Orthovanadate Stimulates cAMP Phosphodiesterase 3 Activity in Isolated Rat Hepatocytes through Mitogen-Activated Protein Kinase Activation Dependent on cAMP-Dependent Protein Kinase

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Orthovanadate (vanadate) as well as insulin stimulated phosphodiesterase 3 (PDE3) in the particulate fraction of rat hepatocytes. The vanadate-induced activations of PDE3 and mitogen-activated protein kinase (MAPK) were inhibited by H-89 and PD98059, suggesting that the MAPK activation via cAMP-dependent protein kinase (PKA) and MAPK kinase is involved in the vanadate action. On the other hand, the insulin-induced activations of PDE3 and Akt were inhibited by wortmannin, suggesting involvement of the Akt activation via phosphatidylinositol 3-kinase (PI3K) in the insulin action. The vanadate-induced activations of PKA and PDE3 were inhibited in part by propranolol or genistein, suggesting that vanadate may exert its actions via dual signaling pathways of β-adrenergic receptors and receptor tyrosine kinases of growth factors. Vanadate, in contrast to insulin, did not promote the phosphorylation of insulin receptor substrate-1. The vanadate-induced increase in the phosphorylation of a main isoform of MAPKs, p44 protein, was detected by immunoblotting migration patterns of SDS-PAGE. A partially purified PDE3 activity was increased by addition of MAPK or Akt to the reaction mixture, suggesting that MAPK as well as Akt acts upstream of PDE3. The activation of PDE3 by insulin was independent of a transient increase in the MAPK activity, probably due to the dephosphorylated inactivation mediated by the induced activation of MAPK phosphatases (MKPs). Vanadate did not affect the MKP activity. These results indicate that vanadate stimulates the particulate PDE3 activity by activating mainly p44 MAPK via a PKA-dependent process, and that it differs from insulin with regard to a phosphorylation cascade of PDE3 activation.

Key words orthovanadate; phosphodiesterase 3; mitogen-activated protein kinase; cAMP-dependent protein kinase; hepatocyte; insulin

Orthovanadate (vanadate) mimicks most of the biological effects of insulin on isolated rat fat pads or cells. These actions include the stimulation of glucose uptake,1) the activation of glycogen synthase via an increased tyrosyl phosphorylation of insulin receptor,2) the inhibition of lipolysis,3,4) and an increase in lipoprotein lipase activity.5) In addition, vanadate has adrenergic actions such as the stimulation of adenyl cyclase activity in various membranes isolated from rat adipocytes,6) rat adrenal glands,7) and rabbit ocular ciliary processes.8) Actually, the vanadate-stimulated release of lipoprotein lipase and hepatic lipase activities was due to a transient increase in the intracellular cAMP content accompanied by an activation of cAMP-dependent protein kinase (PKA).9,10) An increase in the cellular lipoprotein lipase activity by vanadate also was induced through mechanisms different from the action of insulin.11) We have recently reported that the incubation of isolated mouse fat pads with vanadate, in contrast to insulin, decreases cellular leptin content and secretion due to increased degradation via a cAMP/PKA-dependent process involving proteasome activation and/or ubiquitination.12,13) Classically, in rat hepatocytes, vanadate inactivates glycogen synthase and activates glycogen phosphorylase.14) Cellular levels of cAMP or cGMP are regulated by the relative activities of their synthesizing enzymes, adenylcyclase and guanylyl cyclase, and hydrolyzing enzymes, cyclic nucleotide phosphodiesterases (PDEs). PDEs regulate many physiological functions, including cardiac contractility, smooth muscle relaxation, platelet aggregation, visual response, fluid homeostasis, and immune responses.15—17) PDE activities are stimulated by a phosphorylation event, the binding of Ca2+/calmodulin, or the interaction of regulatory proteins. PDEs are classified into 11 or more major isoenzyme families. The particulate PDE from rat adipocytes was stimulated by insulin,18,19) and the stimulation of PDE3 was dependent on the activation of Akt but not mitogen-activated protein kinase (MAPK).20—22) We previously reported that vanadate stimulated the particulate PDE 3 activity of rat fat pads through the activation of myelin basic protein kinase.23,24) The dense vesicle and peripheral plasma membrane PDEs from hepatocytes have been reported to be hormone-sensitive enzymes.25,26) The insulin-induced activations of these two PDEs were thought to be achieved by distinct routes.27,28) However, the effects of vanadate on the regulation of the hepatic PDEs through receptor signaling pathways are still unknown. It would be of great advantage to clarifying the cellular events which regulate the PDE activity if we knew the intracellular effects of vanadate on hepatocytes.

