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

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*J Immunol* 2000; 164:4678-4688; doi: 10.4049/jimmunol.164.9.4678

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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

Faiyaz Ahmad, Li-Na Cong, Lena Stenson Holst, Ling-Mei Wang, Tova Rahn Landstrom, Jaclyn H. Pierce, Michael J. Quon, Eva Degerman, and Vincent C. Manganiello*

Wild-type (F/B), constitutively active (F/B*), and three kinase-inactive (F/B−, F/Bb, F/Bc) forms of Akt/protein kinase B (PKB) were permanently overexpressed in FDCP2 cells. In the absence of insulin-like growth factor-1 (IGF-1), activities of PKB, cyclic nucleotide phosphodiesterase 3B (PDE3B), and PDE4 were similar in nontransfected FDCP2 cells, mock-transfected (F/V) cells, and F/B and F/B− cells. In F/V cells, IGF-1 increased PKB, PDE3B, and PDE4 activities ~2-fold. In F/B cells, IGF-1, in a wortmannin-sensitive manner, increased PKB activity ~10-fold and PDE3B phosphorylation and activity (~4-fold), but increased PDE4 to the same extent as in F/V cells. In F/B* cells, in the absence of IGF-1, PKB activity was markedly increased (~10-fold) and PDE3B was phosphorylated and activated (3- to 4-fold); wortmannin inhibited these effects. In F/B* cells, IGF-1 had little further effect on PKB and activation/phosphorylation of PDE3B. In F/B− cells, IGF-1 activated PDE4, not PDE3B, suggesting that kinase-inactive PKB behaved as a dominant negative with respect to PDE3B activation. Thymidine incorporation was greater in F/B* cells than in F/V cells and was inhibited to a greater extent by PDE3 inhibitors than by rolipram, a PDE4 inhibitor. In F/B cells, IGF-1-induced phosphorylation of the apoptotic protein BAD was inhibited by the PDE3 inhibitor cilostamide. Activated PKB phosphorylated and activated rPDE3B in vitro. These results suggest that PDE3B, not PDE4, is a target of PKB and that activated PDE3B may regulate cAMP pools that modulate effects of PKB on thymidine incorporation and BAD phosphorylation in FDCP2 cells. *The Journal of Immunology, 2000, 164: 4678–4688.

There is considerable interest in identifying downstream targets and effectors of PKB action. Glycogen synthase kinase was the first physiological substrate identified (12). Cardiac 6-phosphofructo-2 kinase, a critical enzyme in the regulation of glycolysis, was also reported to be phosphorylated and activated by PKB in vitro (13). BAD, a member of the Bcl-2 family that promotes cell death, is phosphorylated in intact cells and in vitro by PKB. IL-3-induced phosphorylation of BAD is thought to result in its functional inactivation by promoting its binding to 14-3-3 (10). PKB is also thought to promote cell survival by phosphorylating Forkhead family transcription factors, resulting in their association with 14-3-3 proteins, retention in the cytosol, and functional inactivation (14). In endothelial cells, PKB increases NO production and release by phosphorylation and activation of endothelial NO synthase (15).

More recently, in oocytes (8), rat adipocytes (16, 17), and 3T3-L1 adipocytes (18), PDE3 isoforms have been suggested to be downstream targets and effectors of PKB actions. In adipocytes, insulin-induced activation of PDE3B, via PI3-K and PKB-dependent signals, is a critical component in the antilipolytic action of insulin (19–21). Activation of PDE3 by PKB is thought to be involved in the resumption of meiosis in quiescent frog oocytes (8).

FDCP2 promyeloid cells have been utilized to study the mitogenic actions of insulin and IL-4, and to establish the role of insulin receptor-substrate proteins (IRS-1, IRS-2) in these processes (22). In these cells, activation of insulin, IGF-1, or IL-4 receptors generates some common signals (23, 24). FDCP2 cells require IL-3 for growth; IL-4 cannot replace IL-3, but enhances its effects (25). In FDCP2 cells, IL-3 and IL-4 interact with different types of...
receptors and induce tyrosine phosphorylation of different proteins. For example, IL-4, not IL-3, stimulates tyrosine phosphorylation of IR-2 (25, 26). FDCP2 cells also contain PDE4 as well as PDE3B, and recently we found that in these cells, IL-4 activates both PDE3B and PDE4, whereas IL-3 and PMA selectively activate PDE4 (26). IL-4, IL-3, and PMA activate PDE4 via mitogen-activated protein kinase (MAP kinase)-dependent signals, whereas, similar to insulin in rat adipocytes (16, 21), IL-4 activates PDE3B via MAP kinase-independent signals (26).

The goal of this study was to evaluate the role of PKB in the activation of PDE3B by IGF-1 in FDCP2 cells and to determine whether PDE3B was a downstream effector and regulator of some PKB actions. For this purpose, we generated permanently transfected FDCP2 cells that overexpress wt (F/B), constitutively active (F/B*), and three kinase-inactive (F/Ba, F/Bb, F/Bc) forms of PKB (5, 27). In the absence of IGF-1, activities of PKB, PDE3B, and PDE4 were similar in nontransfected FDCP2 cells, cells transfected with control vector (F/V), and F/V cells. In F/V cells, IGF-1 increased PKB, PDE3B, and PDE4 activities ~2-fold. In F/B cells, however, IGF-1, in a wortmannin-sensitive manner, increased PKB ~10-fold and PDE3B activity (~4-fold) and phosphorylation, but increased PDE4 only ~2-fold (as in F/V cells). In F/B* cells, the absence of IGF-1, PKB was increased ~10-fold and PDE3B was activated (~3–4-fold) and phosphorylated. Wortman nin inhibited activation of PKB and phosphorylation/activation of PDE3B. In F/B* cells, IGF-1 had little further effect on PKB and activation/phosphorylation of PDE3B, and increased PDE4 to the same extent as in F/V cells. In cells transfected with kinase-inactive forms of PKB, IGF-1 activated PDE4, not PDE3B, suggesting that kinase-inactive PKB functioned as a dominant negative with respect to activation of PDE3B. Activated PKB phosphorylated and activated rPDE3B in vitro. Proliferation of F/B* cells and IGF-1-induced phosphorylation of BAD in F/B cells was more sensitive to inhibition by PDE3 inhibitors than the PDE4 inhibitor rolipram. These and other results are consistent with the idea that in FDCP2 cells, PDE3B, not PDE4, is a target, if not substrate, of activated PKB and a downstream effector of some actions mediated by PKB.

