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Multisite phosphorylation of adipocyte and hepatocyte phosphodiesterase 3B

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Abstract

Phosphodiesterase 3B (PDE3B) is an important component of insulin and cAMP-dependent signalling pathways. In order to study phosphorylation of PDE3B, we have used an adenoviral system to express recombinant flag-tagged PDE3B in primary rat adipocytes and H4IIE hepatoma cells. Phosphorylation of PDE3B after treatment of cells with insulin, cAMP-increasing agents or the phosphatase inhibitor, calyculin A were analysed by two dimensional tryptic phosphopeptide mapping and mass spectrometry. We found that PDE3B is multisite phosphorylated in adipocytes and H4IIE hepatoma cells in response to all these stimuli. Several sites were identified; serine(S)273, S296, S421, S424/5, S474 and S536 were phosphorylated in adipocyte as well as H4IIE hepatoma cells whereas S277 and S507 were phosphorylated in hepatoma cells only. Several of the sites were phosphorylated by insulin as well as cAMP-increasing hormones indicating integration of the two signalling pathways up stream of PDE3B, maybe at the level of protein kinase B.

1. Introduction

Cyclic nucleotide phosphodiesterases (PDEs) constitute eleven families of cAMP and cGMP hydrolyzing enzymes. PDE3B, one of two members in the PDE3 family, is highly expressed in brown and white adipose tissue, liver and pancreatic- β cells [1], and has been shown to have an important role in the regulation of lipid and glucose metabolism in intact cells as well as in vivo [2]. Although it is well established that insulin as well as hormones that increase cAMP induce activation of PDE3B in adipocytes and hepatocytes, mechanisms involved in the regulation of PDE3B have been studied mainly in adipocytes. Thus, insulin-stimulation of adipocytes induces serine phosphorylation and activation of PDE3B which involves tyrosine phosphorylation of insulin receptor substrates (IRS), activation of phosphoinositide 3-kinase (PI3K), and generation of phosphatidylinositol 3,4/3,4,5 phosphate [3, 4]. Downstream of PI3K, protein kinase B (PKB) appears to be an important insulin-stimulated kinase phosphorylating PDE3B [5-7]. Stimulation of adipocytes with isoproterenol and other cAMP-increasing agents also leads to serine phosphorylation and activation of PDE3B which is believed to be important in feedback regulation of cAMP.

Regarding the identity of sites phosphorylated in PDE3B in response to hormone stimulation of cells, the results from adipocytes are incomplete and partly contradictory, and there is to our knowledge no information on the phosphorylation of the 135 kDa PDE3B in hepatocytes. Two-dimensional tryptic phosphopeptide mapping of endogenous PDE3B from primary rat adipocytes identified S302 (corresponding to S296 in mouse) as a major site phosphorylated in adipocytes in response to both insulin and isoproterenol [8]. In another study using a site-directed mutagenesis approach in 3T3-L1 mouse adipocytes, S273 was shown to be important for insulin-mediated activation of PDE3B whereas S296 was demonstrated to be important for isoproterenol but not insulin-mediated activation of the enzyme [7]. The small amount of phosphorylated protein/peptide that can be obtained from endogenous phospho-PDE3B is a limiting factor for detailed studies on PDE3B phosphorylation. In this work we have developed and used an adenoviral system to express flag-tagged PDE3B in primary rat adipocytes and cultured H4IIE hepatoma cells. We show that PDE3B in adipocytes as well as hepatocytes is subjected to multisite phosphorylation, and a number of new sites have been identified.

2. Materials and methods

2.1. Material

Dulbecco's modified Eagle medium (DMEM) and foetal calf serum (FBS) were obtained from GIBCO BRL (Praisley, UK); ^{32}P i was purchased from Amersham Bioscience (Little Chalfont, UK); anti-flag M2 affinity gel and bovine serum albumin (BSA) from Sigma (Saint Louis, USA); calyculin A from Alexis Biochemicals (Läufelfingen, Switzerland); sequencing grade modified trypsin from Promega (Madison, USA); $\text{C}_{13}\text{E}_{12}$ from Berol Kemi AB (Stenungssund, Sweden); insulin from Novo Nordisk (Copenhagen, Denmark); BCA protein assay reagent from Pierce (Rockford, USA) H4IIE rat hepatoma cells from ATCC (Manassas, USA) and Sprague-Dawley rats from B&K Universal (Stockholm, Sweden). OPC3911 was a kind gift from Otsuka America Pharmaceutical (Rockville, USA). The β -galactosidase adenovirus was kindly provided by Drs. C. B. Newgard and H. Mulder and the flag-tagged PDE3B adenovirus were produced as described by L. Härndahl et al [9]. The PDE3B antibodies were polyclonal and raised in rabbit against the peptide CGYYGSGKMFRRPSLP from PDE3B.

2.2. Preparation, infection, stimulation and ^{32}P i-labeling of primary rat adipocytes

Adipocytes were prepared from epididymal adipose tissue of 36-43-day old male Sprague-Dawley rats according to Rodbell [10] with modifications according to Honnor et al [11]. The cells (12.5 % cell suspension) were suspended in DMEM with 5 % FBS and 0.5 % BSA and infected with β -galactosidase adenovirus (Ad β -gal, 5.0×10^7 virus/ml) or murine flag-tagged PDE3B adenovirus (AdPDE3B, 6.5×10^7 virus/ml) for 6 or 14 hours at 37°C. The infected cells were washed and subjected to subcellular fractionation as described by Simpson et al [12] with modifications as described by Göransson et al [13], or resuspended in Krebs-Ringer medium pH 7.5, 25 mM HEPES, 200 nM adenosine, 2 mM glucose and 1 % BSA (0.3-1.5 ml 10 % cell suspension). For ^{32}P i-labeling, the medium was supplemented with 2.5 % BSA, 600 μM KH_2PO_4 (2-4 ml 10 % cell suspension) and ^{32}P i (0.2-0.5 mCi/ml). The resuspended cells were stimulated at 37°C, homogenized at room temperature in 0.6-2 ml of buffer A consisting 50 mM TES, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1 mM EGTA, antipain (10 $\mu\text{g}/\text{ml}$), leupeptin (10 $\mu\text{g}/\text{ml}$) and pepstatin A (1 $\mu\text{g}/\text{ml}$), and placed on ice. Homogenates from labeled cells was centrifuged at $175\,000 \times g$ for 1 h at 4°C and the fat cake and supernatant removed. The crude membrane fraction was rehomogenized in 0.75-1 ml buffer A supplemented with 1% $\text{C}_{13}\text{E}_{12}$, 80 mM NaF

and 80 mM NaCl, put on ice for 1 h and centrifuged ($8\,000 \times g$, 10 min, 4°C). The supernatant was mixed with 50-70 μl packed anti-flag M2 affinity agarose for 2.5-4 h at 4°C . The gel was washed five times with 500 μl of buffer A and flag-PDE3B was either eluted by boiling the gel for 15 min with 90 μl of buffer A supplemented with 1.6 % SDS and 1 mg/ml BSA, or by adding trypsin as described below.

