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online data

SUPPLEMENTARY ONLINE DATA

Differential regulation of adipocyte PDE3B in distinct membrane compartments by insulin and the β_3-adrenergic receptor agonist CL316243: effects of caveolin-1 knockdown on formation/maintenance of macromolecular signalling complexes

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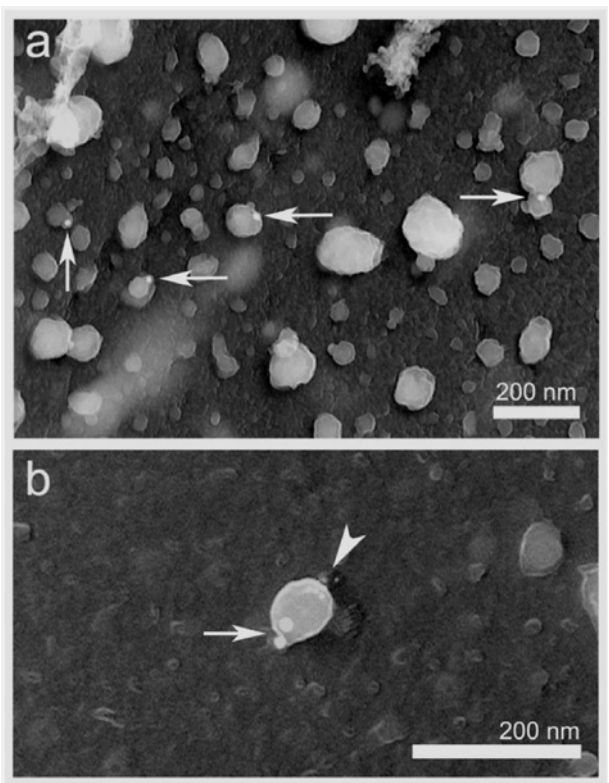

Figure S1 Immunogold labelling of PDE3B and cav-1 at the intracellular face of the PM

TEM of PM sheets from primary human adipocytes were prepared on grids and labelled with antibodies against PDE3B (arrows) and cav-1 (arrowhead), followed by labelling with secondary antibodies conjugated to colloidal gold particles (15 nm, PDE3B; 6 nm, cav-1). Images have been contrast-inverted.

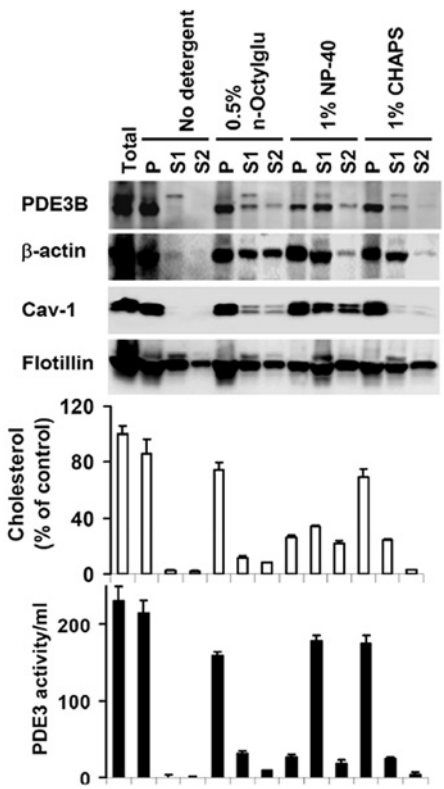

Figure S2 Efficiency of several detergents for solubilization of PDE3B from 3T3-L1 adipocyte membranes

PDE3B was solubilized from 3T3-L1 adipocyte membrane fractions (~5 mg/ml protein) using different detergents. Solubilization efficiency was estimated from the analysis of Western blot scans, PDE3 activity measurements and cholesterol content. Cholesterol content is presented as the percentage of cholesterol in individual fractions relative to original membranes. A representative Western blot is shown ($n = 2$). PDE3 activity (pmol of cAMP hydrolysed/min per ml) is presented as mean + half of the range ($n = 2$ experiments). For each experiment, cells were harvested from six plates for solubilization with each detergent; PDE3 activity assays were carried out in duplicate. NP-40, Nonidet P40.

MATERIALS AND METHODS

Isolation and incubation of primary human adipocytes

Subcutaneous fat was obtained during elective abdominal surgery, under general anaesthesia. Informed consent was obtained from all participating individuals; the procedures were approved by the local ethics committee. Human adipocytes, isolated by collagenase digestion [1], were allowed to float by gravity, and were incubated overnight [2]. Before analysis, adipocytes were incubated (15 min at 37 °C) with 10 nM phenylisopropyladenosine and 0.5 unit/ml adenosine deaminase.