Materials and Methods

Transferrin and senescence were purchased from Collaborative Research (Boston, MA); [3H]AMP, from New England Nuclear (Boston, MA); anti-human PKB-CT, anti-mouse BAD-NT, and anti-rat p70S6 kinase polyclonal Abs, from Santa Cruz Biotechnology (Santa Cruz, CA); anti-human extracellular signal-related kinase (ERK)-1 mAb, from Sigma Transduction Laboratories (Lexington, KY); anti-rat ERK1-CT polyclonal (MAP kinase) Ab, from Upstate Biotechnology (Lake Placid, NY); the PI3-K inhibitor wortmannin, from Biomol Research Laboratories (Plymouth Meeting, PA); MEK-1 inhibitor PD90859, from New England Bio- labs (Beverly, MA); phosphoros-32 as H3PO4 (1000 Ci/nmol); and [γ-32P]ATP (3000 Ci/mmol), from ICN Radiochemicals (Costa Mesa, CA); and [3H]thyminidine (22 Ci/nmol), from Amersham (Arlington Heights, IL). K9 (KRRKRLTTLK) and Cross tide (GRPRRTSSFAEG) peptides were synthesized at the Biocommolecular Unit, Lund University (Lund, Sweden). Prestained molecular size markers were from Bio-Rad (Rich mond, CA). Other materials were obtained as indicated and were of the highest grade available.

Cell culture and incubations

The murine, IL-3-dependent hemopoietic cell line, FDCP2, was propagated and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS, 5% WEHI-3B (American Type Culture Collection, Rockville, MD)-conditioned medium (which contains IL-3), and 2 mM glutamine (25). For most experiments, exponentially growing FDCP2 cells (2–5 × 106 cells/ml) were collected, centrifuged (1200 × g, 5 min), and suspended and homogenized (~10–15 strokes in a Dounce homogenizer (Kontes Instruments, Vineland, NJ)) in lysis buffer A (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1% Nonidet P-40 detergent, 1 mM EDTA, 10 mM NaF, 10 mM sodium pyrophosphate, 1 mM Na3V04, 1 mM PMSF (Sigma, St. Louis, MO), 10 μg/ml each of aprotinin, pepstatin, and leupeptin (Boehringer Mannheim, Indianapolis, IN) and 1 mM benzamidine (Sigma), and kept for 30 min at 4°C. Protein was measured using the Bradford assay (Bio-Rad, Richmond, CA) with BSA as standard.

Expression of PKB in FDCP2 cells

The following plasmid vector constructs (4, 27) were transformed into Escherichia coli, amplified, and purified.

pCIIS2 (F/V). A vector that generates high levels of expression (27) was used as parent vector for subsequent constructions.

Akt-WT (F/B). A 1.4-kb XbaI/BamHI fragment containing the cDNA for mouse Akt-1 (28) was blunt ended and ligated in the sense orientation into the HpaI site in the multiple cloning region of pCIIS2.

Constitutively active Akt-myrA (F/B*). A BglII/BamHI fragment containing the cDNA for Akt-1 with a mutation (473A > C) in the regulatory region of Akt (26) was blunt ended and ligated in the sense orientation in the HpaI site in the multiple cloning region of pCIIS2.

Kinase-inactive Akt (F/Ba). A point mutant of Akt-WT with a substitution of alanine for serine at position 179 in the catalytic ATP binding site (F/Ba) was constructed using the mutagenic oligonucleotide-1 (5'-GC TAT TAC CTC AGG ATG TAG GCG ATG ACA G-3') and the mutagenic oligonucleotide-2 (5'-TCT TCG TAC TAC TGC GCA CTT GAT CCA GAG-3') and oligonucleotide-3 (5'-TCT TCG TAC TAC TGC GCA CTT GAT CCA GAG-3'). In addition to introducing the amino acid changes noted above, the mutagenic oligonucleotides also introduced silent mutations that destroyed an XnoI site and created a new BglII site. The presence of the correct mutations was confirmed by direct sequencing. Another kinase-inactive mutant AktAAA (F/Bc) was created by substitution of alanine for serine at position 179, alanine for threonine at position 308, and alanine for serine at position 473 (phosphorylation sites in the regulatory region of Akt) (F/Bc) were constructed using oligonucleotide-2 (5'-CCA TTA GCA AGT TCT TTT CAA GCT AGG ACA G-3') and oligonucleotide-3 (5'-TCT TCG TAC TAC TGC GCA CTT GAT CCA GAG-3'). In addition to introducing the amino acid changes noted above, the mutagenic oligonucleotides also introduced silent mutations that destroyed an 

Incorporation of [3H]Thymidine into DNA in FDCP2 cells

Thymidine incorporation was assayed as previously described (22). Cells in log phase growth were washed twice in PBS and suspended in growth medium; 3 × 106 cells were added to each of 24 wells (final volume, 1 ml) containing RPMI 1640 medium with or without conditioned medium containing IL-3 (5% WEHI) and the indicated concentrations of PDE inhibitors, 8-Br-cAMP, or PGE1, DMSO or ethanol at a final concentration of 0.1% or less was added to appropriate wells as vehicle controls. Cells were preincubated at 37°C for 48 h at 37°C. [3H]Thymidine (22 Ci/nmol) was added to a final concentration of 0.5 μCi/ml, and incubation was continued for 2 h. Cells were harvested (Skatron Cell Harvester) and lysed on glass microfiber filters; unincorporated nucleotide was removed by repeated washing with water. Filters were dried and counted in scintillation fluid. Data are presented as mean ± SEM of cpm in samples from triplicate incubations. For cell survival assays, IL-3-depleted cells were seeded at a density of 3 × 104/ml in RPMI 1640 containing 10% FBS. In some experiments, the ratio of viable to dead cells was determined by trypan blue exclusion.

