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

Svante RESJÖ *[1], Alina OKNIANSKA†, Stanislaw ZOLNIEROWICZ†, Vincent MANGANIELLO‡ and Eva DEGERMAN*

*Section for Molecular Signalling, Department of Cell and Molecular Biology, Lund University, P. O. Box 94, S-221 00 Lund, Sweden, †Intercollegiate Faculty of Biotechnology UG-MUG, Debinki 1, 80-211 Gdansk, Poland, and ‡Pulmonary/Critical-Care Medicine Branch, NHLBI, National Institutes of Health, Bethseda, MD 20892, U.S.A.

Phosphodiesterase type 3B (PDE3B) has been shown to be activated and phosphorylated in response to insulin and hormones that increase cAMP. In order to study serine/threonine protein phosphatases involved in the regulation of rat adipocyte PDE3B, we investigated the phosphorylation and activation of PDE3B *in vivo* in response to phosphatase inhibitors and the dephosphorylation and deactivation of PDE3B *in vitro* by phosphatases purified from rat adipocyte homogenates. Okadaic acid and calyculin A induced dose- and time-dependent activation of PDE3B. Maximal effects were obtained after 30 min using 1 μM okadaic acid (1.8-fold activation) and 300 nM calyculin A (4-fold activation), respectively. Tautomycin and cyclosporin A did not induce activation of PDE3B. Incubation of adipocytes with 300 nM calyculin A inhibited protein phosphatase (PP) 1 and PP2A completely. Okadaic acid (1 μM) reduced PP2A activity by approx. 50 % but did not affect PP1 activity, and 1 μM tautomycin reduced PP1 activity by approx. 60 % but PP2A activity by only 11 %. This indicates an important role for PP2A in the regulation of PDE3B. Furthermore, rat adipocyte PDE3B phosphatase activity co-purified with PP2A but not with PP1 during MonoQ chromatography. As compared with insulin, okadaic acid and calyculin A induced phosphorylation of PDE3B by 2.8- and 14-fold respectively, whereas tautomycin and cyclosporin A had no effect. Both calyculin A and okadaic acid induced phosphorylation on serine 302, the site known to be phosphorylated on PDE3B in response to insulin and isoproterenol (isoprenaline), as well as on sites not identified previously. In summary, PP2A seems to be involved in the regulation of PDE3B *in vivo* and can act as a PDE3B phosphatase *in vitro*. In comparison with insulin, calyculin A induced a dramatic activation of PDE3B and both calyculin A and okadaic acid induced phosphorylation on additional sites, which could have a role in signalling pathways not yet identified.

Key words: calyculin A, insulin signalling, lipolysis, okadaic acid.

INTRODUCTION

By catalysing hydrolysis of cAMP and cGMP, phospho-diesterases (PDEs) regulate intracellular concentrations and biological effects of these second messengers. PDEs comprise a large group of structurally related enzymes that belong to at least nine gene families that differ in their primary structure, affinities for cAMP and cGMP, responses to specific effectors, sensitivities to specific inhibitors and mechanisms of regulation [1–5]. The PDE3 family has two members, PDE3A and PDE3B, that are expressed in a tissue-specific manner [6]. PDE3B is activated and phosphorylated in adipocytes [7–9] and hepatocytes [10] in response to insulin and cAMP-increasing hormones and in FDCP2 myeloid cells in response to insulin-like growth factor I (IGF-I; F. Ahmad, L. N. Cong, L. Stenson Holst, L.-M. Wang, J. Pierce, T. Rahn Landström, M. Quon, E. Degerman and V. Manganiello, unpublished work). PDE3B has also been shown to be activated in FDCP2 cells in response to interleukin 4 (IL-4) [11], in pancreatic β-cells in response to IGF-I [12], leptin [13] and glucagon-like peptide 1 [13], in autoreactive CD4+ T cell clones specific for myelin basic protein in response to antigen stimulation [14] and has also been shown to be involved in

growth-hormone signalling in adipocytes [15]. The effects of insulin, growth hormone, IGF-I and IL-4 on PDE3B have been suggested to be important in antagonizing biological responses induced by cAMP, such as lipolysis in adipocytes [6,15,16], glycogenolysis in the liver [17], insulin secretion in β-cells [12,13] and inhibition of proliferation in FDCP2 cells. The cAMP-mediated activation of PDE3B, presumably catalysed by protein kinase A, has been suggested to be important in feedback regulation of cAMP [18].

Hormone-induced regulation of PDE3B has been studied extensively in primary rat adipocytes where phosphorylation of serine 302 [6–9] and activation of PDE3B is thought to be the major mechanism whereby insulin antagonizes protein kinase A-mediated phosphorylation and activation of hormone-sensitive lipase and lipolysis [16,17,19,20]. Although phosphatidylinositol 3-kinase is most probably involved as an upstream regulator of PDE3B in the insulin-signalling pathway [21], the kinase that phosphorylates and activates PDE3B directly has not been identified. Recent findings imply that it could be protein kinase B [22]. PDE3B is also phosphorylated on serine 302 and activated in response to isoproterenol (isoprenaline). The isoproterenol-induced activation of PDE3B that occurs over the same dose

range and time-frame as activation of protein kinase A and lipolysis is thought to be important in feedback regulation of cAMP and lipolysis [18]. In the presence of insulin and isoproterenol, a synergistic phosphorylation and activation of PDE3B has been demonstrated [8,18].