2.3. Preparation, infection, stimulation and ^{32}P -labeling of H4IIE hepatoma cells

H4IIE hepatoma cells were propagated and maintained in DMEM medium supplemented with 10% FBS, and 2 mM glutamine. The cells (1×10^7 cells in 100 mm petri dishes) were infected with AdPDE3B (2×10^9 virus/ml) or Ad β -gal (2×10^9 virus/ml) in DMEM medium without serum for 3 h before the medium was supplemented with 10% FBS and cultured for another 45 h. After infection, H4IIE cells were washed 3 times with PBS and incubated for 16 h in DMEM without serum. The cells were then incubated for 2 h with serum-free DMEM containing 300 μM KH_2PO_4 and ^{32}P (0.2 mCi/ml), before stimulation. The H4IIE cells were suspended in 3-4 ml 50 mM HEPES (pH 7.5), 250 mM sucrose, 1mM EDTA, 10 mM pyrophosphate, 5 mM NaF, 100 mM NaCl, 1 mM Na_3VO_4 , Roche protease inhibitor cocktail, and homogenized in a Dounce homogenizer containing 1% Nonidet P-40. Homogenates were sonicated (1 x 5 pulses, output 2, 30% cycle) at 4°C , kept on a rotator for 30 min at 4°C , and centrifuged ($12\,000 \times g$, 20 min, 4°C) to remove insoluble material. Protein was measured using BCA protein assay reagent with BSA as standard. Approximately 3-8 mg proteins isolated from H4IIE cells infected with Ad β -gal or AdPDE3B were immunoprecipitated with 50 or 150 μl of anti-flag M2 affinity gel overnight. The precipitate was washed four times and eluted by boiling in Laemmli sample buffer for further analysis.

2.4. Trypsinization, peptide extraction and two dimensional (2-D) phosphopeptide mapping of tryptic ^{32}P -labeled flag-PDE3B from primary rat adipocytes and H4IIE hepatoma cells

Adipocyte ^{32}P -labeled PDE3B bound to anti-flag M2 affinity gel was trypsinized at 37°C for 16 h with shaking in 210 μl containing 48 mM ammonium bicarbonate, 48 μM hydrogenchloride and 4.8 $\mu\text{g}/\text{ml}$ trypsin. The tryptic digests were removed and dried by vacuum centrifugation. Gel bands of $\sim 1.5 \times 6$ mm containing approximately 200-300 ng immunoprecipitated PDE3B from H4IIE hepatoma cells were washed with HPLC grade water, HPLC grade methanol and finally

with 50% acetonitrile (ACN) (750 μ l/tube with gentle shaking for 2-3 min). The gel pieces were dried for 30 min in a SC 110A SpeedVac[®] Plus (ThermoSavant) and incubated in 80 μ l containing 100 mM ammonium bicarbonate and 5.0 μ g/ml trypsin at 37°C. After 3 h, additional trypsin was added to the final concentration 9.0 μ g/ml, and the gel pieces were incubated for 12 h at 37°C. The digest solution was transferred to a polypropylene tube and extracted with 60 μ l of 0.1% TFA and 50% ACN for 30 min at 37°C with shaking. The extracts were evaporated under vacuum and analyzed with MS/MS analysis after nano-liquid chromatography separation or subjected to 2-D tryptic phosphopeptide mapping exactly as described in [8].

2.5. Mass spectrometry (MS) analysis of phosphorylated adipocyte PDE3B peptides from 2-D tryptic phosphopeptide maps

Phosphopeptides from the 2-D tryptic phosphopeptide maps (originating from 12 ml 10% adipocyte cellsuspension) were scraped out and mixed with 200 μ l 20% ACN and 0.1% formic acid for 10 min and subjected to an ultrasonic bath for 10 min. The samples were centrifuged (40 000 x g for 20 min) and the supernatants vacuum centrifuged to complete dryness. The dried peptide fractions were dissolved in 8 μ l 1% formic acid in H₂O and 6 μ l were subjected to High Performance Liquid Chromatography (HPLC)-tandem MS (MS/MS) analysis. In some cases, the remaining 2 μ l were mixed with 1 μ l MALDI matrix (5 mg alpha-cyano-4-hydroxycinnamic acid/ml in 50% ACN, 0.1% trifluoroacetic acid) and spotted on MALDI target plates. During HPLC-MS/MS the peptides were separated on an Atlantis dC18 NanoEase column (0.075 x 150 mm, 3 μ m particle size) connected to a CapLC pump coupled to a Q-ToF Ultima mass spectrometer (Waters UK Ltd., Manchester, UK), using a 40 minute linear gradient from 6% ACN to 55% ACN in H₂O, 0.1% formic acid with a flow rate of 0.25 μ l/min. The samples spotted on MALDI target plates were analyzed using a Finnigan LTQ linear ion trap mass spectrometer equipped with a vMALDI ion source controlled by Excalibur 1.4 (Thermo Electron Corporation, San José, CA, USA). The instrument was set to perform an MS survey scan (m/z 900-2000) followed by MS/MS scans of the 15 most abundant ions in each sample and to automatically switch to MS³ when detecting a neutral loss of 98 Da, thus performing both MS/MS and MS³ on potential phosphopeptides. The acquired MS/MS data from the Q-ToF Ultima was converted to PKL files using ProteinLynx 2.2 (Waters UK Ltd., Manchester, UK). The PKL files were then searched using Mascot 2.0 (Matrix Science Ltd, London, UK). The tolerance for the precursor ion mass was set to 0.2 Da and the tolerance for the fragment ion mass was set to 0.1 Da. The data from the LTQ was processed using Bioworks Browser 3.2

(Thermo Electron Corporation, San José, CA, USA) and searched using the Sequest search algorithm incorporated in Bioworks Browser. The tolerance for both the precursor and fragment ion masses was set to 1 Da. All searches were performed against the mus musculus subset of the NCBI nr database, using cysteic acid and methionine sulfone as constant modification and phosphorylation of serine, threonine and tyrosine as variable modifications. The MS/MS spectra of identified phosphopeptides were then inspected manually to see if the identification was correct and to ensure correct assignation of phosphorylation sites.