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Table 1. Effects of IGF-1 on PDE3 and PDE4 activities in FDCP2 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total PDE</th>
<th>PDE3</th>
<th>PDE4</th>
<th>Total PDE</th>
<th>PDE3</th>
<th>PDE4</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (n = 8)</td>
<td>12.5 ± 1.0</td>
<td>5.7 ± 0.7</td>
<td>6.2 ± 1.1</td>
<td>21.0 ± 2.3</td>
<td>11.0 ± 1.7</td>
<td>10.8 ± 0.5</td>
</tr>
<tr>
<td>F/V (n = 8)</td>
<td>13.4 ± 1.5</td>
<td>5.9 ± 0.9</td>
<td>6.6 ± 1.1</td>
<td>24.6 ± 4.7</td>
<td>12.9 ± 2.9</td>
<td>11.8 ± 2.5</td>
</tr>
<tr>
<td>F/Ba (n = 3)</td>
<td>13.8 ± 0.8</td>
<td>5.9 ± 0.6</td>
<td>6.1 ± 0.9</td>
<td>18.9 ± 0.4</td>
<td>6.2 ± 1.3</td>
<td>10.9 ± 0.6</td>
</tr>
<tr>
<td>F/Bbb (n = 3)</td>
<td>13.4 ± 1.5</td>
<td>5.9 ± 0.5</td>
<td>6.5 ± 0.5</td>
<td>22.1 ± 1.5</td>
<td>6.0 ± 1.0</td>
<td>13.2 ± 0.7</td>
</tr>
<tr>
<td>F/Bc (n = 3)</td>
<td>13.4 ± 1.0</td>
<td>6.0 ± 0.9</td>
<td>6.6 ± 0.4</td>
<td>21.0 ± 1.5</td>
<td>5.8 ± 0.3</td>
<td>12.3 ± 0.9</td>
</tr>
<tr>
<td>F/B* (n = 10)</td>
<td>32.5 ± 5.2</td>
<td>21 ± 3.7</td>
<td>8.3 ± 2.1</td>
<td>32.5 ± 1.9</td>
<td>21.1 ± 1.9</td>
<td>10.9 ± 1.0</td>
</tr>
</tbody>
</table>

* Cells were incubated without or with 10 nM IGF-1 for 10 min before total PDE and PDE3 and PDE4 activities in cell lysates were assayed as described in Materials and Methods. Activity represents cAMP hydrolysis inhibited by cistolamide (a selective PDE3 inhibitor); PDE4, that activity inhibited by rolipram (a selective PDE4 inhibitor).

PDE activities are reported as pmol cAMP hydrolyzed/min/mg (mean ± SEM) from the indicated number (n) of experiments and include data from Figs. 2–4.

** p < 0.001; * p < 0.02; * p > 0.05, compared to activities in F/V cells. PDE activities in nontransfected FDCP2(F) cells were not significantly different from those in F/V cells.

**cAMP PDE assay**

Samples of cell lysates (usually 100 μl) were incubated for 10 min at 37°C in a total volume of 0.3 ml containing 50 mM HEPES, pH 7.5, 8.3 mM MgCl2, 0.1 mM EDTA, and 0.1 μM 3H[cAMP (25–35,000 cpm) as substrate (31). After dephosphorylation of 3H-AMP to 3H-adenosine with Crotalus atrox venom (Sigma), the product was separated from substrate that hydrolysis of substrate was usually less than 20%. PDE3 activity is that inhibited by 0.5 mM cilostamide, a specific PDE3 inhibitor, and PDE4 activity, that inhibited by 0.5 mM rolipram, a specific PDE4 inhibitor (32).

**Immunoprecipitation and immunoblotting**

For most experiments, portions of FDCP2 lysates (2–3 mg protein) were first cleared by incubation with 1 μg of preimmune mouse IgG for 1 h at room temperature, and then with 60 μl of protein G-Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 30 min before centrifugation (2800 0 x g, 4°C, 5 min). Cleared cell lysates were incubated with specified Abs for 2 h at room temperature, followed by incubation with fresh protein G-Sepharose for 30 min before centrifugation (2800 x g, 4°C, 5 min). Immunoprecipitates were washed three times with lysis buffer A containing 0.1% Nonidet P-40, boiled in Laemmli buffer (Bio-Rad, Richmond, CA) (33), and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes in Tris-glycine buffer (25 mM Tris-base and 192 mM glycine at pH 8.3), containing 20% methanol. Membranes were incubated in blocking buffer (150 mM NaCl, 0.5% (w/v) Nonidet P-40, 0.01% NaN3, and 10 mM Tris, pH 7.4), containing BSA (50 mg/ml) and OVA (10 mg/ml), for 1 h at room temperature, followed by additional 2 h in the same solution containing the appropriate Abs. Membranes were washed three times (10 min each) in blotting buffer without Abs, followed by incubation with 125I-labeled protein A or protein G (2 μg) (Amersham Life Sciences, Arlington Heights, IL) for 1 h at room temperature, followed by three washes (10 min each) with blotting buffer. Immunoreactive proteins were visualized by phosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

For some immunoblots, instead of 125I-labeled protein A, HRP-labeled second Ab (mouse, rat, rabbit, or goat) and enhanced chemiluminescence (ECL) reagents 1 and 2 (Amersham) were used; immunoreactive proteins were visualized by exposing nitrocellulose filters to Bio-max Kodak film and developed in a Standard Medical Imaging (Columbia, MD) developer.

