Role of PDE3B in insulin-induced glucose uptake, GLUT-4 translocation and lipogenesis in primary rat adipocytes

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Abstract

In adipocytes, phosphorylation and activation of PDE3B is a key event in the antilipolytic action of insulin. The role of PDE4, another PDE present in adipocytes, is not yet known. In this work we investigate the role of PDE3B and PDE4 in insulin-induced glucose uptake, GLUT-4 translocation and lipogenesis. Inhibition of PDE3 (OPC3911, milrinone) but not PDE4 (RO 20-1724) lowered insulin-induced glucose uptake and lipogenesis, especially in the presence of isoproterenol (a general β-adrenergic agonist), CL316243, a selective β3-adrenergic agonist, and pituitary adenylate cyclase-activating peptide. The inhibitory effect of OPC3911 was associated with reduced translocation of GLUT-4 from the cytosol to the plasma membrane. Both OPC3911 and RO 20-1724 increased protein kinase A (PKA) activity and lipolysis. H89, a PKA inhibitor, did not affect OPC3911-mediated inhibition of insulin-induced glucose uptake and lipogenesis, whereas 8-pCPT-2'-O-Me-cAMP, an Epac agonist which mediates PKA independent cAMP signaling events, mimicked all the effects of OPC3911. Insulin-mediated activation of protein kinase B, a kinase involved in insulin-induced glucose uptake, was apparently not altered by OPC3911. In summary, our data suggest that PDE3B, but not PDE4, contributes to the regulation of insulin-induced glucose uptake, GLUT-4 translocation, and lipogenesis presumably by regulation of a cAMP/ Epac signalling mechanisms.

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs)[1] are important regulators of signal transduction processes mediated by cAMP and cGMP. Eleven PDE families (PDE1-11) have been identified with regard to their substrate affinities, biochemical and physical properties, mechanisms whereby they are regulated, and different sensitivities to inhibitors [1, 2]. In adipocytes, PDE3B (the PDE3 family consists of two members, PDE3A and PDE3B) plays a key role in the antilipolytic action of insulin [3]. Insulin-induced phosphorylation and activation of PDE3B leads to increased hydrolysis of cAMP, lowering of protein kinase A (PKA) activity and thereby a decrease in PKA-dependent phosphorylation and activation of hormone sensitive lipase and lipolysis [4].

In adipocytes, modulators of cAMP regulate insulin-induced glucose uptake, a process known to involve translocation of insulin sensitive glucose transporters (GLUT-4) from an intracellular pool to the plasma membrane [5]. However, different effects of these agents have been reported. Thus, cAMP-increasing β-adrenergic agonists have been shown to increase [6, 7], to have no effect on [8, 9], or to decrease [10-12] insulin-induced glucose uptake in adipocytes. Studies using PDE3 inhibitors, OPC3911 and cilostamide, showed impaired, [13, 14] or unaffected [15] insulin-induced glucose uptake in adipocytes. The role of PDE4, which is also expressed in adipocytes, is not known. RO 20-1724, a PDE4 inhibitor, was found to suppress insulin-induced glucose uptake in myocardium [16], and another PDE4 inhibitor, rolipram, decreased glucose uptake *in vivo* in brain, heart, and skeletal muscle [17].

In this study we evaluated the role of PDE3B and PDE4 in insulin-induced glucose uptake, GLUT-4 translocation, and lipogenesis. We found that inhibition of PDE3B, but not PDE4, resulted in lowering of all these insulin-induced processes. Furthermore, overexpression of PDE3B resulted in increased lipogenesis. On the other hand, inhibition of PDE3B and PDE4,

respectively, increased PKA activity and lipolysis to similar extent. An Epac agonist was found to mimic, whereas H89, a PKA inhibitor did not interfere with the ability of OPC3911 to inhibit insulin-induced glucose uptake, GLUT-4 translocation and lipogenesis. These results indicate that PDE3B is involved in regulation of insulin-induced glucose uptake, GLUT-4 translocation and lipogenesis, presumably by regulating cAMP/ Epac signalling events.

2. Materials and methods

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 [18, 19]. Lund University Ethic Committee approved the study (permission number: M170-03).

2.2. Lipolysis assay

Glycerol release was measured as described previously [22]. Four hundred microliter aliquots of 5% (v/v) adipocytes in KRH were incubated as indicated in "Results" for 30 min at 37° C with shaking. Reactions were stopped by putting the tubes on ice for 20 min. One milliliter of hydrazine buffer containing 50 mM glycine (pH 9.8), 0.05% hydrazine hydrate, 1 mM $MgCl_2$, supplemented with 0.75 mg/ml ATP, 0.375 mg/ml nicotinamide adenine dinucleotide, 25 µg/ml glycerol-3-phosphate dehydrogenase, and 0.5 µg/ml glycerokinase was added to 200 µl of collected media. After incubation for 40 min in room temperature, OD_{340} was measured and glycerol release was calculated.