Very little is known about serine/threonine-specific protein phosphatases (PPs) involved in the regulation of the phosphorylation and activation of PDE3B. Adipocyte homogenates have been shown to contain approximately equal activities of PP1 and PP2A, lower activity of PP2C and very low activity of PP2B [23]. In addition to the PPs listed above [24,25], molecular cloning has revealed the presence of PP4, PP5, PP6 and PP7 in mammalian cells [26–29]. According to human-genome nomenclature, the five subfamilies PP1, PP2A/PP4/PP6, PP2B, PP5 and PP7 are grouped in the PPP family, whereas PP2C belongs to the distinct PPM family of PPs [25–29] (the designations PPP and PPM were assigned by a nomenclature committee formed at the FASEB Summer Research Conference on Protein Phosphatases in 1992). So far, no information on the presence of PP4, PP5, PP6 and PP7 in adipocytes has been reported. The catalytic subunits of PP1, PP2A and PP2B exist *in vivo* as dimeric or trimeric complexes with variety of regulatory subunits [24–28,30]. Incubation of isolated rat adipocytes with okadaic acid, an inhibitor of PP2A and PP1, has been shown to lead to the activation of PDE3B [31], suggesting that one or both of these phosphatases are involved in determining the phosphorylation and activity state of PDE3B.

In this study, rat adipocytes were treated with serine/threonine PP inhibitors of different specificities and the effects on phosphorylation and activation on PDE3B were studied. Under conditions where PP2A but not PP1 was inhibited *in vivo*, increased phosphorylation and activation of PDE3B was observed. Furthermore, adipocyte-derived PP2A but not PP1 could deactivate PDE3B *in vitro*, suggesting an important role for PP2A in regulating the activity state of PDE3B. In comparison with insulin, both okadaic acid and calyculin A induced more extensive phosphorylation and activation of PDE3B, and induced phosphorylation on serine 302 as well as on novel sites.

EXPERIMENTAL

Adipocyte preparation, incubation with insulin and phosphatase inhibitors and preparation of PDE3B

Isolated rat adipocytes were prepared using the collagenase-digestion method [32] with modifications according to Honnor et al. [33]. Packed cell volume was estimated by centrifuging the adipocyte suspension in a haematocrit tube and measuring the proportion of adipocytes in the tube after centrifugation. The adipocytes were resuspended in a buffer consisting of 25 mM Hepes (pH 7.5), 2 mM glucose, 200 nM adenosine, 1% BSA, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 4.8 mM KCl and 1.2 mM KH_2PO_4 for non-radioactive experiments. For experiments where the cells were labelled with ^{32}P-phosphate the same buffer was used except for 3.5% BSA and 300 μM KH_2PO_4. For labelling, 0.3–1 mCi ^{32}P-phosphate/ml of cell suspension was used. Adipocyte suspensions (1.5–2 ml of 10–12% cells) were incubated with insulin (Novo Nordisk, Copenhagen, Denmark), calyculin A, okadaic acid (both from Alexis Corporation, Läufelfingen, Switzerland), tautomycin or cyclosporin A (both from Calbiochem), as indicated. After the incubation, the cells were washed with 10 ml of 50 mM Tes (pH 7.4)/250 mM sucrose/1 mM EDTA/0.1 mM EGTA/5 mM NaF/40 mM phenyl phosphate/10 μg/ml antipain/10 μg/ml leupeptin/1 μg/ml pepstatin A (homogenization buffer) and homogenized in 1 ml of the same buffer at room temperature with 10 strokes in a

glass/Teflon pestle homogenizer. The homogenate was centrifuged at 30000 g for 45 min at 4 °C, and the crude membrane fractions were resuspended in 0.5 ml of the homogenization buffer for determinations of PDE3B activity or solubilized in 0.5 ml of the same buffer containing 1% $C_{13}E_{12}$ [a non-ionic alkyl poly(oxyethylene glycol) detergent from Berol Kemi AB, Stenungsund, Sweden] for subsequent immuno-isolation.

Determination of PDE activity

Resuspended crude adipocyte membranes were assayed according to Manganiello et al. [34] at 30 °C in a total volume of 300 μl of 50 mM Hepes (pH 7.4)/0.1 mM EGTA/8.3 mM $MgCl_2$/ 0.5 mM cAMP/1 μCi/ml [^{3}H]cAMP/0.6 μg/ml ovalbumin. Under these conditions, PDE3B represented > 90% of the total PDE activity in the membrane fraction [7,8,18].