**Phosphorylation of PDE3B and BAD**

PDE3B or BAD proteins were identified in FDCP2 cell lysates by immunoprecipitation/immunoblotting with anti-PDE3B-NT or anti-PDE3B-AT Abs (rabbit Abs raised against peptides derived from deduced sequences in the N-terminal (RKDERERDTPAMRSS, aa 2–19) and C-terminal (NLKQLVDNASLPQADE, aa 1078–1092) portions of rat (RPDE3B) (34), or anti-BAD-NT Abs, respectively. Immediately before experiment, FDCP2 cells transfected with wt (F/V) or constitutively active (F/V*) PKB, or vector alone (F/V) were washed twice, suspended in serum-free RPMI 1640 medium containing TSB, and incubated (3 ml cells, 7 x 106 cells/ml) in six-well Costar plates (25) for 1–2 h at 37°C. Cells were then incubated in RPMI 1640 medium containing 300 mM KH2PO4 and 1% BSA with or without 32PO4 (40 μCi/ml) for 90–100 min at 37°C, and finally with or without 10 nM IGF-1 for 10 min. PDE3B and BAD were immunoprecipitated from cleared lysates with anti-PDE3B-NT or anti-BAD-NT Abs, respectively. SDS-PAGE was then conducted, and dried gels were scanned on a PhosphorImager (Molecular Dynamics), as described above. Immunoprecipitates from cells incubated without 32PO4 were prepared similarly and immunoblotted with anti-PDE3B or anti-BAD Abs. PKB activity was assayed by phosphorylation of Crotiside in PKB immunoprecipitates.

**PKB assays with Crotiside, K9 peptide, and histone 2B substrates**

Immunoprecipitation with anti-PKB-CT Ab (50 μl/10 μg IgG) was conducted for 2 h at room temperature or overnight at 4°C, as described above, and washed proteins were suspended in 30–40 μl of reaction buffer (20 mM HEPES, pH 7.4, 1 mM DTT, 10 mM MgCl2, 10 mM MgCl2, 5 μM ATP, 10 μCi [γ32P]ATP, 5 μM cAMP protein kinase inhibitor) containing either 13 μg of K9 peptide (KKRRNTLTK), 1 μg of Crotiside (GRPRSTSSFAEG), or 2.5 μg of histone-2B (Boehringer Mannheim) as substrate (17, 35, 36). As recently described (26), after incubation for 15 min at 30°C, assay reactions with K9 or Crotiside peptides were terminated by addition of 10 μl of 1% BSA, 1 mM ATP, pH 3, and 5 μl of 30% TCA. For reaction blanks, the stopping solution was added to immunoprecipitated samples before assay buffer. Samples were centrifuged, and 25 μl of supernatant was applied to phosphocellulose (P81; Whatman, Tewksbury, MA) paper (2 × 2 cm squares), which were washed three times (5 min each) with phosphoric acid (7.5 mg/ml), and once with acetone (5 min) before radioassay of 32P incorporated into substrate.

With histone 2B as substrate for PKB, reactions were terminated by addition of Laemmli buffer and boiling. Proteins were separated by SDS-PAGE, the gel was dried, and 125I-labeled histone 2B was detected and quantified by PhosphorImager (Molecular Dynamics), as recently described (26).

**MAP kinase and p70S6 kinase assays**

Activation of MAP kinase was assessed as recently described (26) by measuring phosphorylation of myelin basic protein (MBP) peptide in immunoprecipitates prepared with anti-ERK1-CT Ab (21 μg/10 μg IgG; Upstate Biotechnology). Samples of immunoprecipitates were incubated at 30°C for 15 min in 50 mM Tris (pH 7.5), 0.4 mM EGTA, 0.4 mM Na3VO4, 25 mM MgCl2, 150 μM ATP, 10 μCi [γ32P]ATP (10 Ci/mmol), 5 μM cAMP protein kinase inhibitor (Calbiochem, San Diego, CA), and 5 μM MBP peptide (APRTPGGR) substrate (Upstate Biotechnology), in final volume of 50 μl. Reactions were terminated, and phosphocellulose squares were washed and analyzed, as described for PKB assays. p70S6 kinase was detected in lysates from F/V and F/V* cells after separation of proteins (~30 μg) by SDS-PAGE and immunoblotting with polyclonal p70S6 kinase Ab. Activated kinase exhibited a lower electrophoretic mobility than inactive kinase.
Results and Discussion

PDE3 and PDE4 enzymes exhibit high affinity for cAMP (31, 32) and, as shown in Table I, with 0.1 μM [3H]cAMP as substrate, account for most of the cAMP hydrolytic activity in FDCP2 cell lysates (26). Two PDE3 isoforms, PDE3A and PDE3B, have been identified (16); PDE3B mRNA was amplified by RT-PCR from FDCP2 cell RNA (31) (data not shown). PDE3B protein in FDCP2 lysates was identified by immunoprecipitation/immunoblotting with anti-PDE3B-NT Ab (34) (data not shown).

Characterization of FDCP2 cells transfected with PKB constructs

Several independently isolated lines were generated from a single transfection of FDCP2 cells with constructs that expressed vector alone (F/V) and wt PKB (F/B), constitutively active (F/B*), and kinase-inactive (F/Ba, F/Bb, F/Bc) forms of PKB, were incubated with or without 10 nM IGF-1 for 10 min. A and D, Proteins (~30 μg) in lysates of control and transfected cells were separated by SDS-PAGE and immunoblotted with anti-PKB-CT Ab. PKB activity was assessed by phosphorylation of (B and E) Crosstide (mean cpm ± SEM, n = 3) or histone 2B (not shown) with PKB immunoprecipitates from lysates (~2.5 mg protein) of control and IGF-1-stimulated cells. C and F, MAP kinase activity was assessed by phosphorylation of MBP peptide (mean cpm ± SEM, n = 3) with MAP kinase immunoprecipitates from cell lysates (~2.5 mg protein). In this and all other figures, when not present, SEMs were too small to be displayed graphically.