2.3. Glucose uptake assay

2-deoxy-D-[1-^{3}H]-glucose uptake was measured as described [20]. One hundred microliters of 30% (v/v) adipocytes in Krebs-Ringer bicarbonate-HEPES (KRBH) buffer (120 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM $NaHCO_3$, 30 mM HEPES, pH 7.4) with 1% bovine serum albumin (BSA) was added to 100 µl of KRBH buffer supplemented as indicated in "Results" and incubated for 10 min at 37 °C. Fifty microliter aliquots of KRBH buffer containing 0.5 mM 2-deoxyglucose, 0.5 µCi 2-deoxy-D-[1-^{3}H]-glucose (Amersham) and either 60 µM cytochalasin b in DMSO or DMSO alone were added. After 10 min reactions were stopped with 10 µl 1.5 mM cytochalasin b. Cells were separated from the buffer by centrifugation (6000xg) through dinonyl phtalate for 1 min. 2-deoxy-D-[1-^{3}H]-glucose uptake was determined by scintillation counting.

2.4. Lipogenesis assay

Lipogenesis was measured as previously described [21]. 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 10 min (fresh adipocytes) or for 30 min (adipocytes infected with adenovirus carrying cDNA for β galactosidase or Flag-PDE3B) 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.

2.5. PKA assay

One milliliter aliquots of 6% (v/v) adipocytes in KRH buffer, pH 7.4 with 1% BSA were incubated as indicated in "Results" for 1 min at 37°C. After addition of 200 µl of ice-cold homogenization buffer (5 mM Tris, 5 mM EDTA, 1.5 mM IBMX, 50 µM OPC 3911, 5 µg/ml pepstatin A, 50 µg/ml antipain, 50 µg/ml leupeptin, pH 7.4) the cells were homogenized and centrifuged (15500xg for 10 min at 4°C). Ten microliters of infranatant was incubated with 5 µl of phosphorylation mix (20 mM TES ($C_6H_{15}NO_6S$), 250 mM sucrose, 50 mM $MgSO_4$, 0.2 mM ATP, 5 mM dithioerythriol, 4 mg/ml kemptide, and 5 µCi [γ-^{32}P]ATP with or without 10 µM protein kinase A inhibitor) for 30 min at 30°C. Reactions were stopped with 10 µl of stop-mix (1% BSA, 1 mM ATP, pH 3.0). Proteins were precipitated with 30% trichloroacetic acid for 15 min on ice. After centrifugation (9200xg) at 4°C for 3 min, 10µl aliquots of each sample were transferred to P-81 membranes. The membranes were washed three times in 75 mM H_3PO_4 and once in acetone. The amount of incorporated ^{32}P was determined by scintillation counting.

2.6. Subcellular fractionation of adipocytes

Seven 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 3 ml of homogenization buffer (40 mM HEPES, 10 mM NaF, 1 mM dithioerythriol, 1 mM phenylmethylsulphonylfluoride, 0.25 mM sodium orthovanadate, 10 µg/ml antipain, 10 µg/ml leupeptin and 1 µg/ml pepstatin A, pH 7.4) and centrifuged at 16600xg for 15 min at 4°C. The solidified fat was removed and the infranatant was used to prepare the microsomal fraction. The 16600xg pellet was resuspended in 1.5ml of homogenization buffer, layered on top of a 1.5ml sucrose cushion

(1.12M sucrose, 20 mM Tris PH 7.4, 1 mM EDTA) and centrifuged at 104000xg for 70 min at 4°C. Plasma membranes, collected at the sucrose-buffer interface, were centrifuged at 48000xg for 45 min at 4°C and resuspended in 200 µl of homogenization buffer. The initial infranatant was centrifuged at 48000xg for 20 min at 4°C, yielding a high density microsomal (HDM) fraction which was resuspended in 200 µl of homogenization buffer. By centrifugation of the supernatant at 216000xg for 70 min at 4°C a low density microsomal (LDM) fraction was obtained. The pellet was resuspended in 200 µl of homogenization buffer.

2.7. SDS-PAGE and western blot analysis

After protein concentration had been measured (Bradford [23]), the samples were mixed with Laemmli sample buffer and subjected to polyacrylamide gel electrophoresis (PAGE) (10% acrylamide) and western blot analysis. After the electrotransfer of proteins onto a polyvinylidene difluoride membrane (Millipore) or Hybond-C membrane (Amersham Biosciences), 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 1:2000 dilution of anti-GLUT4 antibodies (Santa Cruz Biotechnology), a 1:1000 dilution of anti-GEF antibodies (Santa Cruz Biotechnology), anti P-Ser473PKBα antibodies (a kind gift from Professor Lars Rönnstrand) or a 1:1000 dilution of anti-PDE3B antibodies (raised in rabbits as described, see 24). After washing in TBS-T buffer membranes were incubated for 1h with 1:5000 dilution of anti-rabbit antibodies linked with Horseradish peroxidase (Amersham), 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).

2.8. Adenovirus infection and cell treatment

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 for16 hours 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 (AdFlag-PDE3B) was constructed as previously described [25].

2.9. Determination of PDE3 activity

Primary adipocytes infected for 16 hours with AdV β -gal or AdVPDE3B-Flag were washed in KRH buffer and homogenized (10 strokes at room temperature) in homogenization buffer (50 mM N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES), 40 mM p-nitro-phenyl-phosphate, 2 mM EGTA, 1 mM EDTA, 250 mM sucrose, 1 mM dithioerytritol, 0.05 mM vanadate, 10 μg/ml antipain, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, pH 7.4). The cell homogenates were centrifuged at 33000 x g for 45 min at 4°C and the crude membrane fractions were resuspended in homogenization buffer. PDE3 activity was measured as described previously [26]. Assays were performed at 30°C for 15 min.