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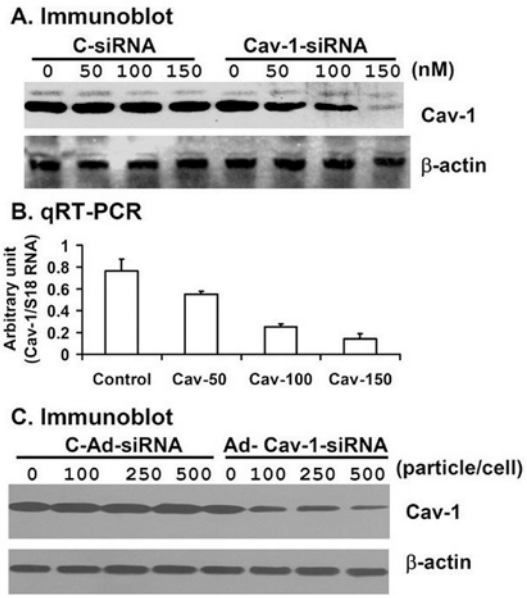

Figure S3 Synthetic and Ad siRNA-mediated KD of *CAV-1* expression in 3T3-L1 adipocytes

(**A**) Differentiated 3T3-L1 adipocytes were transfected with the indicated concentrations of duplex siRNA targeted against *CAV-1* (cav-1 siRNA) or a scrambled sequence (control siRNA, C). At 72 h after transfection, cell lysates were prepared, and proteins were analysed by Western blotting with anti-cav-1 and β-actin antibodies. One representative Western blot is shown ($n = 2$). (**B**) After transfection with the indicated concentrations of siRNA, *CAV-1* KD was confirmed by qRT-PCR analysis. Values are the means + half of the range ($n = 2$). For each experiment, RNA was isolated from three different plates in each experimental group and qRT-PCR was assayed in duplicate. (**C**) Cells were infected with a different number of *CAV-1* siRNA and control siRNA Ad particles (as described in the Materials and methods section), to determine the optimal concentration required for KD of *CAV-1*. At 72 h after transfection, cell lysates were prepared, and proteins were analysed by Western blotting. One representative Western blot is shown ($n = 2$).

Electron microscopy of adipocyte PM (plasma membrane) sheets

PM sheets were prepared as described previously [3]. Human primary adipocytes were attached to poly-L-lysine/Formvar-coated nickel grids and flushed with ice-cold 150 mM KCl and 1.9 mM Tris/HCl buffer (pH 7.4). PMs remaining on the grids were fixed by immersion [20 min at room temperature (20 °C)] \ break in ice-cold 0.1 M sodium cacodylate, containing 0.1 M sucrose, 3% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde. Membranes were blocked with 0.05 M glycine (15 min at room temperature), and with 5% (w/v) BSA-c, 0.1% (w/v) gelatin and 1% normal goat serum (60 min at 37 °C). Membrane sheets were incubated (overnight at 4 °C) with primary antibodies against PDE3B and cav-1 (catalogue number 610058; BD Biosciences) in phosphate buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl and 27 mM KCl) containing 1% (w/v) BSA-c and 0.2% normal goat serum. Membranes were rinsed in fresh buffer before labelling with secondary gold-conjugated antibodies (15 and 6 nm gold particles), and incubated (overnight at 4 °C). Membranes were then rinsed and post-fixed (10 min at room temperature) in 2% (v/v) glutaraldehyde (in 0.1 M sodium cacodylate, containing 0.1 M sucrose, pH 7.5), and followed by 1% (w/v) OsO_4 in 0.1 M sodium cacodylate, containing 0.1 M sucrose, pH 7.5 (20 min at room temperature). Grids were frozen, freeze-dried and coated with 2 nm tungsten by magnetron sputtering directly in the freeze-dryer [4]. TEM (transmission electron microscopy) was with a Jeol EM1230 instrument. No labelling was observed in the absence of primary antibody.