Role of PKB in IGF-1-induced activation of PDE3B in FDCP2 cells

In the absence of IGF-1, activities of PDE3B and PDE4 were similar in nontransfected FDCP2 cells, F/V cells, F/B cells, and F/Ba, F/Bb, F/Bc forms of PKB, were incubated with or without 10 nM IGF-1 for 10 min. PDE3 and PDE4 enzymes exhibit high affinity for cAMP (31, 32) and, as shown in Table I, with 0.1 μM [3H]cAMP as substrate, account for most of the cAMP hydrolytic activity in FDCP2 cell lysates (26). Two PDE3 isoforms, PDE3A and PDE3B, have been identified (16); PDE3B mRNA was amplified by RT-PCR from FDCP2 cell RNA (31) (data not shown). PDE3B protein in FDCP2 lysates was identified by immunoprecipitation/immunoblotting with anti-PDE3B-NT Ab (34) (data not shown).

Role of PKB in phosphorylation/activation of PDE3B in FDCP2 cells

We next evaluated the effects of IGF-1 and PKB on phosphorylation/activation of PDE3B in 32P-labeled FDCP2 cells. As shown in Fig. 3, in F/B cells overexpressing wt PKB, neither PDE3B (C) nor PKB (D) was activated in the absence of IGF-1, and PDE3B was not phosphorylated (A). As shown in Fig. 3, however, after incubation of F/B cells with 1 or 10 nM IGF-1 for 10 min, phosphorylation of PDE3B was markedly increased (A), as was PDE3B.
FIGURE 2. Effect of rapamycin and PD98059 on PDE, PKB, MAP kinase, and p70S6K activities in F/V and F/B* cells. FDCP2 cells, transfected with vector alone (F/V) or constitutively active PKB (F/B* cells), were incubated with or without 20 nM rapamycin (rap) (A–C) or 10 μM PD98059 (PD) (D–F) for 30 min. A. Lysates (~30 μg protein) from F/V and F/B* cells were separated on SDS-PAGE and immunoblotted with anti-p70S6K Ab. B and E. Portions of lysates from cells incubated with or without rapamycin (B) or PD98059 (E) were assayed for total PDE (M and E) and PDE4 (f) activities (mean cpm ± ½ range, n = 2) in F/V and F/B* cells was assessed by phosphorylation of MBP peptide with MAP kinase immunoprecipitates from cell lysates (~2.5 mg protein). B and E. Portions of lysates from cells incubated with or without rapamycin (B) or PD98059 (E) were assayed for total PDE, PKB, MAP kinase, and p70S6K activities in F/V and F/B* cells. FDCP2 cells, transfected with vector alone (F/V) or constitutively active PKB (F/B* cells), in the absence of IGF-1, PKB activity was ~10-fold greater than that in F/V cells (Fig. 4D). PDE3B was phosphorylated (Fig. 4A) and activated (~3- to 4-fold) (Fig. 4C) without change in the amount of immunoreactive PDE3B (Fig. 4B). In F/B* cells, IGF-1 had little additional effect on activation of PKB (see Fig. 1) or on phosphorylation/activation of PDE3B (data not shown, Table I). As also shown in Fig. 3, wortmannin, in a concentration-dependent manner, blocked the effects of IGF-1 on PKB (Fig. 3D), phosphorylation/activation of PDE3B (Fig. 3, A and C), and activation of PDE4 (Fig. 3C). As shown in Fig. 4, in F/B* cells, wortmannin, in a concentration-dependent manner, almost completely blocked IC50 ~25 nM) the elevated phosphorylation/activation of PDE3B (Fig. 4A) and PKB activity (Fig. 4D). In nontransfected FDCP2 cells, wortmannin also blocked the activation of PDE3B and PDE4 by IL-4 (26). Beyond PI3-K, however, IL-4 effects on PDE3B and PDE4 diverged. IL-4-induced activation of PDE3B was apparently independent of MAP kinase and p70S6 kinase and not blocked by PD98059 (MEK-1 inhibitor) or rapamycin (p70S6 kinase inhibitor). Activation of PDE4 by IL-4 was, however, dependent on MAP kinase and blocked by PD98059 (26). In FDCP2 cells expressing wt MEK, IL-3 activated PDE4, not PDE3B; in FDCP2 cells expressing constitutively activated MEK, PDE4, not PDE3B, activity was increased in the absence of IL-3 (26).

Because phosphoinositide-dependent kinases (PDK) are thought to phosphorylate and activate PKB directly (1–3), the PI3-K inhibitor wortmannin, as expected, blocked IGF-1-induced activation of PKB and phosphorylation/activation of PDE3B in F/B cells (Fig. 3). The mutant PKB expressed in F/B* cells, however, is not activated intrinsically by alteration at the catalytic site, rather it contains an N-terminal myristoylation sequence that targets it to membranes, where it is presumably continuously or constitutively activated in response to basal production of phosphoinositides with activation of PDK isoforms or to a specific pool of active PI3-K. In F/B* cells (Fig. 4), wortmannin could inhibit constitutively activated PKB by blocking production of phosphoinositides involved in PKB association with membranes and/or in the activation of specific PDK isoforms. Whether activated PKB contributes to feedback activation of PI3-K or sensitization of PDK to phosphoinositides is not known, but other workers have also reported that wortmannin or LY294002 inhibits constitutively activated PKB (activated by targeting to membranes) in 3T3-L1 adipocytes (38) or HEK293 cells (37).