Data analysis

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

3. Results

3.1. PDE3B regulates insulin-induced glucose uptake, GLUT-4 translocation to the plasma membrane and lipogenesis

As shown in Fig 1, isoproterenol-induced lipolysis was potentiated both in the presence of the PDE3 inhibitor OPC3911 and the PDE4 inhibitor RO 20-1724. On the other hand, insulin-induced decrease in lipolysis was blocked in the presence of OPC3911 but not RO 20-1724, indicating a key role for PDE3B but not PDE4 in the anti-lipolytic action of insulin, in agreement with previous results [15, 27, 28]. Very little is known regarding the role of PDE3B and PDE4 in other metabolic processes in adipocytes. As shown in Fig. 2A, insulin-induced glucose uptake in adipocytes was inhibited by the PDE3 inhibitor OPC3911 in the presence of isoproterenol, a cAMP increasing β-adrenergic agonist (by 60%). In the absence of isoproterenol, OPC3911-induced inhibition of insulin-induced glucose uptake appeared not to be significant. Similar results were obtained with milrinone, another PDE3 inhibitor (data not shown). A PDE4 inhibitor, RO 20-1724, did not affect insulin-induced glucose uptake.

We also tested the effects of OPC 3911 and RO 20-1724 on insulin-induced glucose uptake in the presence of pituitary adenylate cyclase-activating peptide (PACAP) and CL316243 (β3-adrenergic receptor agonist), which through interaction with G-protein linked receptors increase cAMP and lipolysis [29, 30]. The inhibitory effect of OPC3911 on insulin-induced glucose uptake was potentiated in the presence of both PACAP and CL316243 (data not

shown) as was the case for isoproterenol. On the other hand, the inhibitory effect of OPC3911 was not potentiated by adenosine deaminase (ADA) which increases cAMP by degradation of adenosine (leading to removal of adenosine receptor mediated inhibition of adenylate cyclase).

Having shown that OPC3911 is a potent inhibitor of insulin-induced glucose uptake and lipogenesis, we next investigated whether the inhibitory effect was related to changes in GLUT-4 translocation from the cytosol to the plasma membrane. As shown in Fig. 3, stimulation of adipocytes with insulin resulted in an increased amount of GLUT-4 in the plasma membrane, as detected by western blot analysis. This increase was reduced in the presence of OPC3911, especially in combination with isoproterenol. In agreement with the functional studies, isoproterenol alone did not have any inhibitory effect on insulin-induced GLUT-4 translocation (data not shown). In both high density membranes (HDM) and low density membranes (LDM) the amount of GLUT-4 significantly decreased after stimulation by insulin. However, this decrease did not occur in the presence of OPC3911, especially in combination with isoproterenol. Thus, OPC3911-mediated inhibition of insulin-induced glucose uptake correlated with inhibition of translocation of GLUT-4 to the plasma membrane.

Insulin-induced glucose uptake is an important first step in several pathways leading to the storage of nutrients in the form of triglycerides (lipogenesis). As shown in Fig. 2B, insulin-induced lipogenesis was inhibited in the presence of OPC3911 (by 45%). The inhibitory effect of OPC3911 was further enhanced in the presence of isoproterenol, resulting in an approximately 70% inhibition of insulin-induced lipogenesis. On the other hand, isoproterenol alone had a small potentiating effect on insulin-induced lipogenesis as it was previously

shown [29]. Milrinone, another PDE3 inhibitor, inhibited insulin-induced lipogenesis in the same manner as OPC3911 (data not shown). RO 20-1724 had little or no effect on insulin-induced lipogenesis. Lipogenesis was also studied in adipocytes overexpressing PDE3B. Infection of 3T3-L1 adipocytes with AdVFlag-PDE3B led to a 35-fold overexpression of PDE3B (Fig. 4A, B), which resulted in almost 4-fold increase in lipogenesis as compared to control cells infected with AdVβ-gal (Fig. 4C). These findings are in agreement with reduced lipogenesis in adipocytes treated with PDE3 inhibitors.

As shown in Fig. 5, OPC3911 and RO 20-1724 increased PKA activity both in the absence and presence of isoproterenol, whereas RO 20-1724 was slightly more efficient than OPC3911. Thus, different abilities of OPC3911 and RO 20-1724 to inhibit insulin-induced glucose uptake (in the presence of isoproterenol) and lipogenesis (both in the presence and absence of isoproterenol) do not correlate with their effects on total PKA activity. A minor role of PKA in mediating the effects of OPC3911 to inhibit insulin-induced glucose uptake (data not shown) and lipogenesis (Fig. 6) is further supported by the findings that H89, a PKA inhibitor, did not reverse these effects.