2.6. MS analysis of flag-PDE3B extracted peptides from H4IIE hepatoma cells

Peptide extracts from flag-PDE3B isolated from H4IIE hepatoma cells were analyzed using a Micromass Q-TOF Ultima Global (Micromass, Manchester, UK) in electrospray mode interfaced with an Agilent HP1100 CapLC (Agilent Technologies, Palo Alto, CA) prior to the mass spectrometer. Five to eight μ l of each digest was loaded onto a Vydac C18 MS column (100 x 0.15 mm; Grace Vydec, Hesperia, CA) and chromatographic separation was performed at 1 ml/min using the following gradient: 0-10% B over 5 min; gradient from 10-40% B over 60 min; 40-95% B over 5 min; 95% B held over 5 min (solvent A: 0.2% formic acid in water; solvent B: 0.2% formic acid in ACN). Data was processed using the MassLynx software package (version 3.5) to generate peak list files prior to in-house licensed Mascot 1.8 search at <http://biospec.nih.gov> (MatrixScience Ltd., London, UK). For ESI-MS/MS data, a tolerance of 1.0 Da was chosen for fragments. The combined NCBIInr database was searched with species restriction of mammalia. Carbamidomethyl (C), oxidation (M), phospho (ST) and phospho (Y) were chosen as variable modifications.

2.7. Additional analytical procedures

Homogenates from adipocytes and flagPDE3B-immunoprecipitates from 32 Pi-labelled adipocytes and H4IIE hepatoma cells were boiled in Laemmli sample buffer and subjected to sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE), 7-8% acrylamide. The gel was either stained with Simply Blue™ safe stain (Invitrogen) for 15 min or analyzed by digital imaging (Fuji FLA-3000, Fuji Inc., Tokyo, Japan) for 32 Pi detection. The proteins were in some experiments electrotransferred to hybond-C membranes and incubated with PDE3B antibodies as described previously [14]. PDE3 activity and lipolysis were measured as previously

described [15, 16], in duplicates. The statistical significance of differences were counted using a paired Student's t-test. Results are presented as mean \pm standard error of the mean (SEM).

3. Results

3.1. Characterization of recombinant PDE3B overexpressed in primary rat adipocytes and H4IIE hepatoma cells

Incubation of primary rat adipocytes with flag-PDE3B adenovirus (AdPDE3B) (6 or 14 hours) resulted in overexpression of PDE3B (10 or 25 fold) compared to cells incubated with β -galactosidase adenovirus (Ad β -gal) (figure 1A, B, C). Overexpression of PDE3B in H4IIE hepatoma cells after AdPDE3B infection (48 h) was 50-100 fold (figure 3A, B). The recombinant PDE3B was characterized with regard to location, regulation and biological effects. Adipocytes expressing flag-PDE3B were subjected to subcellular fractionation, and as seen in figure 1C recombinant and endogenous PDE3B (from adipocytes infected with Ad β -gal) showed identical distribution between the different subcellular compartments. Adipocytes and H4IIE cells expressing flag-PDE3B were stimulated with insulin, cAMP-increasing agents (forskolin, isoproterenol or/and OPC3911) or a phosphatase inhibitor (calyculin A), and PDE3 activity was measured in the homogenates (endogenous PDE3B in adipocyte and H4IIE cells constituted 10 % and 1% of total PDE3 activity, respectively, in all conditions). As shown in figure 2A and 3A flag-PDE3B was activated both in adipocytes and H4IIE cells stimulated with the different agents, as is the case for endogenous PDE3B [17-20]. For the analysis of PDE3B phosphorylation in adipocytes and H4IIE cells expressing flag-PDE3B, cells were incubated with 32 Pi, and flag-PDE3B was immunoprecipitated using anti-flag M2 affinity gel. As shown in figure 2B and 3C, insulin, cAMP increasing agents and calyculin A increased phosphorylation of flag-PDE3B as compared to non-stimulated adipocytes and H4IIE cells. This has previously been shown for endogenous PDE3B in adipocytes [8, 18, 19], but to our knowledge not for the 135 kDa PDE3B in hepatocytes. Finally, lipolysis was measured in AdPDE3B and Ad β -gal infected primary rat adipocytes to investigate the ability of flag-PDE3B to mediate regulation of lipolysis. As shown in figure 4, lipolysis was lower in AdPDE3B infected cells as compared to Ad β -gal infected cells. Furthermore, in AdPDE3B infected cells insulin-induced inhibition of isoproterenol mediated lipolysis was enhanced. Thus, at 0.1 nM insulin isoproterenol-induced lipolysis was lowered by 24.0 +/- 7.1 % and 40.9 +/- 4.2 % (p=0.0014) in Ad β -gal and AdPDE3B infected cells, respectively. At 0.3 nM insulin the lowering of lipolysis was 47.5 +/- 7.3 % and 55.8 +/- 5.1 % (p=0.058) in Ad β -gal and AdPDE3B infected cells, respectively. In the following, flag-PDE3B phosphorylated in response to different agents in adipocytes and H4IIE cells were used as starting material for studies of PDE3B phosphorylation.

3.2. PDE3B is subjected to multisite phosphorylation in response to insulin, cAMP increasing agents and calyculin A in primary rat adipocytes and H4IIE hepatoma cells

As a first step to study the sites phosphorylated in PDE3B in adipocytes and hepatoma cells, we generated 2-D tryptic phosphopeptide maps of flag-PDE3B phosphorylated in response to different stimuli. Primary rat adipocytes and H4IIE cells expressing flag-PDE3B were labelled with ^{32}P i and treated with insulin, cAMP-increasing agents or calyculin A. Detergent-treated membrane fractions were applied to an anti-flag M2 affinity gel. After extensive washing flag-PDE3B attached to the anti-flag M2 affinity gel was subjected to trypsin digestion (adipocyte derived material) or eluted from the column (H4IIE cells and adipocyte derived material). Eluted PDE3B was subjected to SDS-PAGE, detected by autoradiography (seen in figure 2B and 3C), cut out and digested with trypsin. The PDE3B tryptic phosphopeptides (tPP) from both adipocytes and H4IIE cells were separated in two dimensions using thin layer electrophoresis (TLE) and thin layer chromatography (TLC). Several tPPs denoted tPP1-13 were generated from the adipocyte PDE3B (figure 5A-E) and H4IIE cell PDE3B (figure 5G-J) in response to the different agents. Figure 5F represents a summary of 40 adipocyte and H4IIE PDE3B maps showing a merged picture of the tPPs reproducibly observed with the different agents. tPP2, tPP3 and tPP8-12 appear to be present in all maps from adipocytes independent of the activating stimuli. On the other hand, tPP1, which is present after insulin as well as isoproterenol +/- OPC3911 stimulation, is not present after calyculin A stimulation. Furthermore tPP7 is present only in maps from adipocytes stimulated with isoproterenol +/- OPC3911, and tPP4-6 and tPP13 appear only in maps from calyculin A stimulated cells. When comparing the two cell types, tPP2-6, tPP9-10 and tPP13 appear to be present in maps from adipocytes as well as H4IIE cells. The major differences between the adipocyte and H4IIE PDE3B maps are the lack of tPP1, tPP7 and tPP8 in the H4IIE maps. The lack of tPP1 can be explained by the fact that the tPP corresponding to tPP1 in PDE3B H4IIE maps contains one additional phosphorylation and two additional arginine (see MS/MS results below) which gives the peptide a different mobility during the TLE and TLC. Unfortunately we were not able to locate the H4IIE tPP corresponding to adipocyte tPP1 in the H4IIE maps. In the PDE3B maps generated from H4IIE cells, forskolin, which increases cAMP in the cell, interestingly stimulated similar PDE3B phosphorylation pattern as insulin (figure 5I vs. 5H).