Taken together, our previously reported data (26) and that presented in this report support the idea that in FDCP2 cells, IGF-1 activates PDE3B and PDE4 via PI3-K-dependent signals. Downstream of PI3-K, PDE3B is phosphorylated/activated by activated PKB, whereas PDE4 is activated via MEK/MAP kinase signals.

Effects of PKB on phosphorylation/activation of PDE3B in vitro

Because effects of IGF-1 and wortmannin on phosphorylation/activation of PDE3B in F/B or F/B* cells do not prove that PDE3B is a direct substrate for PKB, we tested the ability of PKB to directly...
phosphorylate and activate recombinant mouse (M)PDE3B. Previous experiments have demonstrated that native RPDE3B was phosphorylated on serine 302 in intact rat adipocytes (analogous to serine 296 in MPDE3B) incubated with insulin (16), and that RPDE3B was also phosphorylated in vitro by partially purified rat adipocyte PKB (17) or rPKB (18). Available evidence suggests that phosphorylation is not required to maintain, but does increase, basal PDE activity. As shown in Fig. 5, activated PKB, in a time- and concentration-dependent manner, phosphorylated and increased the activity of wt rMPDE3B synthesized in Sf-9 insect cells. As shown in Fig. 6, activated PKB did not, however, phosphorylate or increase the activity of M3BΔ604, a truncated rMPDE3B from which the N-terminal 604 aa (including putative phosphorylation sites for PKB and PKA (16, 18)) were removed. The higher sp. act. of M3BΔ604 compared with that of wt MPDE3B may be related to higher levels of expression or accumulation of a larger proportion of active M3BΔ604 molecules. It is also possible that the N-terminal portion of PDE3B contains an autoinhibitory domain, the constraints of which are released by phosphorylation or removal of the N-terminal portion of the molecule. Taken together, these data suggest that PKB not only has a role as an upstream regulator, but also directly acts on PDE3B and could thereby be an important determinant in control of intracellular cyclic nucleotide concentrations.

Effects of PDE inhibitors and cAMP on BAD phosphorylation and thymidine incorporation in F/V and F/B* cells

Activated PKB has been reported to increase cell proliferation/survival (9, 11, 40), and to activate PDE3 and trigger meiosis in Xenopus oocytes (8). PKB-catalyzed phosphorylation of BAD may be involved in the antiapoptotic effects of IL-3 (10). As shown in Fig. 7A, in 32P-labeled F/V and F/B cells, but not in F/Bc cells, IGF-1 increased phosphorylation of BAD, which was blocked by incubation of F/B cells with 8-Br-cAMP or the PDE3 inhibitor cilostamide (Fig. 7B). Rolipram, a specific PDE4 inhibitor, was much less effective in inhibiting IGF-1-stimulated phosphorylation of BAD (Fig. 7B). These agents did not block IGF-1-induced activation of PKB (assessed by phosphorylation of Crosstide peptide by immunoprecipitated PKB) (data not shown).
as shown in Fig. 8, in complete medium with IL-3, [3H]thymidine incorporation was ~50% higher in F/B* cells than F/V cells. In the absence of IL-3, thymidine incorporation by F/B* cells was comparable with that in F/V cells grown in complete medium. Whereas F/B* cells were able to proliferate and survive in the absence of IL-3, F/V cells were IL-3 dependent (as previously reported for nontransfected FDCP2 cells (25)).

Increases in intracellular cAMP, brought about by agents that activate adenyl cyclase or by cAMP analogues that activate PKA, have been reported to induce apoptosis and/or inhibit proliferation in some cells, including lymphoid cells (41–44), granulosa cells (45), tumor cells (46–48), and cultured smooth muscle cells (49). PDE inhibitors, by presumably increasing cAMP, have also been reported to inhibit proliferation/induce apoptosis of lymphoid cells (50–53), cultured vascular smooth muscle cells (54), and tumor cells (55). As shown in Fig. 8, the PDE3 inhibitors

![Diagram](http://www.jimmunol.org/)

**FIGURE 5.** PKB-induced phosphorylation and activation of wt PDE3B in vitro. Recombinant wt PDE3B (−10 μg SF-9 cell lysate protein) was incubated at 30°C for 15 min in the indicated concentrations of commercial PDE3 inhibitor (Upstate Biotechnology), activated PKB (0.3 μg/μl) for 15 min (A) or for the indicated times with (□) or without (○) activated PKB (Upstate Biotechnology) PDE3B (−1 μg) (B). The reaction mixtures (with or without [32P]ATP) contained 50 μM ATP, 5 μM PKA inhibitor, and 5 μM okadaic acid (A and B). Reactions (without [32P]ATP) were terminated by dilution with PDE assay buffer and immediately assayed for PDE activity. Data are mean ± SEM of three experiments, assayed in duplicate. To assess phosphorylation of PDE3B (middle panels), reaction mixtures were boiled immediately in Laemmli buffer, and analyzed by PhosphorImager after SDS-PAGE. Portions of reaction mixtures (without [32P]ATP) were immunoblotted with anti-PDE3B Ab (bottom panels).

![Graph](http://www.jimmunol.org/)

**FIGURE 6.** Effects of PKB on phosphorylation and activation of wt PDE3B and a truncated PDE3B mutant in vitro. Recombinant wt PDE3B and PDE3B-Δ604 (a PDE3B from which the first 604 aa were deleted) were incubated (~10 μg SF-9 cell lysate protein) at 30°C for 15 min with or without 20 μM of activated PKB (immunoprecipitated from lysates of F/V, F/B, and F/B* cells after activation by incubation of cells for 10 min in the presence of 50 nM IGF-1) with or without [32P]ATP in reaction buffer containing 50 μM ATP, 5 μM PKA inhibitor, and 5 μM okadaic acid. To assess PDE3B phosphorylation (A), reaction mixtures were boiled immediately in Laemmli buffer, and analyzed by PhosphorImager analysis after SDS-PAGE. Reactions (without [32P]ATP) were terminated by dilution with PDE assay buffer and immediately assayed for PDE activity (B). Data are mean ± SEM of three experiments, assayed in duplicate. Portions of reaction mixtures were also immunoblotted with anti-PKB-CT Ab (C), or anti-PDE3B-CT Ab (34) (D).