3.2. Epac is expressed in adipocytes and 8-pCPT-2'-O-Me-cAMP lowers insulin-induced glucose uptake, GLUT-4 translocation to the plasma membrane and lipogenesis

PKA independent cAMP signalling pathways have been described [31, 32] and cAMP has been shown to interact with GTP exchange factors, GEFs or Epacs (Exchange Proteins directly Activated by cAMP). Epac-mediated signaling was recently found to play a role in exocytosis [33], and in the activation of protein kinase B (PKB) [34], two events highly relevant for insulin-induced glucose uptake. As shown in Fig. 7, presenting western blots

from total homogenate and subcellular fractions of adipocytes, Epac is expressed in adipocytes. In order to test the possibility that the inhibitory effect of OPC3911 on glucose transport and lipogenesis might be mediated by regulation of a PKA-independent pathway, an Epac agonist, 8-pCPT-2'-O-Me-cAMP was used. As shown in Fig. 8, 8-pCPT-2'-O-Me-cAMP mimicked the ability of OPC3911 to reduce insulin-induced glucose uptake and lipogenesis. We then examined the influence of 8-pCPT-2'-O-Me-cAMP on GLUT-4 translocation and as shown in Fig. 9, the Epac agonist was able to impair translocation of GLUT-4 from an intracellular pool to the plasma membrane suggesting that the PKA-independent effects of OPC3911 could be mediated via cAMP/ Epac signalling.

Activation of PKB by insulin has been suggested to have an important role in glucose uptake and GLUT4 translocation [35] and Epac has recently been found to be involved in PKB activation [34]. However, as shown in Fig. 10, insulin-induced phosphorylation of PKB was not reduced in the presence of OPC3911, either without or with isoproterenol. It has also been demonstrated that insulin induces activation of the MAP kinase cascade, as well as cross-talk between β-adrenergic receptors and the MAP kinase in adipocytes [36]. However, addition of PD98059, an inhibitor of MEK (MAP kinase kinase), did not interfere with the ability of OPC3911 to inhibit insulin-induced glucose uptake or lipogenesis (data not shown).

4. Discussion

In this work we provide evidence that PDE3B, a key enzyme in the antilipolytic action of insulin [3], is also involved in the regulation of insulin-induced glucose uptake, translocation of GLUT-4 to the plasma membrane and lipogenesis in primary rat adipocytes. Treatment of adipocytes with selective PDE3 inhibitors OPC3911 and milrinone, but not RO 20-1724, a

PDE4 inhibitor, resulted in lowering of insulin-induced glucose uptake and lipogenesis. These effects were potentiated by several different agonists known to stimulate receptor-mediated production of cAMP. This is in agreement with a previous study showing that the cAMP analogue, 8-bromo-cAMP, potentiated the ability of OPC3911 to inhibit insulin-induced glucose uptake [13]. On the other hand, ADA which increases cAMP by catalyzing degradation of adenosine [37] and therefore leads to removal of adenosine receptor mediated inhibition of adenylate cyclase, did not potentiate OPC3911 mediated lowering of insulin-induced glucose uptake and lipogenesis, suggesting that PDE3B controls specific pools of cAMP. In agreement with some studies [2], but in contrast to others [38, 39], we found no inhibition of insulin-induced glucose uptake by the addition of isoproterenol alone. Rather, with regard to insulin-induced lipogenesis, we found a slight potentiation by isoproterenol, in agreement with previous results [29]. Differential effects of various cAMP-modulating agents, and especially the finding that OPC3911 but not RO 20-1724 had effects on insulin-induced glucose uptake and lipogenesis, support the hypothesis of cAMP compartmentalization. Different pools of cAMP could regulate different downstream effects in both, PKA-dependent and independent manners. As for PKA-dependent processes, PKA anchoring proteins (AKAPs) [40] have been suggested to recruit PKAs as well as other proteins (*e g*. PDE4) [41] to different intracellular locations, resulting in compartmentalization of PKA signaling events. Our results using the Epac agonist, 8-pCPT-2'-O-Me-cAMP, which mediates PKA-independent cAMP signalling [31,32] and H89, a PKA inhibitor, suggest that the inhibitory effects of OPC3911 on insulin-induced glucose uptake and lipogenesis could occur by regulation of cAMP/ Epac signaling events. It should be noticed that inhibition of either PDE3 or PDE4 results in potentiation of isoproterenol-induced cAMP increase [42] and activation of PKA in adipocytes. Differential effects of PDE3/PDE4 inhibitors have also been demonstrated for insulin-induced inhibition of lipolysis; inhibition of PDE3, but not PDE4,

blocks the antilipolytic effect of insulin [this study, 15, 27, 43]. Most likely PDE3B which is a membrane associated enzyme [3] regulates a cAMP pool different from a pool regulated by PDE4, an enzyme found mainly in the cytosolic fraction of adipocytes [28]. In a number of other cell types [44-46] and in several PDE3 and PDE4 knock out mice models [47-49] it has been demonstrated that different PDE family members have unique and non-overlapping functions presumably due to the regulation of different pools of cAMP. Although new methods are being developed in different laboratories for measurements of cAMP in real time and space in living cells, it is still unclear how cAMP, in response to different stimuli, triggers different cellular responses in one cell.

A correlation between cAMP-mediated inhibition of insulin-induced glucose uptake and inhibition of translocation of GLUT-4 to the plasma membrane has been shown in some studies [8] but not in others [6]. Isoproterenol-induced inhibition of insulin-induced glucose uptake in the presence of ADA was suggested to be caused by trapping of GLUT-4 in occluded vesicles associated with the plasma membrane during exocytosis, rather than by reduction of total plasma membrane GLUT-4 content [6]. Activity of the plasma membrane associated GLUT-4 transporter could explain some of the discrepancy between insulin-induced glucose uptake and the amount of GLUT-4 in the plasma membrane. GLUT-4 has been shown to be phosphorylated [7, 50], and its phosphorylation was associated with the transport activity [7]. Our findings show a clear correlation between total plasma membrane, HDM, and LDM GLUT-4 content and inhibition of insulin-induced glucose uptake caused by OPC3911.