Ad (adenoviral) siRNA (small interfering RNA) KD (KD) of *CAV-1* (caveolin-1)

Ad-*CAV-1* siRNA vectors were kindly supplied by Joel S. Pachter (Department of Pharmacology, University of Connecticut Health Center, Farmington, CT, U.S.A.), and were was used as described previously [5]. In constructing the siRNA vector, a mouse *Cav-1* siRNA oligonucleotide (5′-GGAGAUUGACCUGGUCAA-3′), which was selected according to siRNA oligonucleotide design criteria (BD knockout RNAi systems; BD Clontech), was targeted to a sequence in *Cav-1* mRNA (GenBank® accession no. NM_007616) that started at position 221. Ad siRNA encoding the luciferase gene was used as a control [5]. Adenovirus constructs were amplified in HEK (human embryonic kidney)-293A cells and purified as described previously [6]. Adenovirus particles were quantitated by measuring absorbance at $A_{260\ nm}$, and infection efficiency was determined by immunostaining with an anti-adenovirus hexon protein. Adipocytes were infected (3 h) with adenovirus Ad-*Cav-1*si or control Ad-Lucsi (500 viral particles/cell) in complete DMEM (Dulbecco's modified Eagle's medium) before the medium was supplemented with 10% (v/v) FBS (fetal bovine serum), and incubated for another 46 h. Infection efficiency was determined by immunostaining with anti-adenovirus hexon protein using the Adeno-x kit (Clontech). *Cav-1* KD was confirmed via immunoblotting.

KD of *Cav-1* with a second synthetic siRNA

An siRNA duplex oligonucleotide, (5′-AACCAGAAGGGAC-ACACAGUU-3′) targeted to a sequence in murine *Cav-1* mRNA which started at position 301, and a control, non-targeting, scrambled siRNA oligonucleotide (5′-AATT-AAAAAAACCCCCGGGGG-3′), used as a negative control, were purchased from Dharmacon. Blast searches confirmed that the *Cav-1* and scrambled siRNA sequences have no overlap with other proteins. Optimal conditions for siRNA KD involved transfecting adipocytes with 150 nM synthetic siRNA using MBS (modified bovine serum) mammalian transfection reagent (Stratagene) in DMEM, following the manufacturer's protocols. After 10 h, fresh complete DMEM/10% (v/v) FBS was added, and adipocytes were further incubated for 46 h. *Cav-1* KD was confirmed via immunoblotting and qRT-PCR (quantitative real-time-PCR) assay.

qRT-PCR

Adipocytes, in 10-cm dishes, were lysed using 1 ml of QIAzol lysis reagent (Qiagen). Lysates were collected, transferred into a microtube, and pipetted up and down until the samples were homogeneous. After incubation of samples (5 min at room temperature), chloroform (300 μl) was added to each sample and the tubes were shaken vigorously. After 5 min, the solution was centrifuged (20000 ***g*** at 4 °C for 15 min). The upper aqueous phase was transferred to a new tube and 1 vol. of 70% ethanol was added. The solution was immediately applied to RNeasy mini spin columns (RNeasy mini kit), and total RNA was purified with on-column DNase digestion (RNase-Free DNase Set; Qiagen), according to the manufacturer's protocol. Total RNA (100 ng) in triplicate was subjected to qRT-PCR, using the HT7900 Sequence Detection System (Applied Biosystems) and QuantiTect SYBR green RT-PCR kit (Qiagen), according to the manufacturer's protocols. *Cav-1* was normalized to S18 and cyclophilin A, which served as internal controls for every sample. The sequences of primers for *Cav-1* are 5′-CCAGCTTCACCACCTTCACT-3′ and

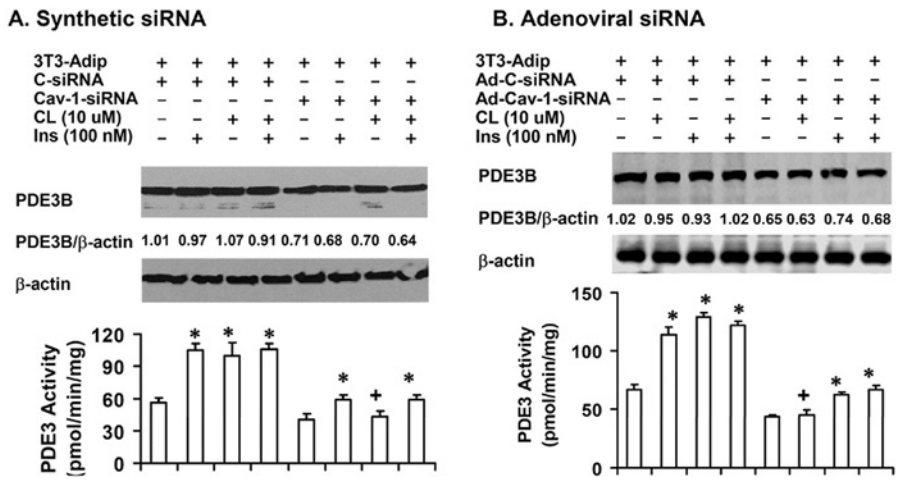

Figure S4 KD of *CAV-1* is associated with reduced PDE3B expression and impaired activation of PDE3B