![Graph](http://www.jimmunol.org/)

**FIGURE 7.** BAD phosphorylation in PKB-transfected cells. Left, Effects of IGF-1 in F/V, F/B, and F/Bc cells. Right, Effects of cilostamide, rolipram, PGE, and cAMP on IGF-1-induced BAD phosphorylation in F/B cells. FDCP2 cells, transfected with empty vector (F/V), kinase-inactive (F/Bc), or wt (F/B) PKB, were incubated at 37°C in RPMI 1640 medium with 50 μM KH2PO4 and TSB, then with (A) B without G and (C, D, E, and F) added according to the following: 32PO4 (100 μCi/ml) for 90–100 min, then as indicated for 20 min with or without the PDE3 inhibitor cilostamide (10 μM), PDE4 inhibitor rolipram (50 μM), adenylate cyclase activator PGE1 (5 μM), and 8-Br-cAMP (500 μM), and finally for 10 min with or without 10 nM IGF-1. DMSO or ethanol at final concentrations of 0.1% or less was added to appropriate wells as vehicle controls. A and B, BAD-32P was immunoprecipitated with anti-BAD-NT Ab, and dried gels were scanned on a PhosphorImager. C and D, Immunoprecipitates from cells incubated without [32P]ATP were prepared and immunoblotted with anti-BAD-NT Ab E and F, Proteins (~30 μg) in lysates of control and PKB-transfected cells were separated by SDS-PAGE and immunoblotted with anti-PKB-CT Ab.
trequinsin (A) and cilostamide (B) inhibited, in a concentration-dependent manner, the enhanced thymidine incorporation in F/B* cells grown in complete medium with IL-3, with little or no effect in F/B* cells grown without IL-3. Rolipram (Fig. 8B), a specific PDE4 inhibitor, was less effective than the PDE3 inhibitors. Although PGE1 (which activates adenyl cyclase) (Fig. 8D) was rather ineffective in inhibiting thymidine incorporation, higher concentrations of 8-Br-cAMP did inhibit thymidine incorporation by F/V and F/B* cells in complete medium, but had little effect in F/B* cells grown without IL-3 (Fig. 8C). As shown in Fig. 9, however, at concentrations that were not very effective alone, combinations of PGE1 and cilostamide or cilostamide and rolipram had additive or synergistic inhibitory effects on thymidine incorporation in F/B cells and F/B* cells grown in complete medium, and especially in F/B* cells grown without IL-3 (Fig. 8C). As shown in Fig. 9, however, at concentrations that were not very effective alone, combinations of PGE1 and cilostamide or cilostamide and rolipram had additive or synergistic inhibitory effects on thymidine incorporation in F/B cells and F/B* cells grown in complete medium, and especially in F/B* cells grown without IL-3. The combination of PGE1 and rolipram was not as effective as PGE1 and cilostamide or cilostamide and rolipram. These results suggest that, although increases in cAMP brought about by either activation of adenyl cyclase/PDE3 inhibition or inhibition of both PDE3 and PDE4 can markedly inhibit thymidine incorporation (Fig. 9), the role of PDE3 appears predominant.

The detailed mechanism(s) by which cAMP inhibits cell proliferation/survival has not been completely identified and may involve inhibition of autocrine growth factors and cytokines such as IL-2 (50, 56) or direct interruption of mitogenic signaling pathways. Sustained elevation of intracellular cAMP can induce arrest during G1 or G2 phases of the cell cycle (42, 46, 47). cAMP was reported to inhibit Ras/Raf-1 activation of ERK signaling as well as cyclin kinase 4, c-Jun N-terminal kinase, c-Jun, NF-kB, and Stat 1 expression (56–61). Activation of the type I PKA by cAMP analogues inhibits proliferation of lymphocytes (44) and meiotic maturation of mouse oocytes (62). Although it is highly likely that other downstream targets of PKB, e.g., BAD, directly participate in the antiapoptotic effects of PKB (10), PKB-catalyzed phosphorylation/activation of PDE3B, presumably resulting in a decrease in cAMP, could block some of these inhibitory effects of cAMP and thus enhance downstream actions of PKB. Another signaling kinase, p70S6 kinase, is also apparently activated in cells expressing constitutively activated PKB ((38), this study) and could also function as an effector for PKB. There is, however, no direct evidence to suggest that in intact cells p70S6 kinase is a substrate for PKB. cAMP, however, was also reported to inhibit PI3-K-induced activation of p70S6 kinase in IL-2-responsive cells (63). Specific cell

FIGURE 8. Thymidine incorporation into DNA in F/V and F/B* cells. FDCP2 cells transfected with vector alone (F/V) (□, ●), or constitutively active PKB (F/B*) (○, △, ▲) were grown as described in Materials and Methods without (△, ▲) or with (○, △, ▲). 5% WEHI (containing IL-3) and the indicated concentrations of 8-Br-cAMP, PGE1, or PDE inhibitors (cilostamide, trequinsin, and rolipram) for 48 h. [3H]Thymidine was then added, and incubation was continued for 2 h before cells were harvested and DNA was collected on glass microfiber filters for radioassay, as described in Materials and Methods. Data (mean ± SEM of values from triplicate determinations) are representative of two or three separate experiments.