Determination of phosphatase activity

Phosphatase activity was measured as described previously in [35] using [^{32}P]phosphorylase a as substrate. PP1 activity was measured as the phosphatase activity towards phosphorylase a in the presence of 5 nM okadaic acid, and PP2A activity calculated by subtracting the PP1 activity from the total phosphatase activity towards phosphorylase a. Phosphorylase b and phosphorylase kinase were purified from rabbit skeletal muscle as described by Krebs and Fischer [36] and Cohen [37] respectively. ^{32}P-Labelled phosphorylase a was prepared as described in [38].

MonoQ purification and concentration of rat adipocyte PP1 and PP2A

Rat adipocytes (2.7–4.0 ml packed cell volume) were washed in 15 ml of a buffer composed of 50 mM Tris/HCl (pH 7.4), 0.1 mM EDTA, 0.1% β-mercaptoethanol, 5% glycerol and 100 mM NaCl, resuspended in 8 ml of the same buffer with 1 μg/ml pepstatin, 10 μg/ml leupeptin and 10 μg/ml antipain, homogenized in a glass/glass homogenizer, immediately cooled to 4 °C, and centrifuged (10000 g, 10 min, 4 °C). After having removed the solid layer of fat on top of the homogenate, the homogenate was homogenized in the presence of 1% $C_{13}E_{12}$, placed on ice for 30 min and centrifuged (10000 g, 10 min, 4 °C). The solubilized supernatant was filtered through a 0.2 μm Acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI, U.S.A.). The volume of the supernatant after filtration was 5.5 ml, of which 5 ml was applied to a 1-ml MonoQ column (Pharmacia Amersham Biotech) equilibrated in 10 ml of 50 mM Tris/HCl (pH 7.4), 0.1 mM EDTA, 0.1% β-mercaptoethanol, 5% glycerol, 0.03% $C_{13}E_{12}$ and 100 mM NaCl (MonoQ buffer) and washed with 10 ml of the same buffer. The column was eluted with a 40-ml NaCl gradient from 100 to 600 mM (in MonoQ buffer) at 1 ml/min, and 1.1-ml fractions were collected. The fractions were analysed for PP1, PP2A and PDE3B activities. The fractions containing PP1 and PP2A activities were concentrated from 1.1 ml to approx. 90 μl using Centricon YM-30 centrifugal filter devices (Millipore), and the PP1 and PP2A activities after concentration were determined.

Dephosphorylation of activated PDE3B by adipocyte phosphatases purified by MonoQ chromatography

Concentrated MonoQ fractions (35 μl) were mixed with calyculin A-activated PDE3B (20-μl membrane fraction from 14–18-μl packed cell volume) in the absence or presence of 100 nM calyculin A (DMSO was used as vehicle). The mixture was

incubated for 30 min at 30 °C and calyculin A was added to a final concentration of 100 nM to the samples that had received vehicle, and vehicle was added to the samples that had recieved calyculin A. The samples were placed on ice immediately and PDE3B activity was determined. For each MonoQ fraction, the decrease in PDE3B activity in the sample incubated without calyculin A (compared with the corresponding sample incubated with calyculin A) was expressed as percentage deactivation, where 100 % deactivation for a given fraction was the PDE3B activity in the incubation with calyculin A minus the PDE3B activity in the control incubation (membranes from unstimulated adipocytes mixed with MonoQ buffer).

Immunodetection of PP1 and PP2A

Protein samples were analysed on SDS/PAGE, transferred on to PVDF membranes by Western blotting and the membranes were probed with antibodies towards the catalytic subunits of PP1 and PP2A (PP1c and PP2Ac respectively). PP1 antibodies directed against a peptide corresponding to residues 316–330 of human PP1c were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Antibodies against the C-terminus of PP2Ac were generated by injecting rabbits with a 9-mer phosphopeptide (TRRTPD[P]YFL) coupled to keyhole limpet haemocyanin and purified from sera by Protein A–Sepharose chromatography using standard procedures. (These antibodies recognized unphosphorylated PP2Ac, whereas there is no evidence at present that they could have reacted with the tyrosine-307-phosphorylated form of the phosphatase).

Immuno-isolation of PDE3B

Crude adipocyte membranes were resuspended in homogenization buffer containing 1 % $C_{13}E_{12}$, incubated at 4 °C for 1 h and centrifuged at 10 000 g in a table-top centrifuge for 10 min at 4 °C. The supernatant containing the solubilized PDE3B was mixed with rabbit antiserum raised against a peptide corresponding to rat adipose-tissue PDE3B amino acids 423–440 [6,39], incubated on a rocking table at 4 °C overnight. The PDE3B–antibody complex was then precipitated with Protein A–Sepharose beads (Pharmacia). The immunoprecipitate was washed four times in 137 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4/0.1 % N-laurylsarcosine and analysed by SDS/PAGE [40]. The gel was either dried or subjected to Western blotting in a buffer consisting of 96 mM glycine, 12.5 mM Tris and 5 % methanol (pH not adjusted). The radioactive contents of the gel or membrane were visualized using a Fujix BAS 2000 PhosphorImaging system.