At 72 h after transfection with synthetic duplex siRNA (150 nM) (**A**) or Ad siRNA (500 viral particles/cell) (**B**), total membrane fractions were prepared from control and *CAV-1*-KD adipocytes incubated without or with CL (10 μM at 15 min), insulin (100 nM at 10 min) or both. Membrane proteins were solubilized by homogenization (using a Dounce homogenizer) and sonication of membrane pellets in buffer B containing 1% (v/v) Nonidet P40, and, after incubation/rotation by centrifugation (10000 ***g*** for 30 min at 4 °C), solubilized membrane proteins were assayed for PDE3 activity, or subjected to SDS/PAGE (30 μg) and Western blot analysis. In (**A**) and (**B**), one representative Western blot is shown ($n = 2$). The ratio of PDE3B/β-actin (mean $\pm$ S.E.M.) for the experiment in (**A**) is 0.99 ± 0.04 ($n = 4$) and 0.68 ± 0.02 ($n = 4$) for siRNA control and *CAV-1*-KD adipocytes respectively; in (**B**) 0.98 ± 0.02 ($n = 4$) and 0.67 ± 0.02 ($n = 4$) respectively. PDE3 activity data is presented as the mean + half of the range ($n = 2$). For each experiment, cells were harvested from two plates and PDE3 activity assays were carried out in duplicate.

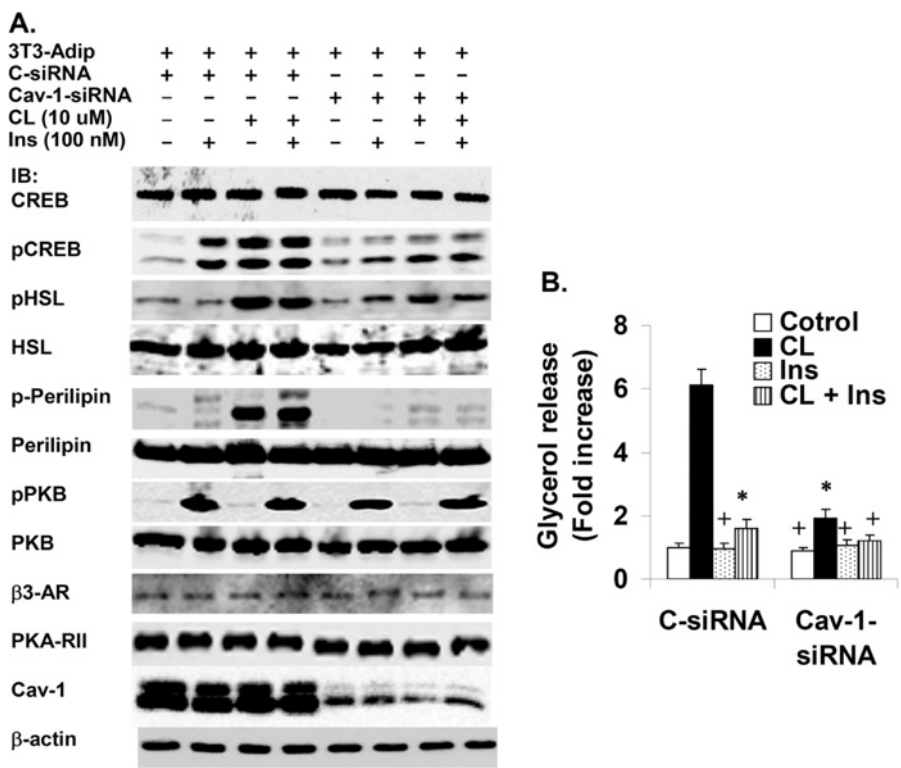

Figure S5 Effects of *CAV-1*-KD on lipolysis, and insulin- and CL-stimulated phosphorylation of PKB and PKA substrates

3T3-L1 adipocytes were transfected with 150 nM siRNA targeted against *CAV-1* or non-targeting S-siRNA. After 72 h, adipocytes were incubated without or with CL or insulin (Ins) for 15 min, or a combination of the two. (**A**) Cell lysates (30 μg) were subjected to SDS/PAGE, and immunoblots were probed with antibodies as indicated. One representative experiment is shown ($n = 2$). (**B**) Adipocytes were incubated (in triplicate) without (control) or with indicated concentrations of insulin (Ins, 100 nM) or CL (100 nM) for 1 h. Lipolysis (glycerol release) was measured. Values (fold increase in glycerol release) represent the mean + half of the range ($n = 2$).