3.3. Identification of sites phosphorylated in PDE3B in primary rat adipocytes and in H4IIE hepatoma cells

In order to identify sites phosphorylated in PDE3B from primary rat adipocytes we scraped out tPP:s from 2-D tPP maps of flagPDE3B, in most cases originating from calyculin A or isoproterenol + OPC3911 stimulated adipocytes (figure 5D and E). The tPPs were identified using mass spectrometry (HPLC-MS/MS) as described in materials and methods. To this end six out of the 13 tPP:s summarised in figure 5F have been identified (table 1). All identifications were performed using spectra from the Q-ToF Ultima. In most cases the phosphorylation site(s) of each peptide was unambiguously assigned. Flag-PDE3B tPP1-6 were found to contain phosphate on S273, S296, S421, S424/5 (peptide phosphorylated on S421 and either S424 or S425), S474 and S536, respectively (table 1). As shown in figure 5A-E, S273, S296 and S421 were phosphorylated in response to stimulation of rat adipocytes with insulin as well as isoproterenol +/- OPC391 whereas S424/5, S474 and S536 were phosphorylated mainly in response to calyculin A.

To identify sites phosphorylated in hepatocyte PDE3B, flag-PDE3B expressing H4IIE cells were stimulated with insulin or calyculin A and the recombinant enzyme was immunoisolated with anti-flag M2 affinity gel and subjected to SDS-PAGE. The 135 kDa PDE3B band detected after staining with Simply BlueTM safe stain (figure 3B) was cut out and subjected to in-gel trypsin digestion. Digested PDE3B was analysed using mass spectrometry (HPLC-MS/MS) as described in materials and methods. As shown in table 2, insulin induces phosphorylation of S273, S277, S296 and S421. In addition to those sites calyculin A induces phosphorylation of S474, S507 and S536.

4. Discussion

In this work we have identified a number of new PDE3B phosphorylation sites, all of which are localized in the regulatory domain of the enzyme (figure 6A). The results presented extensively extend the knowledge of PDE3B phosphorylation in adipocytes and are also, to our knowledge, the first report on phosphorylation of the 135 kDa PDE3B in hepatocytes (table 2). Previous studies on endogenous PDE3B phosphorylated in adipocytes indicated multisite phosphorylation of the enzyme; however, identification of several of the sites was not possible due to the limited amount of phosphorylated PDE3B [8, 19]. In order to generate sufficient amount of material for PDE3B site analysis, in this study mouse PDE3B was overexpressed in adipocytes as well as rat H4IIE cells. Importantly, the overexpressed PDE3B was carefully characterized with regard to localization, regulation and function. Thus, overexpressed PDE3B was localized to the same subcellular compartment as endogenous, i.e. in the plasma membrane and high density membrane fraction. Other important arguments with regard to the relevance for our model are that the overexpressed PDE3B is activated in response to various stimuli in the same manner as the endogenous PDE3B and mediates regulation of lipolysis. Finally, we get similar results from both adipocytes and H4IIE cells regarding phosphorylation of the overexpressed PDE3B which also support the relevance for the sites identified.

Multisite phosphorylation of PDE3B suggests complex and intricate regulatory mechanisms involving phosphorylation-dependency with regard to docking with other proteins, control of catalytic activity, subcellular localization, and protein degradation. With regard to activity control, phosphorylation of S273 was previously shown to be important for insulin-induced activation whereas S296 was shown to be important for isoproterenol-induced activation of PDE3B in 3T3-L1 adipocytes using a site directed mutagenesis strategy [7]. In the present study however, we show that both S273 and S296 are phosphorylated in response to insulin as well as isoproterenol. In agreement with these results, S302 (corresponding to S296 in mouse) in endogenous PDE3B was previously shown to be phosphorylated in response to insulin as well as isoproterenol stimulation of primary rat adipocytes [8]. As shown in the present work, insulin (table 2) as well as forskolin induced phosphorylation of S296 in H4IIE hepatoma cells (figure 5I) indeed supporting an important role for this site as a target for insulin as well as cAMP action. Also in 3T3-L1 adipocytes S296 as well as S273 seem to be important targets for insulin-induced PDE3B phosphorylation. Thus, Onuma et al [21] have demonstrated insulin dependent interactions between PDE3B and 14-3-3 proteins in 3T3-L1 adipocytes, which was disrupted by

adding PDE3B peptides phosphorylated at S296 or S273. All together these results support the importance of both S273 and S296 PDE3B phosphorylation in response to either insulin or isoproterenol in primary rat as well as mouse 3T3-L1 adipocytes. With regard to PDE3B interactions in addition to 14-3-3, we have shown insulin-dependent interactions in 3T3-L1 adipocytes between phosphorylated PDE3B and a number of insulin signalling components such as IRS-1, PI3K p85 and PKB (Ahmad et al, unpublished data). PDE3B has also been shown to interact with the insulin receptor in a hormone dependent manner in human adipocytes [22] and in a hormone insensitive manner with PI3K γ in mouse heart [23], caveolae in rat adipocytes [14], and a 47 kDa protein of unknown function in 3T3-L1 adipocytes [24, 25]. The interactions of PDE3B with several other proteins both in a hormone sensitive and hormone insensitive manner are probably very important. We suggest that PDE3B is regulated by an intricate system of phosphorylations some of which are activity controlling whereas others regulate interactions with other proteins.

Exactly which kinases are responsible for the phosphorylations of PDE3B is not known. The finding that some of the sites in adipocytes and hepatocytes, respectively, can be phosphorylated in response to a broad range of stimuli indicate signal integration of signalling pathways upstream of PDE3B, maybe at the level of PKB. Indeed, PKB, which has been suggested to be a PDE3B kinase, is known to be phosphorylated/activated both by insulin, β -adrenergic agonists and phosphatase inhibitors in adipocytes [26-28] supporting a role for PKB in mediating, at least partly, the isoproterenol effect on PDE3B. This could thus give a mechanistic explanation to why isoproterenol and insulin induce phosphorylation of a set of sites that are the same. On the other hand, isoproterenol, but not insulin, induces the appearance of tPP7 in adipocyte PDE3B maps indicating the presence also of isoproterenol-dependent but PKB-independent control of PDE3B phosphorylation. Unfortunately we have not been able to identify tPP7 (radio sequencing indicates that amino acid 3 and/or 4 is phosphorylated). We did not detect tPP7 in PDE3B maps from forskolin stimulated H4IIE cells which represents one of the few differences when comparing sites phosphorylation in PDE3B in adipocytes and hepatocytes. When performing a search for possible PKB phosphorylation sites within PDE3B using Scansite 2.0, which utilizes short sequence motifs to predict possible cell signalling interactions based on phage-display and oriented peptide library [29] we found with medium stringency four PKB phosphorylation sites with similar scores S273, S296, S474 and S378. Thus, three out of four sites localized within a consensus sequence for phosphorylation by PKB were identified in this work namely S273, S296 and S474 (figure 6B).