Two-dimensional tryptic phosphopeptide mapping of PDE3B

Two-dimensional tryptic phosphopeptide mapping of PDE3B was performed essentially as described previously [41] with some modification [9]. Immuno-isolated [^{32}P]PDE3B was subjected to SDS/PAGE and Western blotting. The piece of membrane containing [^{32}P]PDE3B was identified, cut out and incubated for 30 min at 30 °C in a solution of 0.5 % polyvinylpyrrolidone/ 0.6 % acetic acid. Thereafter, the membrane was washed three times with water and finally transferred to 200 μl of trypsinization buffer (50 mM $NaHCO_3$), and 10 μl of trypsin solution (1 mM HCl/0.1 mg/ml trypsin) was added to the tube, which was then incubated at 37 °C overnight. The next day, the tube was vortexed and the tryptic digest was transferred to another tube and subjected to vacuum centrifugation in a Speed-vac concentrator (Savant, Holbrook, NY, U.S.A.). The dry pellet was dissolved in

50 μl of a performic acid solution and placed on ice for 1 h to oxidize cysteine and methionine residues. The performic acid solution was made by mixing 100 μl of 30 % hydrogen peroxide with 900 μl of formic acid and letting the reaction mixture stand at room temperature for 1–1.5 h before adding it to the pellet. After 1 h on ice the sample was diluted with 450 μl of water, immediately frozen at -80 °C for 10 min and then subjected to vacuum centrifugation until the pellet was completely dry. It was then dissolved in 50 μl of trypsinization buffer, 10 μl of trypsin solution (0.1 μg/μl) was added and the sample was incubated overnight. The next day, 140 μl of a buffer consisting of 0.59 M formic acid and 1.36 M acetic acid (FA buffer) was added to the sample, which was then centrifuged for 10 min in a table-top centrifuge at 10 000 g. An aliquot (180 μl) of the supernatant was transferred to a new tube and subjected to vacuum centrifugation. The dried pellet was then dissolved in 10 μl of FA buffer and subjected to vacuum centrifugation again.

For the mapping, the peptides were dissolved in 10 μl of FA buffer and centrifuged for 2 min in a small table-top centrifuge at 8000 g and 8 μl of the supernatant was applied to a cellulose TLC plate (Merck). The dry plate was then moistened with FA buffer and subjected to electrophoresis in the same buffer at 2000 V for 25 min in a Hunter thin-layer peptide-mapping apparatus (CBS Scientific, Del Mar, CA, U.S.A.). The plate was dried and subjected to chromatography in the other dimension in a buffer consisting of 62.5 % isobutyric acid, 27.9 % water, 4.8 % pyridine, 2.9 % acetic acid and 1.9 % butanol. The plate was dried and analysed using a Fujix BAS 2000 PhosphorImaging system.