Mechanisms whereby inhibition of PDE3B induces inhibition of insulin-induced GLUT-4 translocation to the plasma membrane and inhibition of insulin-induced glucose uptake are not known. It is possible that PDE3B regulates a cAMP pool activating Epac-induced signalling events. Furthermore, signaling triggered by PDE3B involve targets downstream

from PKB or on pathways parallel to the PKB signaling network. It might be possible that a PDE3B-controlled cAMP pool is involved in the regulation of proteins important for the transport or docking/ fusion of GLUT-4 exocytotic vesicles or in the regulation of atypical PKCs, kinases believed to play a role in insulin-induced glucose uptake [51].

Whether or not the ability of OPC3911 to inhibit insulin-induced lipogenesis can be explained solely by its effects on glucose uptake remains to be established. Most likely, other events downstream of glucose uptake are influenced by PDE3B inhibition since, in the conditions where OPC 3911 did not lower insulin-induced glucose uptake (Fig. 2A, without ISO), it significantly lowered insulin-induced lipogenesis (Fig 2B, without ISO).

In conclusion, PDE3B controls a cAMP pool, which through Epac-mediated signalling events, negatively regulates insulin-induced glucose uptake, GLUT-4 translocation to the plasma membrane, and lipogenesis. Our results suggest that Epacs or signalling components downstream of Epacs are interesting targets for the development of drugs for the treatment of insulin resistance.

Figures

Fig. 1. Effects of PDE3 and PDE4 inhibitors on lipolysis and insulin-induced inhibition of lipolysis. Adipocytes were treated with 10 nM isoproterenol (ISO) alone or in combination with either 10 μM OPC3911 or 10 μM RO 20-1724, in the absence or presence of 0.1 and 1 nM insulin (ins). Glycerol release was measured after 30 min. Data are expressed as means +/- SEM., n=4; *significantly different from corresponding isoproterenol-stimulated adipocytes (black bar in each panel).

Fig. 2. Inhibition of PDE3 but not PDE4 inhibits insulin-induced glucose uptake and lipogenesis. Adipocytes were treated with 1 nM insulin (ins) alone or in combination with 10 μM OPC3911, 10 μM RO 20-1724, or both, in the presence or absence of 30 nM isoproterenol (ISO) for 10 min, as indicated. 2-[1-^{3}H]-deoxyglucose uptake (A) and lipogenesis (B) were measured and expressed as fold increase in comparison to control cells without or with ISO, respectively [in the ISO groups the control values are, in absolute terms, 8% (A) and 10% (B) lower as compared to the control values in the group without ISO; in a control experiment neither OPC3911 nor RO 20-1724 significantly influenced control values]. Data are expressed as means +/- SEM., n=5 (A), n=15 (B); *significantly different from corresponding insulin-stimulated adipocytes (black bar in each panel).

Fig. 3. Inhibition of PDE3B inhibits insulin-induced translocation of GLUT-4 to the plasma membrane. Adipocytes were stimulated with 1 nM insulin (ins) with or without 10 μM OPC3911 and 30 nM isoproterenol (ISO) for 10 minutes. Representative GLUT4 immunoblots of the plasma membrane (PM), high density membrane (HDM), and low density membrane (LDM) fractions are shown. GLUT-4 bands from 3 experiments were quantified,

and the intensity of each band was related to the intensity of the band in the control lane (for each experiment). Data are expressed as means +/- SEM, n=3; *significantly different from corresponding insulin-stimulated adipocytes.

Fig. 4. Overexpression of PDE3B results in increased lipogenesis. Adenoviral stocks at 1×10^{10} pfu/ml were used to infect adipocytes (150 µl/2.5 ml of 12 % cells) with Adβ-gal and AdFlag-PDE3B, respectively. Crude membrane fractions from control adipocytes and adipocytes overexpressing PDE3B were isolated and subjected to western blot analysis (10 µg of total protein) or PDE3 activity measurements. Shown is a representative western blot out of 5 independent experiments (A), PDE3B activity in membrane fractions expressed as fold increase in comparison to β-gal cells (B) and lipogenesis, also expressed as fold increase in comparison to β-gal cells (C). Data are presented as means +/-SEM.

Fig. 5. Inhibition of PDE3 or PDE4 induces activation of PKA. Adipocytes were pre-treated for 10 min with or without OPC3911 or RO 20-1724, and then stimulated with 30 nM isoproterenol (ISO) or/ and 1 nM insulin (ins) for 1 min. PKA-activity was measured and expressed as fold increase in comparison to control cells without ISO. Data are expressed as means +/- SEM., n=3; *significantly different from untreated (control) adipocytes.

Fig. 6. Effects of H89 on insulin-induced lipogenesis. Adipocytes were pre-treated for 30 min with or without 10 µM H89, and incubated with 1 nM insulin (ins) alone or in combination with 10 µM OPC3911 in the absence or presence of 30 nM isoproterenol (ISO) for 10 min. Lipogenesis was measured and expressed as % of lipogenesis induced by insulin without or with ISO, respectively. Data are presented as means +/-SEM., n=4; *significantly different from insulin-stimulated adipocytes in the isoproterenol panel.