Although one hypothesis is that isoproterenol mediates its effect via PKB at least to some extent, PDE3B could also be a direct target for PKA, a kinase previously shown to phosphorylate rat adipocyte PDE3B on S427 (corresponding to S421 in mouse) in vitro [30]. S421 is a site identified also in this work (phosphorylated by all stimuli) and is situated in a typical PKA phosphorylation consensus sequence (figure 6B). In addition, three other sites situated in consensus sequences for phosphorylation by PKA appeared when performing a search using scansite 2.0, namely S273, S274 and S296. Thus, S273 as well as S296 are potential targets for PKB as well as PKA catalyzed phosphorylations, which is interesting in the context of interplay between insulin and isoproterenol mediated regulation of PDE3B. In this perspective one should also have in mind that β -adrenergic agonists can mediate cAMP-dependent as well as cAMP-independent signalling events and cAMP-dependent signalling can be PKA-dependent as well as PKA-independent, the latter via Epac (GTP exchange factor activated by cAMP).

With regard to S277 (H4IIE cells), S507 (H4IIE cells), S424/425 (adipocytes and H4IIE-tPP4), and S536 (adipocytes and H4IIE cells), kinases and phosphatases involved in controlling the phosphorylation state of these sites are completely unknown. We have previously shown that PP2A has an important role in the phosphorylation and activation of PDE3B [19]. In this study it is interesting to observe that pre-treatment of adipocytes and H4IIE cells with calyculin A, which inhibits PP1 and PP2A [19], generates almost identical phosphorylation patterns of PDE3B in the two cell-types. In a previous study it was also shown that pre-treatment of rat adipocytes with okadaic acid (a PP2A inhibitor) and calyculin A induced slightly different phosphorylation patterns of endogenous PDE3B which is in agreement with findings from overexpressed PDE3B (data not shown). Thus, the role for different phosphatases and kinases in the regulation of PDE3B phosphorylation will be further evaluated. Finally, we have not yet been able to identify the sites phosphorylated in tPP 7-13.

We conclude that PDE3B is subjected to multisite phosphorylation in response to hormones in adipocytes as well as hepatocytes, which was recently also reported for PDE3A [31]. The elucidation of the regulation of PDE3B phosphorylation is important as part of understanding the cAMP and insulin signalling networks in adipocytes and hepatocytes which play a key role in the regulation of lipolysis, glucose uptake and lipogenesis in adipocytes and glycogenolysis and glyconeogenesis in hepatocytes.

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References

- [1] E. Degerman, and V. Manganiello, Phosphodiesterase 3B: An Important Regulator of Energy Homeostasis, in: J.A.Beavo, S. Francis, M. Houslay (Eds.), *Cyclic Nucleotide Phosphodiesterases in Health and Disease*, ed., Taylor & Francis CRC Press 2007.
- [2] Y. Shakur, L. S. Holst, T. R. Landstrom, M. Movsesian, E. Degerman, and V. Manganiello, Regulation and function of the cyclic nucleotide phosphodiesterase (PDE3) gene family, *Prog Nucleic Acid Res Mol Biol* 66 (2001) 241-277.
- [3] T. Rahn, M. Ridderstrale, H. Tornqvist, V. Manganiello, G. Fredrikson, P. Belfrage, and E. Degerman, Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes. Studies using the selective inhibitor wortmannin, *FEBS Lett* 350 (1994) 314-318.
- [4] T. Okada, Y. Kawano, T. Sakakibara, O. Hazeki, and M. Ui, Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin, *J Biol Chem* 269 (1994) 3568-3573.
- [5] J. Wijkander, T. R. Landstrom, V. Manganiello, P. Belfrage, and E. Degerman, Insulin-induced phosphorylation and activation of phosphodiesterase 3B in rat adipocytes: possible role for protein kinase B but not mitogen-activated protein kinase or p70 S6 kinase, *Endocrinology* 139 (1998) 219-227.
- [6] F. Ahmad, L. N. Cong, L. Stenson Holst, L. M. Wang, T. Rahn Landstrom, J. H. Pierce, M. J. Quon, E. Degerman, and V. C. Manganiello, Cyclic nucleotide phosphodiesterase 3B is a downstream target of protein kinase B and may be involved in regulation of effects of protein kinase B on thymidine incorporation in FDCP2 cells, *J Immunol* 164 (2000) 4678-4688.
- [7] T. Kitamura, Y. Kitamura, S. Kuroda, Y. Hino, M. Ando, K. Kotani, H. Konishi, H. Matsuzaki, U. Kikkawa, W. Ogawa, and M. Kasuga, Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt, *Mol Cell Biol* 19 (1999) 6286-6296.
- [8] T. Rahn, L. Ronnstrand, M. J. Leroy, C. Wernstedt, H. Tornqvist, V. C. Manganiello, P. Belfrage, and E. Degerman, Identification of the site in the cGMP-inhibited phosphodiesterase phosphorylated in adipocytes in response to insulin and isoproterenol, *J Biol Chem* 271 (1996) 11575-11580.
- [9] L. Harndahl, X. J. Jing, R. Ivarsson, E. Degerman, B. Ahren, V. C. Manganiello, E. Renstrom, and L. S. Holst, Important role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic beta-cell exocytosis and release of insulin, *J Biol Chem* 277 (2002) 37446-37455.
- [10] M. Rodbell, Metabolism of Isolated Fat Cells. I. Effects of Hormones on Glucose Metabolism and Lipolysis, *J Biol Chem* 239 (1964) 375-380.
- [11] R. C. Honnor, G. S. Dhillon, and C. Londos, cAMP-dependent protein kinase and lipolysis in rat adipocytes. I. Cell preparation, manipulation, and predictability in behavior, *J Biol Chem* 260 (1985) 15122-15129.
- [12] I. A. Simpson, D. R. Yver, P. J. Hissin, L. J. Wardzala, E. Karnieli, L. B. Salans, and S. W. Cushman, Insulin-stimulated translocation of glucose transporters in the isolated rat adipose cells: characterization of subcellular fractions, *Biochim Biophys Acta* 763 (1983) 393-407.
- [13] O. Goransson, J. Wijkander, V. Manganiello, and E. Degerman, Insulin-induced translocation of protein kinase B to the plasma membrane in rat adipocytes, *Biochem Biophys Res Commun* 246 (1998) 249-254.