RESULTS AND DISCUSSION

Activation of PDE3B in response to okadaic acid and calyculin A is mediated via inhibition of PP2A in adipocytes
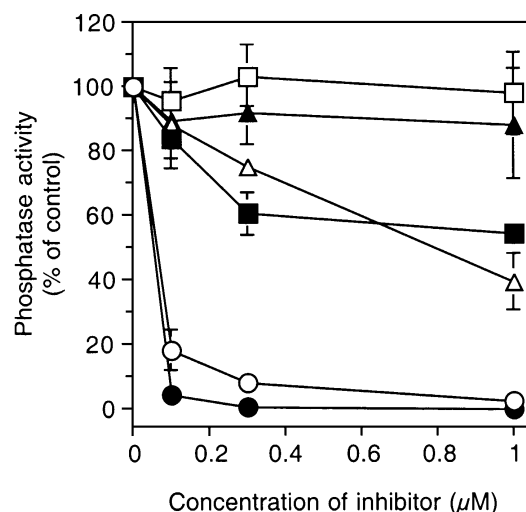
In order to evaluate the role of serine/threonine phosphatases in signalling pathways leading to activation of PDE3B, isolated rat adipocytes were incubated with insulin or phosphatase inhibitors and PDE3B activity was analysed. Incubation with okadaic acid or calyculin A resulted in a dose- and time-dependent activation of PDE3B (Figure 1), whereas tautomycin (1 μM) and cyclosporin A (1 μM) did not have any effect on PDE3B (results not shown). The activation obtained with calyculin A was much higher (4.5-fold) than the activation obtained with okadaic acid (1.8-fold) or insulin (1.5-fold). Calyculin A inhibits PP2A and PP1 with approximately equal specificity for the two types of phosphatases (IC_{50} approx. 0.1 nM) [42], okadaic acid has 100-fold higher specificity for PP2A (IC_{50} approx. 0.1 nM) than for PP1 (IC_{50} approx. 10 nM) [42], and tautomycin has a 10-fold higher specificity for PP1 (IC_{50} approx. 0.1 nM) than for PP2A (IC_{50} approx. 1 nM) [43], whereas cyclosporin A is a PP2B-specific inhibitor [44]. PP2C is only inhibited at micromolar concentrations of okadaic acid, calyculin A and tautomycin [45]. Since calyculin A and okadaic acid but not tautomycin induced activation of PDE3B, we decided to examine the intracellular effects of these inhibitors on phosphatase activities, as the concentrations of the inhibitors used in the intact-cell experiment were high enough to completely block both PP2A and PP1 in vitro. For this purpose, adipocytes were incubated with phosphatase inhibitors as indicated in Figure 2, washed to remove the inhibitors, homogenized, and phosphatase activities in the homogenates were determined. Incubation with 1 μM okadaic acid did not affect PP1 activity at all but inhibited PP2A activity by 46 % compared with the activity in untreated cells; incubation with 1 μM tautomycin inhibited PP1 activity by 60 % but PP2A only by 11 %, and incubation with 300 nM calyculin A was sufficient
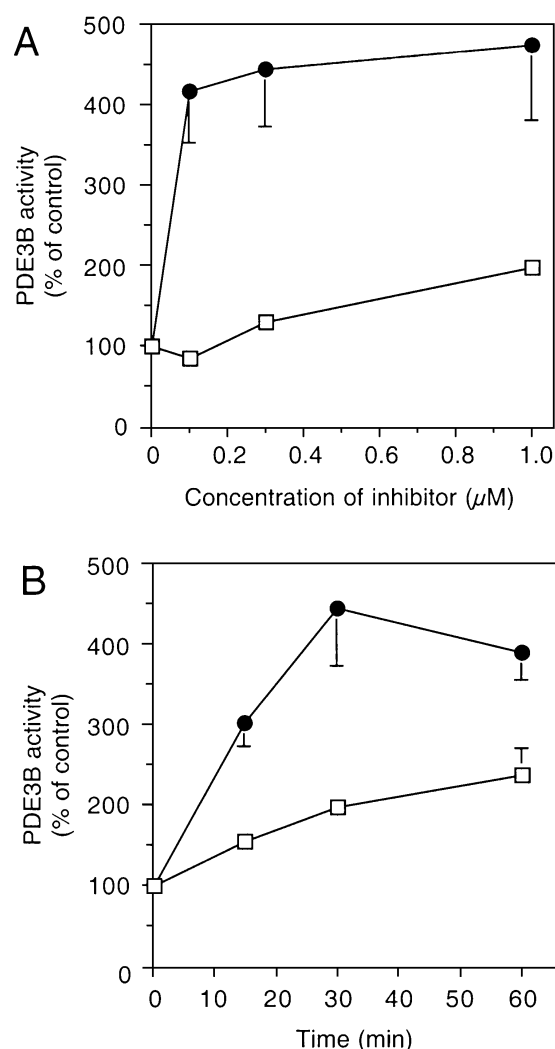
Figure 1   Activation of PDE3B in adipocytes by okadaic acid and calyculin A

(**A**) Rat adipocytes (2 ml, 12% cells) were incubated without or with okadaic acid (□) or calyculin A (●) at various concentrations for 30 min or (**B**) with 1 μM okadaic acid (□) or 300 nM calyculin A (●) for the indicated periods of time. At the end of the incubation the cells were washed with 10 ml of homogenization buffer and homogenized in 1 ml of the same buffer and a crude membrane fraction prepared and assayed for PDE3 activity. PDE3 activity is expressed as percentage of the activity in unstimulated cells (control). The values represent means ± S.D., $n = 3$


Figure 2   Inhibition of PP1 and PP2A in adipocytes in response to okadaic acid, tautomycin and calyculin A

Rat adipocytes (2 ml, 10% cells) were treated with okadaic acid, tautomycin or calyculin A for 30 min at the concentrations indicated in the Figure. After incubation, the cells were washed twice, homogenized and the fat removed. The PP1 and PP2A activities in the homogenates were determined as described in the Experimental section. The phosphatase activity is expressed as percentage of the activity in untreated cells: PP1 activity after treatment with okadaic acid (□), tautomycin (△) and calyculin A (○); and PP2A activity after treatment with okadaic acid (■), tautomycin (▲) and calyculin A (●). The values represent means ± S.D., $n = 3$.

to inhibit both PP1 and PP2A almost completely (Figure 2). Calyculin A binds tightly to the catalytic subunits of both PP1 (K_i, 1.1 nM) and PP2A (K_i, 0.13 nM), okadaic acid binds tightly to PP2Ac (K_i, 0.032 nM) and tautomycin binds tightly to PP1c (K_i, 0.48 nM) [46,47]. It has been shown previously in MCF7 cells that due to the tight binding of the inhibitors to the different phosphatases, cell penetration can be monitored by measuring PP1 and PP2A activities in cell-free extracts [46,47]. One may therefore assume that the complete inhibition of PP1 and PP2A in the homogenates after calyculin-A treatment, the 46% inhibition of PP2A in the homogenates after okadaic acid treatment and the 60% inhibition of PP1 in the homogenates after tautomycin treatment correspond to the conditions in the adipocytes before homogenization. Since okadaic acid binds much less tightly to PP1c (K_i, 147 nM) than to PP2Ac (K_i, 0.032 nM),