This paper presents results showing that vanadate stimulates hepatic PDE3 activity via a phosphorylation cascade different from the action of insulin.

MATERIALS AND METHODS

Animals Male Wistar rats, weighing 250—300 g, were purchased from Shimizu Laboratory Animal Supplies (Kyoto, Japan). They were fed on a commercial pellet diet ad libitum, given free access to water for 1 week according to the Guide for the Care and Use of Laboratory Animals established by Fukuyama University, and starved for 24 h before

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use.

**Materials** Vanadate, insulin, snake venom, and wortmannin were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Genistein, propranolol, and collagenase were obtained from Wako Pure Chemical (Osaka, Japan). H-89 was purchased from Seikagaku Kogyo (Tokyo, Japan). PD98059, rolipram, Ro 20-1724, milrinone, malachite green colorimetric reagent, and MAP(177-189) pT/pY peptide were obtained from Biomol Research (Plymouth meeting, PA, U.S.A.). Bio-Rad Protein Assay Kit was obtained from Bio-Rad (Richmond, CA, U.S.A.). [2,8-3H]cAMP, [γ-32P]ATP, and chemiluminescence reagent were purchased from NEN (Boston, MA, U.S.A.). p42/p44 MAP kinase enzyme assay system was obtained from Amersham Pharmacia Biotech (Little Chalfont, England). Akt substrate was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). PKA Assay Kit, Anti-insulin receptor substrate-1 (IRS-1) IgG, Anti-phosphotyrosine IgG, and Anti-MAP kinase 1/2 IgG were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Protease inhibitor cocktail tablets were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Protein A- Sepharose CL-4B was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Goat anti-rabbit IgG coupled to horseradish peroxidase was obtained from BioMaker (Rehovot, Israel). All other chemicals used were of analytical grade.

**Cell Incubation and Preparation of Subcellular Fractions** Hepatocytes were prepared from the liver of 24-h starved rats using collagenase, essentially according to the method of Berry and Friend.29 The stock solution of vanadate (100 mM, pH 7.4) was diluted with deionized water to the desired concentrations immediately before use. Cells (4×10^6/ml), which were finally suspended in 10 ml of Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mM glucose and 1% bovine serum albumin (KRBGA), were incubated with vanadate or insulin by shaking at 37 °C for the desired periods.24 The incubated cells were homogenized with a microhomogenizer (NS-310E, Nichion Medical Science, Tokyo, Japan) in 1 ml of chilled 10 mM Tris–HCl buffer, containing 250 mM sucrose, 10 mM sodium pyrophosphate, 1 mM benzamidine, and 0.1 mM p-aminido-phenylmethanesulfonyl fluoride (p-APMSF). The homogenate was centrifuged at 650 g for 5 min to remove nuclei and again at 10000 g for 20 min to separate the fraction containing the plasma membrane and mitochondria. The supernatant was further centrifuged at 105000 g for 60 min to separate the particulate and cytosolic fractions.25,30,31 Fractions were prepared without any solubilization procedure and stored at −80°C. The protein concentration was measured using a Bio-Rad Protein Assay Kit.

**PDE Assays** Aliquots (50 μl) of fractions (500 μg protein/ml) were incubated with 125 nm [3H]cAMP in 33 mM Tris–HCl buffer, pH 7.4, containing 4 mM MgCl2 at 30 °C for 20 min, in a total volume of 0.25 ml, as described previously.19,24 The 5′-[^3H]AMP produced was converted to [H]adenosine by the nucleotidase action of the snake venom (50 μg). The reaction mixture (0.5 ml) was applied to a 5.5×30 mm column of Dowex 1×8 (200—400 mesh in chlo-

