CL316243 by iself induced a 5-fold increase in PKB phosphorylation which was decreased by almost 80% in the presence of H89, indicating an important role for PKA in this process. As was the case for insulin- [34] and insulin+CL316243-induced Ser 473/4 phosphorylation of PKB (data not shown), CL316243-induced PKB phosphorylation appeared also to require PI3K, since the effect was inhibited by wortmannin by almost 90% (Fig.1C). Although a PKA-mediated cAMP signalling pathway was dominating in the context of CL316243-induced Ser 473/4 PKB phosphorylation, as seen in Fig. 1C, it was also partly inhibited in the presence of PDE3 and PDE4 inhibitors (by 25% and 20%, respectively).

To further verify the role of PDE3B in the regulation of PKB phosphorylation we overexpressed PDE3B in rat adipocytes using an adenovirus system. After a 5.5 hour-infection, PDE3B was overexpressed about 6-7 fold in comparison to control adipocytes infected with AdVβ-gal. As shown in Fig 2, insulin-induced Ser 473/4 PKB phosphorylation was potentiated by 65% in adipocytes overexpressing PDE3B. On the other hand, no change in CL316243-induced Ser 473/4 phosphorylation of PKB was observed in adipocytes overexpressing PDE3B.

In summary, these data suggest that PDE3B mediates the regulation of Ser 473 PKB phosphorylation. However, the opposite situation also appears to occur as PKB has previously been shown to regulate PDE3B phosphorylation and activation [16, 35]. Therefore, we next investigated whether the conditions that alter Ser 473 PKB phosphorylation also influence PDE3B activity. Thus, as shown in Fig 3, the Epac agonist, which lowered insulin-induced PKB phosphorylation, prevented insulin-induced PDE3B activation. H89, which decreased insulin as well CL316243-induced PKB

phosphorylation, also inhibited insulin and CL316243-induced PDE3B activation. Hence, PKB phosphorylation and PDE3B activation seem to be tightly linked.

3.2 Complex regulation of PKB and PDEB3 by insulin and cAMP mediated pathways; implications for the regulation of lipogenesis

3.2.1 PKB mediates lipogenesis in a PDE3B dependent manner. In a previous study we showed that PDE3B plays an important role in the regulation of insulin-induced lipogenesis, presumably by controlling Epac-mediated cAMP signalling [2]. Furthermore, some other reports indicate that PKB regulates PDE3B phosphorylation and activity. Therefore, we studied PKB phosphorylation in the context of lipogenesis. PKB (constitutively active) was overexpressed in adipocytes approximately 9 fold. As shown in Fig 4, in cells overexpressing PKB, lipogenesis was increased 3-fold in comparison to control cells (overexpressing β-gal). The effect of PKB overexpression on lipogenesis was lowered in the presence of PDE3B inhibitor OPC3911 and by the Epac agonist, 8-pCPT-2'-O-Me-cAMP, by 40% and 10% respectively, suggesting that PKB has an important role in mediating lipogenesis, at least in part via PDE3B.

3.2.2 Activation of PDE3B mediates insulin-induced lowering of ACC phosphorylation. Having shown that PKB and PDE3B are involved in the regulation of insulin-induced lipogenesis, we next investigated possible downstream targets regulated by a cAMP pool controlled by PDE3B. ACC, a key lipogenic enzyme, is known to be phosphorylated and inactivated in response to cAMP-increasing hormones and by AMP kinase [36]. Hence,

we studied ACC phosphorylation under conditions known, according to a recent report [2], to influence regulation of lipogenesis. Thus, OPC3911 and the Epac agonist, 8-pCPT-2'-O-Me-cAMP, prevented insulin-induced ACC dephosphorylation and even potentiated its phosphorylation, by 30% and 10% respectively (as compared to non-stimulated cells), which is in agreement with the ability of OPC3911 and the Epac agonist to inhibit insulin-induced lipogenesis. Isporoterenol, a β-adrenergic receptor agonist, as well as CL316243, potentiated ACC phosphorylation by 40% and antagonized insulin-induced lowering of ACC phosphorylation by 35%. With regard to insulin-induced lipogenesis, isoproterenol or CL316243 did not cause any inhibition of the process; on the contrary, lipogenesis was rather observed to be potentiated [2]. On the other hand, isoproterenol in combination with OPC3911, the condition which had the strongest inhibitory effect on insulin-induced lipogenesis, also efficiently reversed insulin-induced lowering of ACC phosphorylation (Fig 5).

In order to further verify a role of ACC as a downstream component of PDE3B in the regulation of lipogenesis, cells overexpressing PDE3B (6-7 fold) using an adenovirus system were studied. Fig 6 shows that overexpression of PDE3B results in a decreased ACC phosphorylation by 35% and this effect is potentiated in the presence of insulin (up to 50%). In adipocytes overexpressing β-gal, insulin lowered ACC phosphorylation by 40%. These data provide evidence that PDE3B controls a cAMP pool which regulates ACC phosphorylation.