and since tautomycin binds much less tightly to PP2Ac (K_i, 29 nM) than to PP1c (K_i, 0.48 nM) [47], dilution of the cell extracts could lead to the dissociation of the okadaic acid–PP1c and tautomycin–PP2Ac complexes, resulting in an apparent lack of effect of okadaic acid on PP1 and tautomycin on PP2A in the cell-free extracts. However, since a significant amount of PP2A was not inhibited by okadaic acid and since PP1c binds to okadaic acid with a K_i approx. 4600 times higher than PP2Ac, one can assume that PP1 is not inhibited in the intact cell under conditions where PP2A is only partially inhibited [46,47]. Similarly, since a significant amount of PP1 was not inhibited by tautomycin and PP2A binds to tautomycin with a K_i approx. 60 times higher than for PP1, one can also assume that the amount of PP2A inhibition by tautomycin in the intact cell was negligible [46,47]. These results indicate that inhibition of PP2A but not of PP1 is sufficient to induce PDE3B activation. However, we cannot completely exclude the fact that recently identified PPs, such as PP4 and PP5, which have been reported to undergo inhibition by okadaic acid and calyculin A or other as-yet-uncharacterized PPs, could also contribute to the observed effects, since nothing is known about the presence of these phosphatases in adipocytes [48,49].

In order to compare the abilities of rat adipocyte PP1 and PP2A to dephosphorylate and deactivate PDE3B *in vitro*, the phosphatases were partially purified from rat adipocyte homogenates using MonoQ chromatography and incubated with PDE3B activated *in vivo* by calyculin A (Figure 3). The phosphatases were identified by their sensitivity to okadaic acid and by Western blotting. All of the PP1 activity eluted in one peak in fractions 21–22 (276–290 mM NaCl) and the PP2A activity was distributed in two peaks, one in fractions 20–22 (260–290 mM NaCl) and one in fractions 25–28 (331–372 mM NaCl). The PDE3B activity eluted at 470–600 mM NaCl, well separated from the phosphatases. As shown in Figure 3, the PDE3B
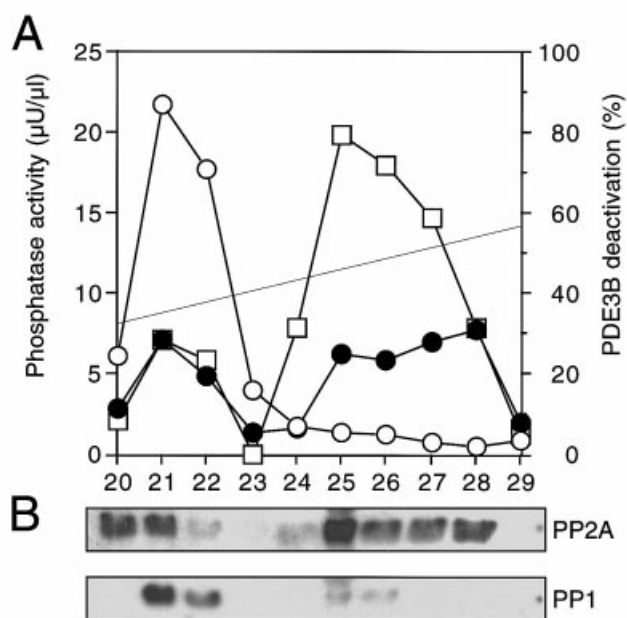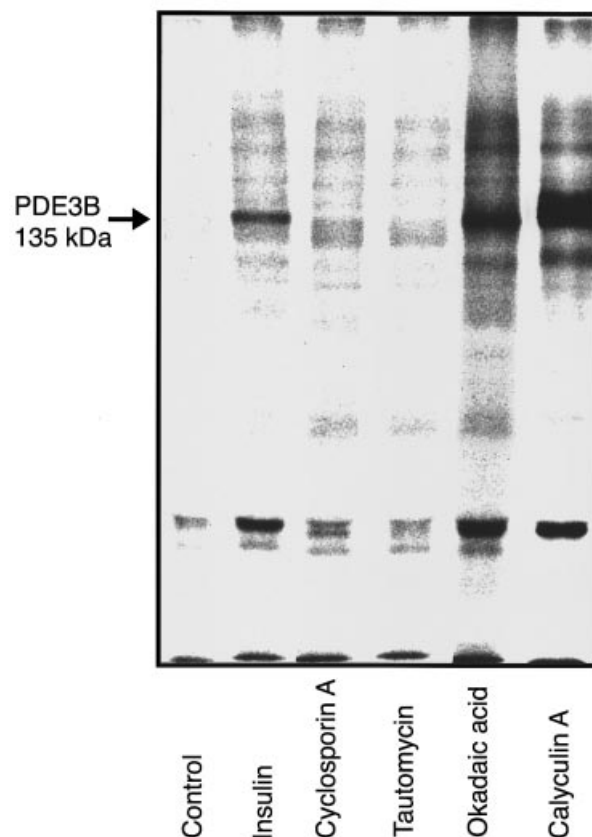
Figure 3   Detection of PDE3B phosphatase activity in MonoQ fractions from an adipocyte homogenate