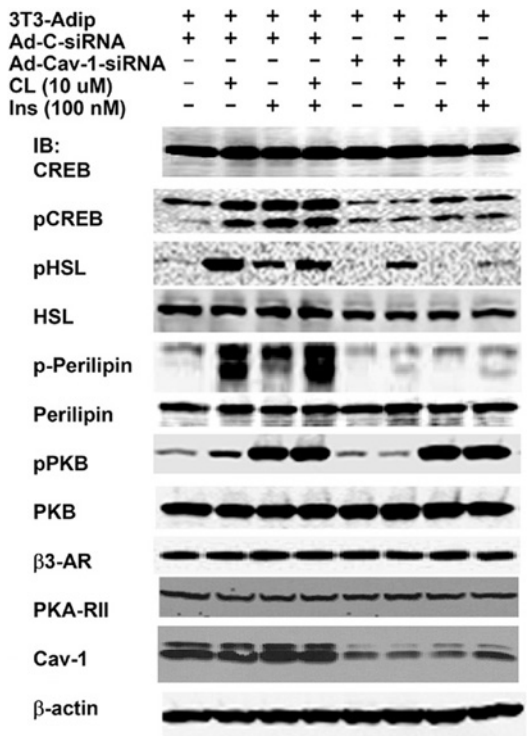

Figure S6 Effects of Ad-siRNA-mediated *CAV-1* KD on insulin- and CL-stimulated phosphorylation of PKB and PKA substrates

3T3-L1 adipocytes were infected with Ad-siRNA (500 viral particles/cell) targeted against *CAV-1* (cav-1 siRNA) or a control Ad siRNA. After 72 h, adipocytes were incubated without or with CL (15 min) or insulin (Ins) (10 min), or a combination of the two. Cell lysates (30 μg) were prepared and resolved by SDS/PAGE, and immunoblots were probed with antibodies as indicated. One representative experiment is shown ($n = 2$).

5′-GCTCTTGATGCACGGTACAA-3′. The sequences of primers for cyclophilin A are 5′-AGCATACAGGTCCTGGCATC-3′ and 5′-TTCACCTTCCCAAAGACCAC-3′. The primers used for S18 are 5′-GATGTGAAGGATGGGAAGTACAG-3′ and 5′-CTTCTTGGATACACCCACAGTTC-3′.

RESULTS

Immunogold labelling of PDE3B and cav-1 at the intracellular face of the PM

TEM of PM sheets from primary human adipocytes indicated that, in human primary adipocytes, PDE3B was found in association with PM/caveolae regions (Supplementary Figure S1). Most of the immunogold labelling of PDE3B in these PM sheets was associated with caveolae [91% of the immunogold particles (415 particles counted in two independent experiments)], indicating that most of the PDE3B in the PM is localized to caveolae domains.

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Efficiency of several detergents for solubilization of PDE3B from 3T3-L1 adipocyte membranes

To identify solubilization conditions for membrane-associated PDE3B, several detergents were screened. Adipocytes were washed and homogenized in a Dounce homogenizer in ice-cold buffer A [50 mM Hepes, 50 mM sucrose, 1 mM EDTA, 10 mM pyrophosphate, 5 mM NaF, 100 mM NaCl, 0.1 μM okadaic acid, 1 mM sodium orthovanadate and Roche protease inhibitor cocktail (pH 7.5)]. After centrifugation at 500 ***g***, total membranes were prepared by centrifugation of supernatants [37600 rev./min (using a SW41 Ti rotor; Beckman), 30 min, 4 °C], as described previously [7]. Membranes were washed once in buffer A and resuspended at $\sim$5 mg/ml protein in buffer A supplemented with the indicated detergents for 30 min on ice. Samples (1 ml) were centrifuged [28000 rev./min (using a SW41 Ti rotor; Beckman) for 30 min at 4 °C]. Pellets were homogenized in buffer A (1 ml each) containing the indicated detergents and recentrifuged. Final pellets were resuspended in buffer A (1 ml) containing the indicated detergents. Portions of supernatants 1 and 2 and final pellets were taken for measurement of protein and cholesterol content and PDE3 activity (pmol of cAMP hydrolysed/min per ml), or subjected to SDS/PAGE and Western blot analysis with antibodies directed against PDE3B, cav-1, flotillin and β-actin, to confirm the efficiency of solubilization of PDE3B and caveolin with different detergents.

Three detergents were studied: n-octyl glucoside, a zwitterionic detergent, CHAPS, and a non-ionic detergent, Nonidet P40. For CHAPS, we tested different concentrations (1–4%), with no significant improvement in solubilization of PDE3B (results not shown). At higher concentrations (4% CHAPS), recovery of PDE3B was reduced. Nonidet P40 was the most efficient in solubilizing PDE3B and caveolin (Supplementary Figure S2).

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