Fig. 7. Expression of Epac in adipocytes. SDS-PAGE and western blot analysis were performed on total homogenate and subcellular fractions of adipocytes. Representative western blots show the content of Epac in total homogenate (H; 60 μg protein/well), plasma membrane (PM; 20 μg protein/well), high density membrane (HD; 10 μg protein/well), low density membrane (LD; 30 μg protein/well) and cytosol (C; 40 μg protein/well).

Fig. 8. 8-pCPT-2'-O-Me-cAMP inhibits insulin-induced glucose uptake and lipogenesis. Adipocytes were pre-treated for 30 min with or without 10 μM 8-pCPT-2'-O-Me-cAMP, and incubated with 1 nM insulin (ins) alone or in combination with 10 μM OPC3911, in the absence or presence of 30 nM isoproterenol (ISO) for 10 min. 2-[1-^{3}H]-deoxyglucose uptake (A) and lipogenesis (B) were measured and expressed as % of glucose uptake and lipogenesis induced by insulin without or with ISO, respectively. Data are presented as means +/- SEM., n=4; *significantly different from corresponding insulin-stimulated adipocytes.

Fig. 9. 8-pCPT-2'-O-Me-cAMP inhibits insulin-induced translocation of GLUT-4 to the plasma membrane. Adipocytes were pre-treated for 30 min with or without 10 μM 8-pCPT-2'-O-Me-cAMP, and incubated with 1 nM insulin (ins) for 10 min. Representative GLUT4 immunoblots of the plasma membrane (PM), high density membrane (HDM), and low density membrane (LDM) fractions are shown. GLUT-4 bands from 2 experiments were quantified, and the intensity of each band was related to the intensity of the band in the control lane (for each experiment). Data are expressed as means +/- STDEV.

Fig. 10. Effects of OPC3911 on insulin-induced phosphorylation of PKB. Adipocytes were incubated for 10 min with 1 nM insulin (ins) alone or in combination with 10 μM OPC3911

or 10 μM RO 20-1724, in the absence or presence of 30 nM isoproterenol (ISO). Cells were homogenized and centrifuged at 19 000g for 45 min. Supernatants were subjected to immunoblot analysis using an antibody recognizing PKB phosphorylated on serine 473. Bands from 3 experiments were quantified using Fuji LAS 1000 Plus system. Results are expressed as means +/- SEM.

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Abbreviations used:

PDE, phosphodiesterase

PKA, protein kinase A

Epacs, Exchange Proteins directly Activated by cAMP

PKB, protein kinase B

ADA, adenosine deaminase

PACAP, pituitary adenylate cyclase-activating peptide
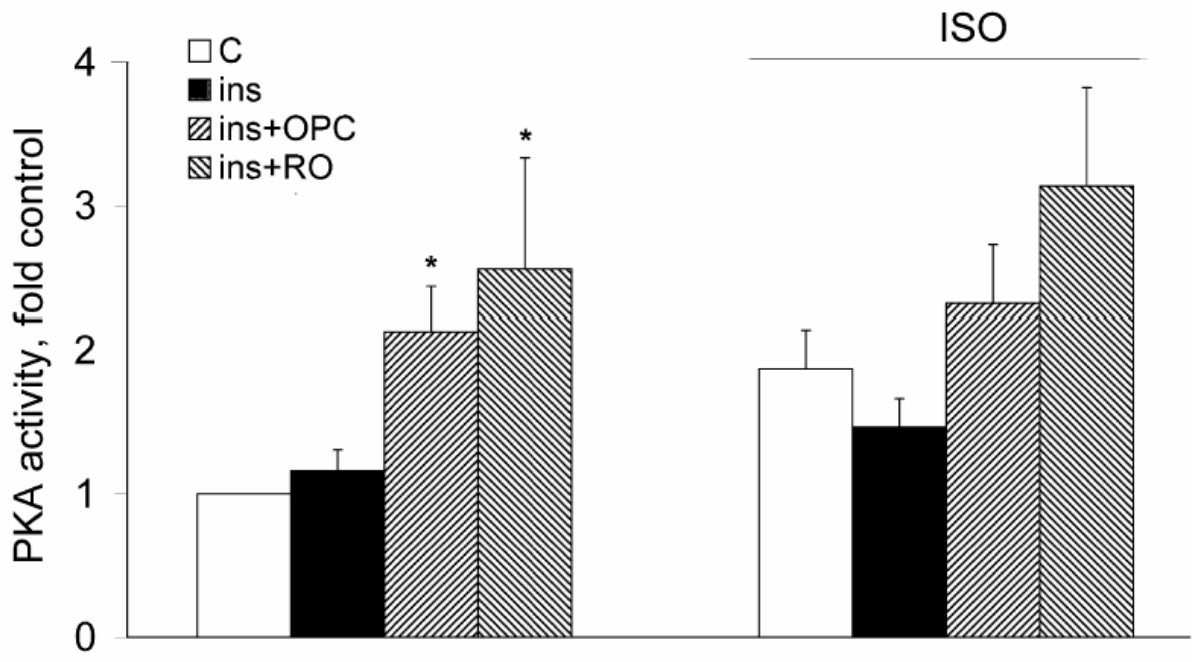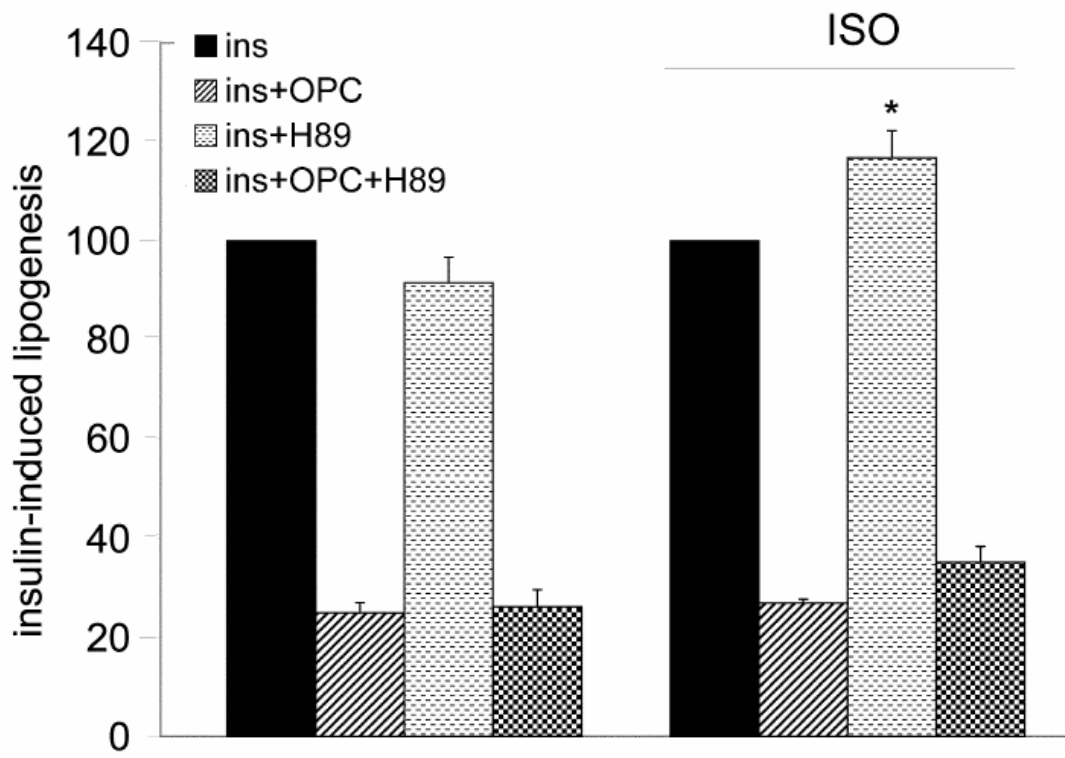
HDM, high density membranes