(**A**) An adipocyte homogenate (from 2.7-ml packed cell volume) was prepared and subjected to MonoQ chromatography as described in the Experimental section. The column was eluted using a 100–600 mM NaCl gradient (indicated by a thin straight line from 260 to 390 mM NaCl in the chromatogram) in MonoQ buffer. The fractions containing PP1 and PP2A activities eluted between approx. 260 and 390 mM NaCl. These fractions were concentrated 12-fold using centrifugal filter devices and assayed for PP1 (○), PP2A (●) and PDE3B phosphatase (□) activity as described in the Experimental section. (**B**) Samples (5 μl) from the concentrated MonoQ fractions were analysed by SDS/PAGE and tranferred on to PVDF membranes by Western blotting. The blots were probed by anti-PP2Ac and anti-PP1c antibodies as described. The chromatogram and Western blots shown are representative of three independent experiments.

Figure 4   Phosphorylation of PDE3B in adipocytes in response to okadaic acid and calyculin A

Rat adipocytes (2 ml, 10% cells) were incubated with ^{32}P-phosphate (0.3 mCi/ml of medium) and treated with 1 nM insulin for 10 min or with 1 μM cyclosporin A, tautomycin, okadaic acid or calyculin A for 30 min. PDE3B was immuno-isolated from solubilized crude membrane fractions as described in the Experimental section. The immunoprecipitates were analysed using SDS/PAGE and the radioactive content of the gel visualized with digital PhosphorImaging.

phosphatase activity co-eluted with the PP2A activity, indicating that PP2A is the main PDE3B phosphatase in adipocytes *in vitro*. It is likely that the PP2A peaks consist of different PP2A holoenzymes and that the broadness of the second peak could indicate that it consists of more than one PP2A isoform. Three families of trimeric holoenzymes, which differ by the presence of variable subunits (B, B′ and B″), as well as a dimeric PP2A$_2$ composed of PP2Ac and the A structural subunit, have been described to exist in mammalian cells, with differential expression in different tissues [24–28,30,50]. It cannot be totally excluded that some of the PDE3B phosphatase activity seen in fractions 20–22 is due to PP1. However, considering the much greater PDE3B deactivation seen in fractions 24–28, where no PP1 activity is detected, it is reasonable to conclude that PP2A is the phosphatase responsible for the PDE3B deactivation seen in fractions 20–22. We are currently isolating different PP2A and PP1 holoenzymes from bovine adipose tissue in order to determine their activities towards phospho-PDE3B.

Insulin, okadaic acid and calyculin A induce phosphorylation of PDE3B on different sites

It has been shown that insulin stimulation of adipocytes results in the phosphorylation of PDE3B on a single site, serine 302, with a maximal estimated stoichiometry of approx. 0.2 mol of phosphate/mol of PDE3B, corresponding to a 1.5–2-fold activ-

ation of PDE3B [7–9]. In order to examine further the mechanism behind the activation of PDE3B induced by phosphatase inhibitors, isolated rat adipocytes preincubated with ^{32}P-phosphate were incubated with insulin or phosphatase inhibitors and PDE3B phosphorylation was analysed. Incubation of adipocytes with insulin, okadaic acid and calyculin A, but not with tautomycin or cyclosporin A, increased the phosphorylation of PDE3B (Figure 4). As compared with PDE3B from insulin-stimulated adipocytes, the extent of phosphorylation of PDE3B from okadaic acid- and calyculin A-treated adipocytes was 2.8 and 14 times higher (based on an average of three measurements), respectively. Treatment with calyculin A but not with okadaic acid resulted in a reduced electrophoretic mobility of PDE3B, indicating that calyculin A induced phosphorylation on sites not phosphorylated in response to insulin or okadaic acid.