- [14] R. Nilsson, F. Ahmad, K. Sward, U. Andersson, M. Weston, V. Manganiello, and E. Degerman, Plasma membrane cyclic nucleotide phosphodiesterase 3B (PDE3B) is associated with caveolae in primary adipocytes, *Cell Signal* 18 (2006) 1713-1721.
- [15] V. C. Manganiello, F. Murad, and M. Vaughan, Effects of lipolytic and antilipolytic agents on cyclic 3',5'-adenosine monophosphate in fat cells, *J Biol Chem* 246 (1971) 2195-2202.
- [16] O. Wieland, [Enzymic method for the determination of glycerin.], *Biochem Z* 329 (1957) 313-319.
- [17] S. Boyes, and E. G. Loten, Purification of an insulin-sensitive cyclic AMP phosphodiesterase from rat liver, *Eur J Biochem* 174 (1988) 303-309.
- [18] E. Degerman, C. J. Smith, H. Tornqvist, V. Vasta, P. Belfrage, and V. C. Manganiello, Evidence that insulin and isoprenaline activate the cGMP-inhibited low-Km cAMP phosphodiesterase in rat fat cells by phosphorylation, *Proc Natl Acad Sci U S A* 87 (1990) 533-537.
- [19] S. Resjo, A. Oknianska, S. Zolnierowicz, V. Manganiello, and E. Degerman, Phosphorylation and activation of phosphodiesterase type 3B (PDE3B) in adipocytes in response to serine/threonine phosphatase inhibitors: deactivation of PDE3B in vitro by protein phosphatase type 2A, *Biochem J* 341 (Pt 3) (1999) 839-845.
- [20] S. J. Beebe, J. B. Redmon, P. F. Blackmore, and J. D. Corbin, Discriminative insulin antagonism of stimulatory effects of various cAMP analogs on adipocyte lipolysis and hepatocyte glycogenolysis, *J Biol Chem* 260 (1985) 15781-15788.
- [21] H. Onuma, H. Osawa, K. Yamada, T. Ogura, F. Tanabe, D. K. Granner, and H. Makino, Identification of the insulin-regulated interaction of phosphodiesterase 3B with 14-3-3 beta protein, *Diabetes* 51 (2002) 3362-3367.
- [22] C. M. Rondinone, E. Carvalho, T. Rahn, V. C. Manganiello, E. Degerman, and U. P. Smith, Phosphorylation of PDE3B by phosphatidylinositol 3-kinase associated with the insulin receptor, *J Biol Chem* 275 (2000) 10093-10098.
- [23] E. Patrucco, A. Notte, L. Barberis, G. Selvetella, A. Maffei, M. Brancaccio, S. Marengo, G. Russo, O. Azzolino, S. D. Rybalkin, L. Silengo, F. Altruda, R. Wetzker, M. P. Wymann, G. Lembo, and E. Hirsch, PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects, *Cell* 118 (2004) 375-387.
- [24] J. A. Chavez, S. Gridley, H. Sano, W. S. Lane, and G. E. Lienhard, The 47kDa Akt substrate associates with phosphodiesterase 3B and regulates its level in adipocytes, *Biochem Biophys Res Commun* 342 (2006) 1218-1222.
- [25] H. Onuma, H. Osawa, T. Ogura, F. Tanabe, W. Nishida, and H. Makino, A newly identified 50 kDa protein, which is associated with phosphodiesterase 3B, is phosphorylated by insulin in rat adipocytes, *Biochem Biophys Res Commun* 337 (2005) 976-982.
- [26] J. Wijkander, L. S. Holst, T. Rahn, S. Resjo, I. Castan, V. Manganiello, P. Belfrage, and E. Degerman, Regulation of protein kinase B in rat adipocytes by insulin, vanadate, and peroxovanadate. Membrane translocation in response to peroxovanadate, *J Biol Chem* 272 (1997) 21520-21526.
- [27] F. C. Mei, J. Qiao, O. M. Tsygankova, J. L. Meinkoth, L. A. Quilliam, and X. Cheng, Differential signaling of cyclic AMP: opposing effects of exchange protein directly activated by cyclic AMP and cAMP-dependent protein kinase on protein kinase B activation, *J Biol Chem* 277 (2002) 11497-11504.
- [28] E. Zmuda-Trzebiatowska, V. Manganiello, and E. Degerman, Novel mechanisms of the regulation of Protein kinase B in adipocytes; implications for Protein kinase A, Epac, Phosphodiesterases 3 and 4, *Cell Signal* (2006).

- [29] J. C. Obenauer, L. C. Cantley, and M. B. Yaffe, Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs, *Nucleic Acids Res* 31 (2003) 3635-3641.
- [30] A. Rascon, E. Degerman, M. Taira, E. Meacci, C. J. Smith, V. Manganiello, P. Belfrage, and H. Tornqvist, Identification of the phosphorylation site in vitro for cAMP-dependent protein kinase on the rat adipocyte cGMP-inhibited cAMP phosphodiesterase, *J Biol Chem* 269 (1994) 11962-11966.
- [31] M. Pozuelo Rubio, D. G. Campbell, N. A. Morrice, and C. Mackintosh, Phosphodiesterase 3A binds to 14-3-3 proteins in response to PMA-induced phosphorylation of Ser428, *Biochem J* 392 (2005) 163-172.

Fig. 1. Expression and subcellular localization of flag-PDE3B in rat adipocytes. Primary rat adipocytes were incubated for 6h with β -galactosidase adenovirus (Ad β -gal) or flag-PDE3B adenovirus (AdPDE3B), homogenized, and analysed either by immunoblotting (20 μ g protein = 2.7 μ l PCV /lane) using anti-PDE3B antibodies (A) or by measuring PDE3 activity (B) (n = 7). (C) primary rat adipocytes were infected for 14 h with Ad β -gal or AdPDE3B, and subjected to subcellular fractionation before PDE3 activity was measured (n = 4). PCV; packed cell volume, results are shown as mean \pm SEM.

Fig. 2. Phosphorylation and activation of flag-PDE3B in rat adipocytes in response to insulin, isoproterenol, OPC3911 and calyculin A. Isolated primary rat adipocytes expressing flag-PDE3B were incubated with (B) or without (A) 32 Pi before stimulation with 2 nM insulin (Ins) for 10 min, 300 nM isoproterenol (Iso) for 10 min, 10 μ M OPC3911 (OPC) for 20 min, 10 μ M OPC for 20 min plus 300 nM iso for the last 10 min, 200nM calyculin A (CalA) for 30 min or without stimuli (Ctrl). (A) the cells were homogenized and PDE3 activity measured (n = 8, from the left p< 0.023, p<0.048, p<0.007, p<0.006 and p<0.003). (B) flag-PDE3B was immunoprecipitated with anti-flag agarose and subjected to SDS-PAGE and autoradiography. One representative gel is shown; quantification of the bands were made using the Fuji LAS 2000 Plus system (lower panel of (B)). The ratios are presented as fold increase in comparison to control, mean \pm SEM (n = 7 except for OPC and CalA n=3, from the left p<0.0023, p<0.043, p<0.14, p<0.0077, p<0.035).