**Protein Kinase Assays** The cytosolic fraction which had been separated from cells treated with insulin or vanadate was used for protein kinase assays. The MAPK activity was determined using a p42/p44 MAP kinase enzyme assay system, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Briefly, a mixture of 15 μl of the cytosolic fraction (500 μg protein/ml), 10 μl of substrate buffer, pH 7.4, containing substrate peptide, and 5 μl of Mg/ATP buffer reagent, pH 7.4, containing Hepes and [γ-32P]ATP was incubated at 30°C for 30 min. The reaction was terminated by the addition of 10 μl of stop reagent containing orthophosphoric acid and carmosine red, and the reaction mixture was centrifuged at 10000 g for 15 s to wash all reagents into the base of the tube. An aliquot of 30 μl was spotted on to binding paper and washed repeatedly, and the radioactivity was measured. The results were expressed in terms of pmol phosphate/min/mg protein. The Akt activity was determined by a slight modification of the method of Wijkander et al.22,23 Briefly, a mixture of an equivalent volume (10 μl) of the cytosolic fraction (500 μg protein/ml) and 11.3 mM Hepes buffer, pH 7.5, containing 130 mM sucrose, 26.6 mM MgCl2, 3.3 mM dithiothreitol, 10 μM H-89, 75 μM [γ-32P]ATP, and 3 μg of peptide PRPAATF as substrate was incubated at 30°C for 20 min. Incubation was terminated by the addition of 15 μl of 1 mM ATP, pH 3.0, containing 1% bovine serum albumin, and 7.5 μl of 30% trichloroacetic acid. After centrifugation at 10000 g for 15 s, 35 μl of the resultant supernatant was applied to phosphocellulose filter paper (Whatman p81) and washed repeatedly. The radioactivity was then measured. The results were expressed in terms of pmol phosphate/min/mg protein. The PKA activity was determined using a PKA Assay Kit, according to the manufacturer’s directions (Upstate Biotechnology). Briefly, a mixture of the cytosolic fraction (5 μg protein), assay dilution buffer (pH 7.2), cAMP, PKC/CaMK inhibitor cocktail, Mg/ATP, [γ-32P]ATP, and kemptide as substrate was incubated at 30°C for 10 min, in a total volume of 60 μl. A 25 μl aliquot of the reaction mixture was applied to phosphocellulose filter paper (Whatman p81) and washed repeatedly. The radioactivity was measured. The results were expressed in terms of nmol phosphate/min/mg protein.

**Phosphatase Assays** The cytosolic fraction which had been separated from the cells treated with insulin or vanadate was assayed for the dual specificity protein phosphatases specific for MAPks, termed MAP phosphatases (MKPs).33 MKP activity was determined using MAP(177-189) pT/pY peptide as substrate by a modified version of the Protein Tyrosine Phosphatase 1B Assay according to the manufacturer’s instructions (Calbiochem). Aliquots (5 μl) of the cytosolic fraction (100 μg protein/ml) and 5 μl of 75 nm MKP substrate were added to 40 μl of an assay buffer, pH 7.2, containing 50 mM Hepes, 1 mM dithiothreitol, 1 mM EDTA, and 0.05% NP-40, in each well and incubated at 30°C for 30 min. After termination of the reaction by adding 100 μl of a malachite green colorimetric reagent, the reaction mixtures were allowed to develop color for 30 min. Amounts of phosphate released were measured as absorbance at 620 nm on a microtiter-plate reader. MKP activity was expressed as nmol phosphate released per min per mg protein.

**Purification of PDE3, MAPK, and Akt** PDE3 was purified partially by chromatography on a DEAE-Sephalcol-
the method of Ueki et al.\textsuperscript{24} The particulate fraction (10 mg protein) was lysed in 1 ml of 0.5% Triton X-100, containing 0.04% 2-mercaptoethanol, 1 mM benzamidine, and 0.1 mM p-APMSF at 4 °C for 1 h and centrifuged at 10000\(g\) for 10 min. The resultant supernatant (1 ml) was applied to a DEAE-Sepharocel column (7.5 ml) previously equilibrated with 10 mM Tris–HCl buffer, pH 7.0, containing 0.1 mM 2-mercaptoethanol, 0.1 mM EDTA, 10% glycerol, and 1 mM benzamidine, and 0.1 mM p-APMSF (column buffer). The column was washed with the column buffer and eluted stepwise with 0.2—0.4 M NaCl in the column buffer. Akt was eluted from an anti-Akt IgG column with a column buffer, pH 3, containing 0.04% 2-mercaptoethanol, 1 mM benzamidine, and 0.1 mM p-APMSF was applied to the column buffer. MAPK was eluted from an anti-MAPK IgG column with a column buffer, pH 11, containing 0.1 M Na\textsubscript{2}CO\textsubscript{3} buffer instead of Tris–HCl buffer. MAPK was eluted stepwise with 0.2—0.4 M NaCl in the column buffer.