LDM, low density membranes
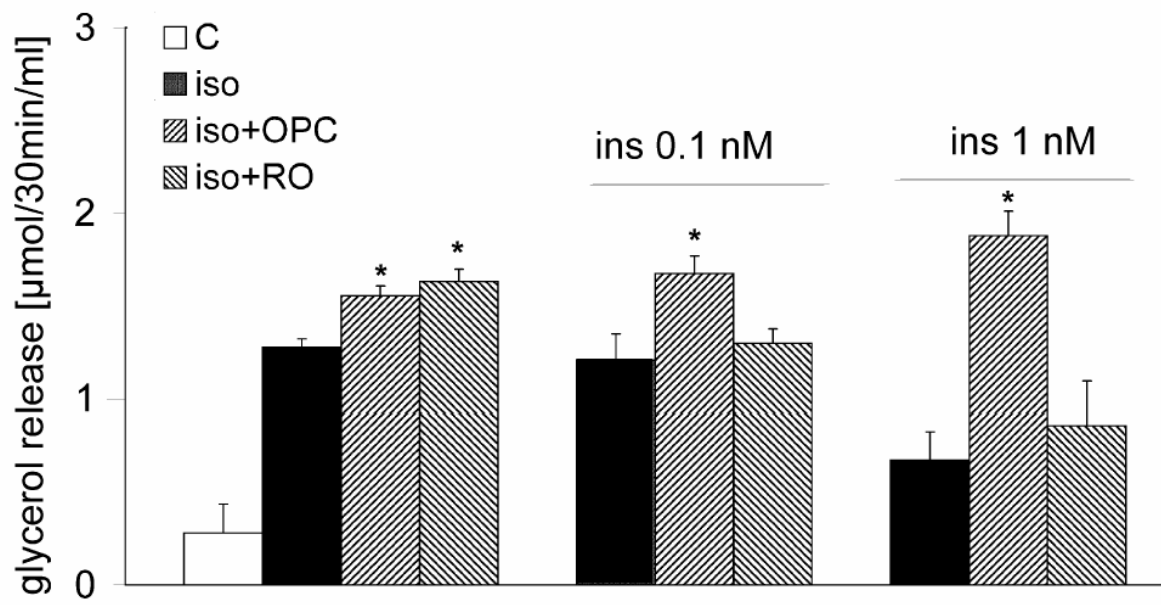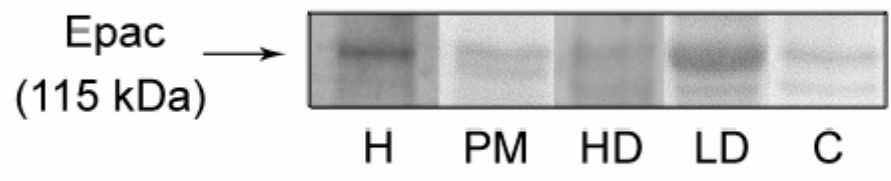
Fig. 1.
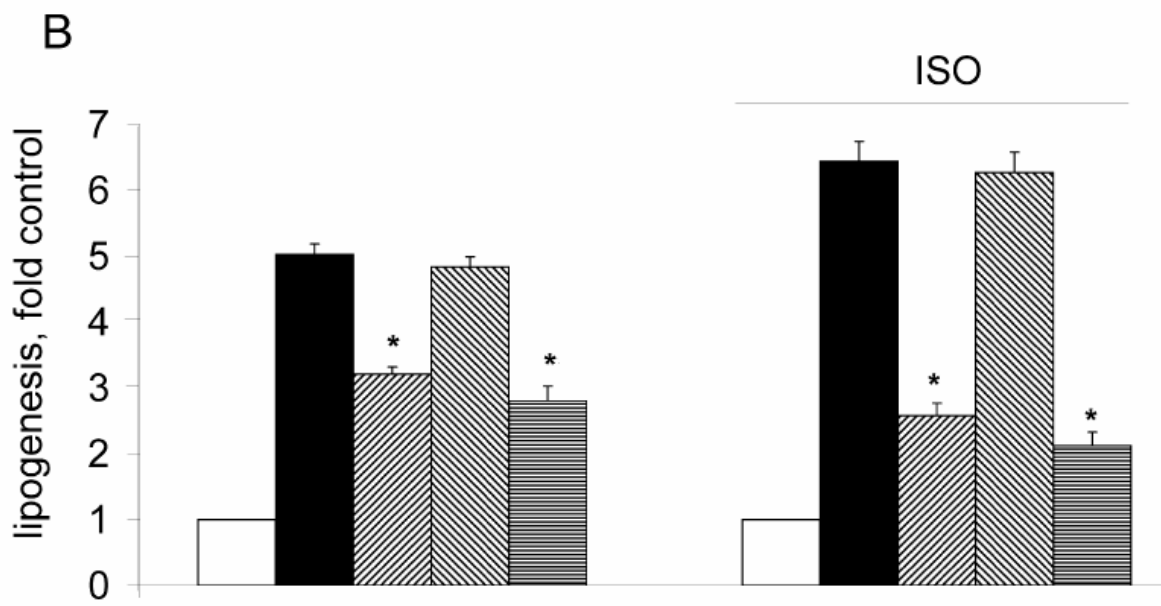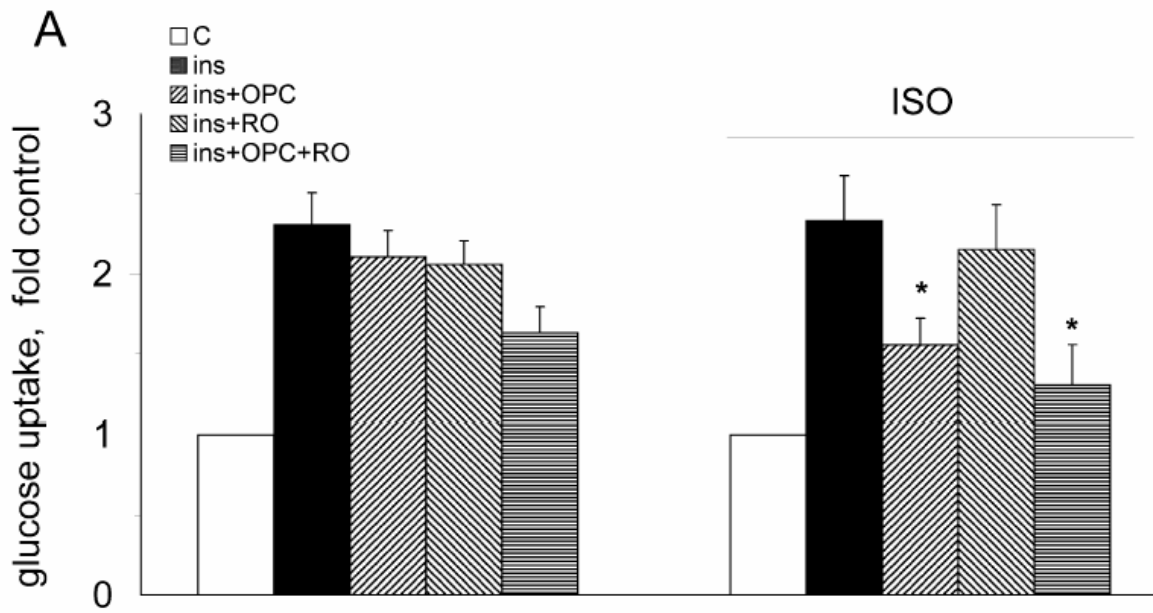
Fig. 2.

Fig.3.

Fig.4.

Fig.5.

Fig.6.

Epac
(115 kDa)

H   PM   HD   LD   C

Fig.7.

Fig.8.

Fig.9.

Fig.10.