4. Discussion

In this work, using a number of cAMP modulating agents and other chemical tools as well as overexpression strategies, we show that insulin and cAMP signalling pathways interact to control PKB phosphorylation (at Ser473/4 in PKB α and β, respectively) and thereby activation in adipocytes. Furthermore, we suggest possible mechanisms for this interaction, which have not yet been described in this cell type. Interestingly, dependently on the stimuli the cells are exposed to, cAMP either stimulates or inhibits PKB phosphorylation, acting through different effectors. Hence, a cAMP pool that activates Epac (exchange protein directly activated by cAMP) and is regulated by PDE3B and PDE4 appears to mediate a decrease in PKB phosphorylation, whereas a cAMP pool that activates PKA seems to have an opposite, i. e. stimulatory, effect on PKB phosphorylation. Regulation of PKB by insulin and cAMP related signalling pathways have previously been studied in a few other cell types. Thus, in skeletal muscle [21] and HEK cells [20] it has also been shown that cAMP can either stimulate or inhibit insulin-induced PKB phosphorylation. In contrast to our results in adipocytes, however, Epac appeared to be a positive, and PKA a negative, regulator of this process. On the other hand, in cardiomyocytes, short term stimulation with adrenaline increased insulin-induced PKB phosphorylation via PKA [37], in agreement with our findings in adipocytes. Also the study on hepatocytes showed possible involvement of PKA in insulin+glucagon or leptin+glucagon-induced PKB activation [20]. These results indicate that when it comes to a crosstalk between insulin and cAMP signalling in the regulation of PKB phosphorylation, cell context seems to be particularly important.

Another component that contributes to complex and differential cAMP signaling, in addition to the presence of different cAMP effectors, is the phosphodiesterases which are

differentially expressed in different cells [7, 8]. Our results indicate that PDE3B and PDE4 control cAMP pools which negatively influence PKB phosphorylation induced primarily by insulin but to certain extent also by CL16243. These pools seem to be related to Epac as the Epac agonist mimicked the ability of the PDE3 and 4 inhibitors to decrease PKB phosphorylation under all tested conditions. cAMP-Epac was first shown to activate Rap1, a small G-protein [38]. It has not yet been shown that Rap 1 is present in adipocytes but on the other hand, Epac might mediate its effects by other proteins there. Interestingly, PDE3B which seems to regulate a pool of cAMP mediating inhibition of PKB phosphorylation, is at the same time a substrate for PKB [35]. Our results show that stimuli that alter PKB phosphorylation, also affect PDE3B activity. Thus, the Epac agonist and the PKA inhibitor caused a decrease in PDE3B activation induced by insulin and CL16243, respectively. Hence, an intricate network to control specific cAMP related events is emerging where a lowering of cAMP as a result of PKB-mediated activation of PDE3B is potentiated, since a decrease in cAMP attenuates Epac's inhibitory effect on PKB phosphorylation.

As for β-adrenergic stimulation in the context of PKB, we found that CL316243, a β3-adrenergic receptor-specific agonist, also by itself increases PKB phosphorylation. This is in agreement with some other studies showing that PKB may be activated by isoproterenol in adipocytes [18] or by forskolin in COS cells [19]. On the other hand, adrenaline alone did not increase PKB phosphorylation in skeletal muscle [21]. The CL316243 effect on PKB phosphorylation in adipocytes appeared to be PKA and PI3K dependent. In other cell types various results have been obtained. In cardiomyocytes [37], isoproterenol-induced phosphorylation of PKB was PKA and PI3K dependent, whereas,

in COS cells [19], cAMP-induced PKB activation was PKA-dependent but PI3K-independent, and in Sertoli cells [39], dbcAMP-induced activation of PKB was PKA-independent but PI3K-dependent. These data strengthen the hypothesis about the specificity of certain signalling events with regard to different tissues. The finding by Moule et al [18], which indicated that isoproterenol-induced activation of PKB in adipocytes was PI3K-independent and not related to phosphorylation, can be explained to some extent with regard to methodology. The band shift assay used in that study is less reliable when it comes to phosphorylation measurements than use of phosphospecific antibodies available nowadays. Mechanisms whereby PI3K and PKA mediate CL316243-induced PKB phosphorylation are not known. One possibility is that PKA phosphorylates and therefore modulates the activity of proteins upstream of PI3K.

Having investigated mechanisms of insulin and cAMP crosstalk in the regulation of PKB phosphorylation, we next investigated its possible implications for adipocyte metabolism. In our previous study we showed that insulin-induced lipogenesis, one of the major metabolic pathways in adipocytes, is positively regulated by PDE3B. The cAMP pool controlled by PDE3B appeared to mediate its inhibitory effects on lipogenesis via Epac [2]. Here we provide evidence that activation of PKB induces lipogenesis and this effect entails PDE3B action. As for possible downstream targets involved in lipogenesis, PDE3B, by degradation of a specific cAMP pool, appeared to be a mediator of insulin-induced dephosphorylation and thereby activation of ACC, a key enzyme in this process. Overexpression of PDE3B caused a significant decrease in phosphorylated ACC. Furthermore, cAMP modulators which appeared to inhibit lipogenesis [2], like the Epac agonist and especially the PDE3 inhibitor OPC3911 in combination with isoproterenol,

showed a robust positive effect on ACC phosphorylation. These data complement each other and give a new insight into the regulation of insulin-induced lipogenesis.