Fig. 3. Phosphorylation and activation of flag-PDE3B in H4IIE hepatoma cells in response to insulin, forskolin or calyculin A. H4IIE hepatoma cells were infected with AdPDE3B or Ad β -gal for 48 h. Cells were incubated with or without 100 nM insulin (Ins) for 10 min, 100 μ M forskolin (Forsk) or 100 nM calyculin A for 15 min and then homogenized. (A) PDE3 activity (mean \pm /-

SEM, $n=5$, $p<0.001$) was measured in the homogenates. (B) flagPDE3B was immunoprecipitated with anti-Flag-agarose. After SDS-PAGE, the gel was stained with Simply Blue™ (Invitrogen) to detect the 135 kDa PDE3B protein. (C) H4IIE cells were incubated with ^{32}P i (200 $\mu\text{Ci}/\text{ml}$) for 120 min at 37°C before stimulation with insulin, forskolin or calyculin A. After PDE3B immunoisolation one portion was used for SDS-PAGE and detection of ^{32}P -phosphorylated PDE3B and another portion for SDS-PAGE and immunoblot analysis.

Fig. 4. PDE3B overexpression in rat adipocytes lowers basal lipolysis, and enhances insulin-induced inhibition of lipolysis. Isolated primary rat adipocytes infected with AdPDE3B or Ad β -Gal adenoviruses for 6 h were incubated with 30 nM isoproterenol (Iso) alone or together with 0.1-0.3 nM insulin (Ins), or without any stimuli (Ctrl) as indicated. Glycerol release was determined after 30 min (mean \pm SEM, $n=7$, $p<0.001$).

Fig. 5. Multisite phosphorylation of PDE3B in rat adipocytes and H4IIE hepatoma cells in response to insulin, cAMP increasing agents and calyculin A. (A-E): Isolated primary rat adipocytes expressing flag-PDE3B were labelled with ^{32}P i and incubated with 2 nM insulin (Ins) for 10 min, 300 nM isoproterenol (Iso) for 10 min, 10 μM OPC3911 for 20 min together with 300 nM isoproterenol (Iso+OPC) during the last 10 min, or 200 nM calyculin A (Cal A) for 20 min or without stimuli (Ctrl). Flag-PDE3B was immunoisolated before trypsin digestion. (G-J): H4IIE hepatoma cells expressing flag-PDE3B were labelled with ^{32}P i and incubated for 15 min with 100 nM insulin (Ins), 100 μM forskolin (Forsk), 100 nM calyculin A (Cal A) or without stimuli (Ctrl). Flag-PDE3B was immunoisolated, subjected to SDS-PAGE, extracted and digested with trypsin, All of the tryptic digests (loaded on the down left corner of each map in the figure) were subjected to thin layer electrophoresis (TLE, 2000 V, 35 min) and thin layer chromatography (TLC, isobutyric buffer); digital imaging was then

performed to detect ^{32}P i. (F) The tryptic phosphopeptides (tPP) from all maps are summarised and denoted 1-13. Representative maps are shown for each condition (n = 6 for A-E, n = 2 for G-J).

Table 1

Identification of sites phosphorylated in mouse PDE3B expressed in primary rat adipocytes

Tryptic phosphopeptides from Flag-PDE3B originating from adipocytes stimulated with calyculin A or Isoproterenol + OPC3911 were extracted from the 2-D phosphopeptide maps and identified. Observed ions used for the assignment of phosphorylation site(s) (ion designation-mass); tPP1: tPP1:b2-255.04, b Δ 98-157.06, b3-406.05, b3 Δ 98-308.05; tPP2: y2-262.14, b3-323.17; tPP3: b2-244.14, b*2-227.11, b $^{\circ}$ 2-226.12, a2-216.14, a*2-199.12, a $^{\circ}$ 2-198.13; tPP4: b2-244.14, b*2-227.11, b $^{\circ}$ 2-226.12, a2-216.14, a*2-199.12, a $^{\circ}$ 2-198.13 y2- y3- y4-; tPP5: b2-175.06 ,b*2-158.06, b $^{\circ}$ 2-157.06, a2-147.07; tPP6: MS2 y11-1294.5, y12-1393.4 MS3 y10-1207.7, y13-1462.5, y14-1519.7.

Table 2

Identification of sites phosphorylated in mouse PDE3B expressed in H4IIE hepatoma cells

Flag-PDE3B from H4IIE hepatoma cells infected with AdPDE3B and stimulated with 100 nM insulin (Ins) for 10 min or 100 nM calyculin A (CalA) for 15 min were isolated by anti-flag agarose, and subjected to SDS-PAGE. The 135 kDa band seen in figure 3C was subjected to trypsin digestion and the peptides were identified. Average total sequence coverage for flag-PDE3B after tryptic analysis were 28, 35 and 44 % in Ctrl, Ins and CalA treated samples respectively (n=3). Observed ions used for the assignment of phosphorylation site(s) (ion

designation-mass); S273, S277: b⁺⁺6-364.68, y6-674.31; S296: b3-323.18, y6-745.40; S421: b2-244.14, b3-313.16; S474: b2-175.07, b3-244.09; S507: y*3-373.18, y6-629.34; S536: b3-258.18, b4-345.18.

Fig. 6. PDE3B phosphorylation and putative kinases involved. (A) The phosphorylation sites identified are localized within the regulatory domain of PDE3B. Two of the serines (S) have been identified only in hepatoma cells (○). NHR (N-terminal hydrophobic region) 1 contains 5 helical transmembrane regions and NHR2 a small hydrophobic region. (B) Sequences surrounding the phosphorylated sites are shown. Some of the sequences contain consensus sequences for phosphorylation by PKB and PKA, and for binding to 14-3-3 proteins, as indicated.