The finding that both okadaic acid and calyculin A stimulated PDE3B phosphorylation more efficiently than insulin prompted us to investigate these phosphorylations further. For this purpose, two-dimensional tryptic phosphopeptide mapping of PDE3B from ^{32}P-labelled adipocytes treated with insulin, okadaic acid and calyculin A was performed. We have shown previously that the peptide map of PDE3B from cells stimulated with insulin and/or isoproterenol contains one main phosphopeptide and
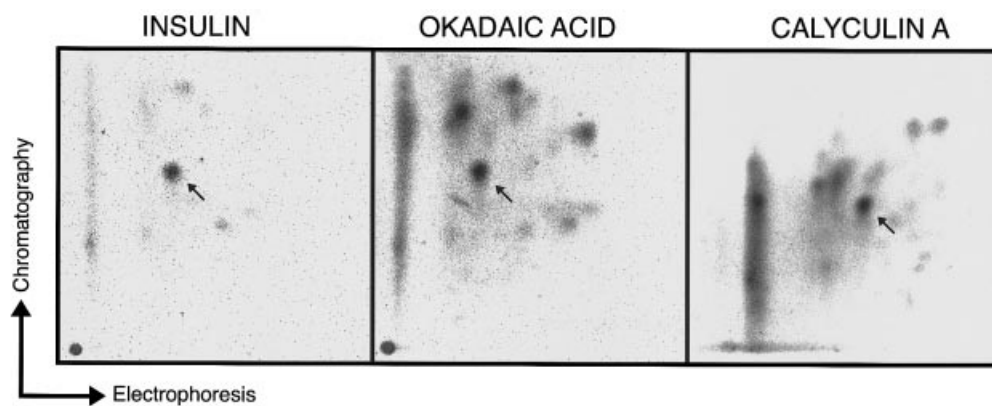
Figure 5   Two-dimensional tryptic phosphopeptide map of PDE3B from ^{32}P-labelled adipocytes treated with insulin, okadaic acid or calyculin A

PDE3B from ^{32}P-labelled adipocytes treated with insulin (1 nM, 10 min), okadaic acid (1 μM, 30 min) or calyculin A (0.3 μM, 30 min) (10–20 ml of 10−12% cells with 1 mCi/ml per condition) was immuno-isolated, subjected to SDS/PAGE, transferred on to a PVDF membrane and digested with trypsin. [^{32}P]Phosphopeptides derived from PDE3B were separated by two-dimensional thin-layer electrophoresis/TLC and visualized with digital PhosphorImaging. The patterns of phosphopeptides are representative of several independent experiments (> 3). The positions of the tryptic peptide containing [^{32}P]serine 302 are indicated with arrows.

that this phosphopeptide is identical to RP[^{32}P]SLPCISR, corresponding to amino acids from 300 to 308 in the rat adipocyte PDE3B sequence with serine 302 phosphorylated [9]. The peptide maps of PDE3B from adipocytes treated with okadaic acid or calyculin A contained a number of novel radioactive peptides (Figure 5). The pattern of peptides was not identical between the maps of PDE3B from okadaic acid and calyculin A, indicating that unique sites are phosphorylated after treatment with each inhibitor. In all maps, however, RP[^{32}P]SLPCISR (indicated with an arrow in Figure 5), identified using co-migration experiments as described by Rahn et al. [9], had the highest content of radioactivity. The identity of the novel peptides and the kinases that catalyse their phosphorylation is presently under investigation. In the peptide map of PDE3B from calyculin-A-treated cells, there are a number of peptides that do not appear to have any counterpart in the map of PDE3B from adipocytes treated with okadaic acid. One or more of these peptides may contain the phosphorylation site or sites responsible for the decreased electrophoretic mobility of PDE3B on SDS/PAGE seen after calyculin-A treatment.

In summary, PP2A but not PP1 was shown to be involved in determining the activity and phosphorylation states of PDE3B *in vivo*. PP2A was also shown to be the major adipocyte PDE3B phosphatase *in vitro*. Thus one explanation for the observation that PP2A inhibition *in vivo* induces PDE3B phosphorylation and activation is that PP2A is acting as a PDE3B phosphatase. In addition, PP2A could also act on upstream signal-transduction components involved in the regulation of PDE3B. The presence of an insulin-stimulated kinase that phosphorylates PDE3B *in vitro* has been reported [21], which recently has been suggested to be protein kinase B [22]. Furthermore, in intact cells, over-expression of protein kinase B has been shown to induce activation of PDE3B (F. Ahmad, L. N. Cong, L. Stenson Holst, L.-M. Wang, J. Pierce, T. Rahn Landström, M. Quon, E. Degerman and V. Manganiello, unpublished work) and PDE3A [51]. It has been shown that okadaic acid treatment results in the activation of protein kinase B and that activated protein kinase B can be deactivated by PP2A *in vitro* [52]. Thus the mechanism whereby PP2A inhibition induces phosphorylation and activation of PDE3B *in vivo* could involve both the deactivation of a PP2A acting directly on PDE3B and the activation of protein kinase B.

The signalling cascade(s) leading to phosphorylations of sites other than serine 302 (okadaic acid and calyculin A) and leading to the dramatic activation and phosphorylation of PDE3B (calyculin A) are not known. Since it has recently been shown that PDE3B is controlled by a variety of hormones in addition to insulin and catecholamines, such as leptin [12,13], IGF-I (F. Ahmad, L. N. Cong, L. Stenson Holst, L.-M. Wang, J. Pierce, T. Rahn Landström, M. Quon, E. Degerman and V. Manganiello, unpublished work), IL-4 [11] and growth hormone [15], it is possible that the new phosphopeptides identified in this work contain sites phosphorylated by kinases activated by these signalling molecules. The identities of the kinases involved and the physiological significance of these phosphorylations are presently being investigated.

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