FIGURE 9. Thymidine incorporation into DNA in F/V and F/B* cells. F/V (□) and F/B* (○, △, ▲) cells were grown as described in Materials and Methods in the absence (□) or presence (○, △, ▲) of 5% WEHI-conditioned medium (containing IL-3) and the indicated concentrations of PGE1, cilostamide, or rolipram, alone or in combination for 48 h. [3H]Thymidine was added and incubation was continued for 2 h before cells were harvested and DNA collected on glass microfiber filters. Data (mean ± SEM of values from triplicate determinations) are representative of three separate experiments.
context may be important in the function of PDE3B and cAMP in the action of PKB on cell proliferation/survival, since, in some cells, cAMP actually promotes growth (48, 64) and prevents or delays apoptosis (65).

The proapoptotic protein BAD, a member of the Bcl-2 protein family, promotes apoptosis by heterodimerization with the survival factors Bcl-2 or Bcl-xL. Bcl or Bcl-xL suppresses apoptosis, at least in part, by preventing release of cytochrome c from mitochondria, and thus blocking activation of caspase proteases (66). Survival factors, including IGF-1, IL-3, nerve growth factor, and other cytokines, induce phosphorylation of BAD on critical serine residues, leading to its interaction with and sequestration by 14-3-3 proteins, its dissociation from the BAD/Bcl complex, and subsequent translocation from mitochondria to the cytosol; free Bcl proteins act as suppressors of apoptosis (66).

Although it is clear that PKB-induced phosphorylation of BAD can play an important role in mechanisms that regulate cell survival/proliferation (10, 67), in some cells other signaling pathways involving Raf-1 and PKA regulate phosphorylation of BAD (68), and PKB-independent pathways involving MEK and MAP kinases suppress apoptosis (68–70). Furthermore, in lymphocytes, activation of the TCR can induce apoptosis via Ca2+-induced activation of the phosphatase calcineurin (PP2b) (71), resulting in dephosphorylation of BAD, its heterodimerization with Bcl proteins, and initiation of apoptosis (72).

Recently, Minishall et al. (73) demonstrated that IGF-1 and IL-4 increased expression of Bcl-2 and survival of FDCP2 cells via PI3-K-dependent pathways. In that study, effects of IGF-1 on PKB and BAD phosphorylation, or of cAMP on the antiapoptotic actions of IGF-1 were not assessed. Our results indicate that in FDCP2 cells, IGF-1 activates PDE3 and PDE4 via PI3-K-dependent pathways (29). Downstream of PI3-K, PDE3 is activated by PKB-dependent and PDE4 by MEK/MAP kinase-dependent (29) signals. In this context, our findings are consistent with the idea that in FDCP2 cells, both the extent and duration of increased intracellular cAMP may be important in regulation of BAD phosphorylation and thymidine incorporation, and that PDEs (especially PDE3B) can influence these parameters and, consequently, cell survival. It is possible that in FDCP2 cells, increased cAMP and activation of PKA could directly or indirectly block association of [32P]BAD with 14-3-3 protein, leading to dephosphorylation of BAD, perhaps by PP2b (calcineurin), and thus allowing its association with Bcl proteins and stimulation of apoptosis (71, 72). The relationship, if any, between effects of cAMP on Bcl-2 expression, BAD phosphorylation, and cell survival of FDCP2 cells has not been established. Such information could help elucidate mechanisms responsible for the proapoptotic and antiapoptotic actions of cAMP in different cells. It would be of further interest to learn whether cAMP and PDE3 inhibitors could block proliferation/survival of ovarian, breast, and pancreatic carcinoma cells in which PKB/Akt is apparently amplified (74, 75).

It is important to identify substrates for PKB. BAD (10), GSK-3 (12), phosphofructokinase (13), and endothelial NO synthase (15) were phosphorylated in vitro by PKB, with accompanying changes in activities of GSK-3 and phosphofructokinase. All four contain an RXRXXS motif with arginine residues at n-3 and n-5 of the phosphorylated serine. The RXRXXS motif, however, also apparently served as a substrate for p70S6 kinase and MAP kinase-activated protein kinase-1 (13). Incubation of intact rat adipocytes with insulin resulted in phosphorylation of serine 302 in endogenous rat adipocyte PDE3B (alogous to serine 296 in MPDE3B) (76), which is in the sequence KMFRPPS, i.e., different from that in GSK-3 (12, 13). Although in their recently published report Kitamura et al. (18) did not identify sites phosphorylated in endodogenous MPDE3B in 3T3L1 adipocytes, they did report that serine 276 (within an RXRXXS motif) in rMPDE3B is phosphorylated by activated PKB. Current studies in our laboratories are attempting to reconcile these apparent differences and identify the site(s) involved in phosphorylation/activation of PDE3B by PKB in vitro and intact cells.

Insulin, IGF-1, or IL-4, each of which utilizes IRS proteins to initiate at least some of its receptor signaling cascades, activates PDE3 in adipocytes (16, 17, 21), FDCP2 cells, pancreatic β cells (77), hepatocytes (78), and Xenopus oocytes (79, 80). In these cells, some effects of the polypeptides are counterregulatory to those of cAMP, which increases lipolysis (19, 20, 81), inhibits cell proliferation/survival (41–55), stimulates insulin secretion (77) and glycogenolysis (82), and blocks meiosis (8), respectively. It is, therefore, tempting to speculate that in these cells, activation of PDE3, which would presumably reduce cAMP and PKA, may play an important role in the counterregulatory effects of the polypeptides, i.e., inhibiting lipolysis (16, 19–21), enhancing cell proliferation, inhibiting insulin secretion (77) and glycogenolysis (82), and stimulating meiosis (8, 79, 80), respectively (Fig. 10). From a broader perspective, these and other studies are consistent with the idea that PDEs comprise a complex group of structurally related and highly regulated enzymes that are critical, if not essential, in influencing specificity, compartmentation, and overall regulation of cyclic nucleotide-mediated processes (16, 83–85).

Acknowledgments

We thank Dr. Martha Vaughan (National Heart, Lung, and Blood Institute for her suggestions and critical reading of our manuscript, and Carol Kosh for excellent secretarial assistance.

References