Table 1

Identification of sites phosphorylated in mouse PDE3B expressed in primary rat adipocytes

Spot	Sequence	Residues (start-end)	Phospho-Serine (S)
tPP1	pSSCaVSLGESAAAGYYGSGK	273-290	S273
tPP2	RPpSLPCaISR	294-302	S296
tPP3	RSpSGASSLLTNEHCaSR	419-434	S421
tPP4	RSpSGAp[SS]LLTNEHCaSR	419-434	S421, S424/5
tPP5	SSpSVSLTHHAGLR	472-484	S474
tPP6	SLGpSVSSAADFHQYLR	533-548	S536

Table 2

Identification of sites phosphorylated in mouse PDE3B expressed in H4IIE hepatoma cells

Phospho-Serine (S)	Sequence	Residues (start-end)	Ins	Cal A
S273, S277	RRpSSCVpSLGESAAAGYYGSGK	271-290	X	X
S296	RPpSLPCISR	294-302	X	X
S421	RSpSGASSLLTNEHCSR	419-434	X	X
S474	SSpSVSLTHHAGLR	472-484	-	X
S507	RAGALPSHLLNSSSHVPVSAGpSLTNR	485-511	-	X
S536	SLGpSVSSAADFHQYLR	533-548	-	X

Figure 1

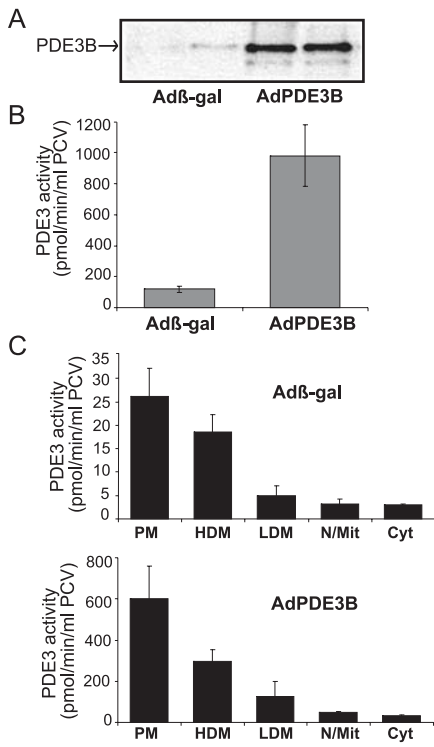


Figure 2

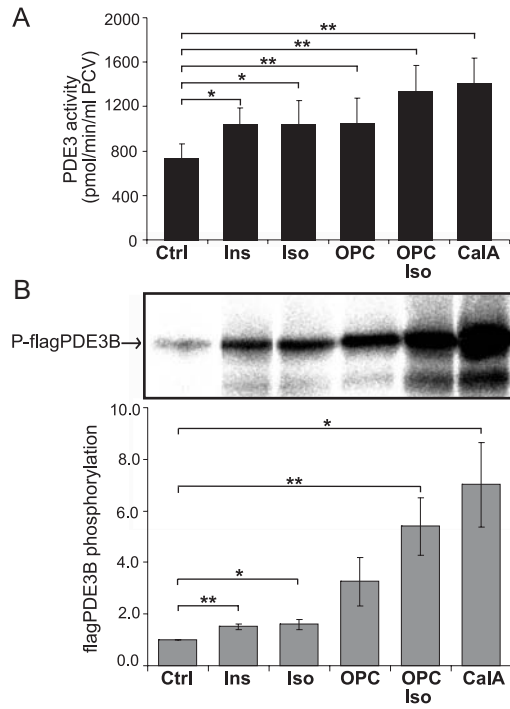


Figure 3

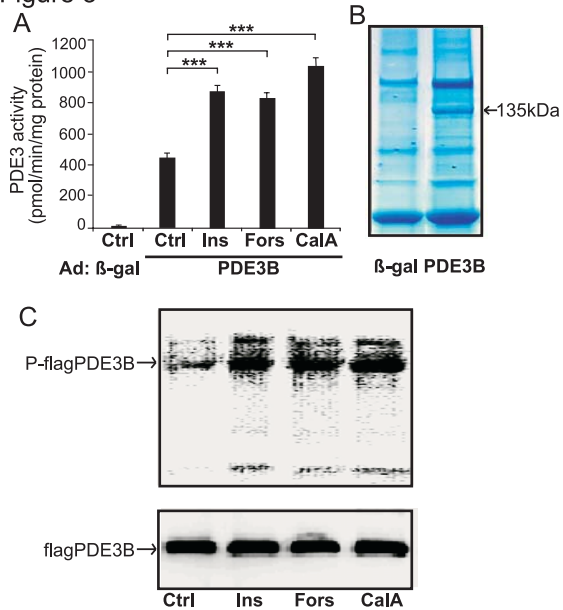


Figure 4

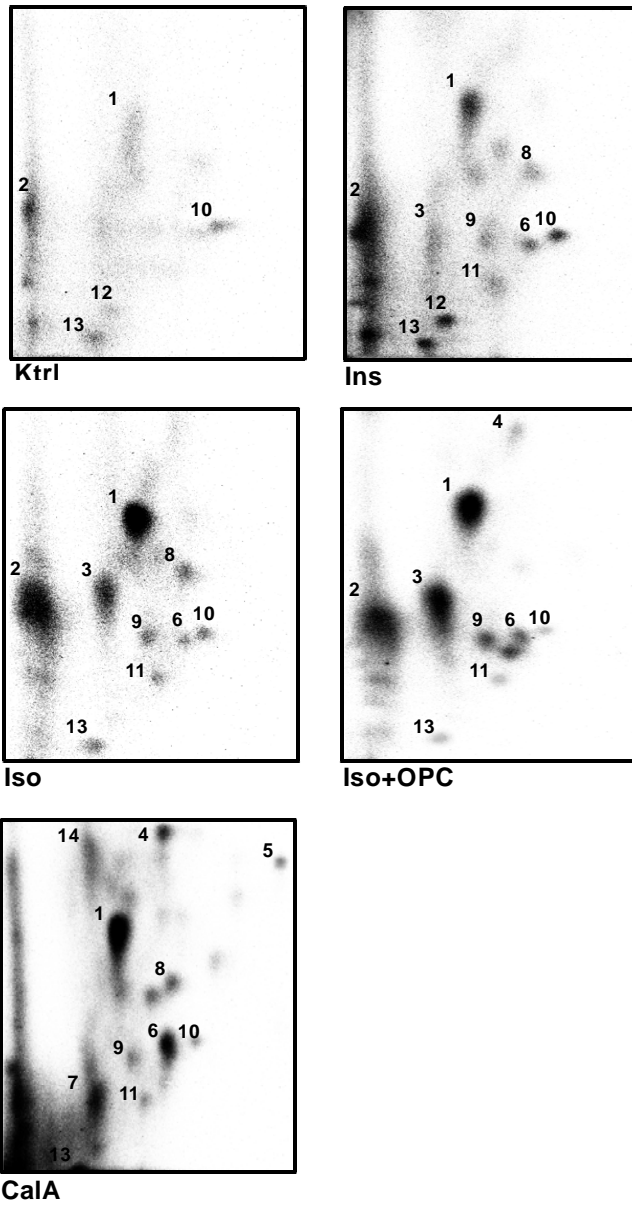


Figure 6