In conclusion, PKB phosphorylation, and therefore activation, in adipocytes is regulated by an orchestrated crosstalk between insulin and cAMP pathways where PDE3B seems to play an important role.

Figures

Fig. 1. Regulation of PKB phosphorylation by insulin- and cAMP-signalling pathways. Adipocytes were stimulated with 0.3 nM insulin (A, B) and 30 nM CL316243 (B, C). As indicated, prior to stimulation with hormones the cells were pretreated with 10 μM OPC3911, 10 μM RO 20-1724 or 100 nM wortmannin for 10 min, and with 10 μM 8-pCPT-2'-O-Me-cAMP (Epac ag.) or 25 μM H89 for 30 min. Representative immunoblots of total cell homogenates and quantification data are shown. Data are expressed as means +/- SEM., n=3-5; *significantly different from corresponding insulin (A), CL (B) or insulin+CL (C) -stimulated adipocytes.

Fig. 2. Overexpression of PDE3B potentiates insulin-induced PKB phosphorylation. Adenoviral stocks at $1x10^{10}$ pfu/ml were used to infect adipocytes (100 l/3 ml of 12.5 % cells) with Ad -gal and AdFlag-PDE3B, respectively. Adipocytes were stimulated with 0.3 nM insulin or 30 nM CL316243 for 10 min and with 10 μM 8-pCPT-2'-O-Me-cAMP (Epac ag.) for 30 min. Representative immunoblots of total cell homogenates and quantification data are shown. Data are expressed as means +/- SEM.,

n=3; *significantly different from corresponding insulin stimulated cells (black bar) in the beta-gal panel.

Fig. 3. PDE3B activation is lowered by a PKA inhibitor and an Epac agonist. Adipocytes were stimulated with 0.3 nM insulin or 30 nM CL316243 for 10 min with or without 10 μM 8-pCPT-2'-O-Me-cAMP (Epac ag.) or 25 μM H89 for 30 min. PDE3 activity was measured in total cell homogenates. Data are expressed as means +/- SEM., n=3-4. *significantly different from corresponding insulin stimulated cells (black bar).

Fig. 4. Overexpression of PKB induces lipogenesis which is lowered by a PDE3 inhibitor. Adenoviral stocks at $1x10^{10}$ pfu/ml were used to infect adipocytes (100 l/3 ml of 12,5 % cells) with Ad -gal and AdVPKBmyr, respectively. Adipocytes were stimulated for 1 h with 1 nM insulin, 10 μM OPC3911 (with 10 min pretreatment) or 15 μM 8-pCPT-2'-O-Me-cAMP (Epac ag.) (30 min-pretreatment). Lipogenesis was measured and expressed as fold increase in comparison to control cells (white bar in the beta-gal panel). Data are expressed as means +/- SEM, n=3. *over the white bar in PKB panel: significantly different from corresponding white bar in the beta-gal panel.*over two last bars: significantly different from non-stimulated PKB-overexpressing cells (white bar in the PKB panel).

Fig. 5. Treatment of adipocytes with a PDE3 inhibitor and an Epac agonist prevents insulin-induced lowering of ACC phosphorylation and reduces lipogenesis. Adipocytes

were treated without or with 1 nM insulin alone or in combination with 10 μM OPC3911 or 10 μM 8-pCPT-2'-O-Me-cAMP (Epac ag.; with 30 min-pretreatment), in the presence or absence of 30 nM isoproterenol for 10 min. Representative immunoblots of total cell homogenates and quantification data are shown. Data are expressed as means +/- SEM., n=3-4. Lipogenesis was measured under the same conditions and expressed as fold increase in comparison to control cells. Data are expressed as means +/- SEM., n=3.

Fig. 6. Overexpression of PDE3B results in decreased ACC phosphorylation. Adenoviral stocks at 1×10^{10} pfu/ ml were used to infect adipocytes (100 μl/ ml of 12,5 % cells) with Adβ-gal and AdFlag-PDE3B, respectively. Adipocytes were stimulated without or with 0.3 nM insulin for 10 min. Representative immunoblots of total cell homogenates and quantification data are shown. Data are expressed as means +/- SEM., n=3 *significantly different from beta-gal (control) cells (white bar).

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Abbreviations used:

PKB, protein kinase B

PKA, protein kinase A

Epacs, Exchange proteins directly activated by cAMP

PDE, phosphodiesterase

ACC, acetyl-CoA carboxylase