**SDS-PAGE and Immunoblotting of IRS-1 and MAPKs**

To prepare the IRS-1 sample, hepatocytes (2 × 10\textsuperscript{6} cells) were incubated with insulin or vanadate at 37 °C for 10 min in 5 ml of KRBGA, washed with 10 mM Tris–HCl buffer, pH 7.4, containing 250 mM sucrose, and lysed in a solubilizing buffer, pH 7.4, containing 20 mM Tris, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 10% glycerol, one fifth of a Protease inhibitor cocktail tablet, 1 mM EGTA, 1 mM vanadate, and 1 mM phenylmethylsulfonyl fluoride at 4 °C for 30 min.\textsuperscript{34} After centrifugation at 10000\(g\) for 10 min, the supernatant was incubated with anti-IRS-1 IgG at 4 °C overnight and then with Protein A-Sepharose CL-4B for 2 h.\textsuperscript{35} Immunoprecipitates were collected by centrifugation, washed twice with 10 mM phosphate buffer, pH 7.2, containing 150 mM NaCl, 50 mM NaF, and 1 mM vanadate, and subjected to SDS-PAGE on 7.5% slab gels.\textsuperscript{36} Immunoblotting procedures were carried out, essentially according to the method of Towbin et al.\textsuperscript{37} Briefly, proteins in the gels were transferred electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked and probed with an anti-phosphotyrosine IgG. Blots were then incubated with a goat anti-rabbit IgG coupled to horseradish peroxidase and subjected to chemiluminescence detection, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Band intensities were estimated using an ATTO Densitograph Software Library Lane Analyzer Ver. 2 (ATTO, Tokyo, Japan).\textsuperscript{12} For immunoblot analysis of the phosphorylation of MAPKs, hepatocytes were preincubated with or without 50 \(\mu\)M PD98059 for 15 min and further incubated with 100 \(\mu\)M vanadate for 15 or 30 min. The cytosolic fraction was subjected to SDS-PAGE with 8% slab gels and then immunoblotting using anti-MAPK antibody that recognizes either the p42 or p44 isoform of MAPK, as described previously.\textsuperscript{24,37}

**Statistical Analysis**

Results are presented as the mean ± S.E. from four separate experiments, each performed in duplicate. The data were analyzed with unpaired Student’s \(t\)-, Dunnet’s, or Bonferroni tests.

**RESULTS**

**Effects of Vanadate and Insulin on PDE Activities in Hepatocytes**

We initially investigated which fractions contain PDE isoforms sensitive to vanadate. A 60-min incubation of hepatocytes with vanadate resulted in a predominant increase in PDE activity of the particulate fraction in a dose-dependent manner, as compared with that of the fraction containing plasma membrane and mitochondria or the cytosolic fraction (Fig. 1). When the hepatocytes were incubated with 100 \(\mu\)M vanadate or 10 \(\mu\)M insulin in KRBGA alone for up to 60 min. The particulate PDE activity was determined. Results are expressed as a percentage of each initial value. Significant differences compared with the control at each time point: \(\ast p<0.05\) and \(\ast\ast p<0.01\).

Fig. 1. PDE Activities of Subcellular Fractions Separated from Vanadate-Treated Hepatocytes

Hepatocytes were incubated with vanadate at the indicated concentrations in 10 ml of KRBGA for 60 min. After the cells were homogenized and fractionated, the fraction containing the plasma membrane and mitochondria (P1), particulate fraction (P2), and cytosolic fraction were assayed for PDE activities using \(^{3}H\)cAMP as substrate. Results are expressed as a percentage of the un-treated group (control). Significant differences compared with the control: \(\ast p<0.05\) and \(\ast\ast p<0.01\).

Fig. 2. Time-Dependent Effect of Vanadate or Insulin on PDE Activity in Hepatocytes

Hepatocytes were incubated with 100 \(\mu\)M vanadate or 10 \(\mu\)M insulin in KRBGA alone for up to 60 min. The particulate PDE activity was determined. Results are expressed as a percentage of each initial value. Significant differences compared with the control at each time point: \(\ast p<0.05\) and \(\ast\ast p<0.01\).
propranolol, H-89, and PD98059, suggesting that PDE3 activity was inhibited by genistein or wortmannin, which it stimulates PDE3 activity.

**Phosphorylation Cascade Involved in Action of Vanadate or Insulin** To confirm whether or not a signal pathway via the insulin receptor is involved in the stimulation of PDE3 activity by vanadate, the phosphorylation of IRS-1 in hepatocytes was examined. A 10-min incubation of hepatocytes with 10 nM insulin resulted in a 165%-increase in the amount of phosphorylated IRS-1 compared to the control group (Table 2). In contrast, no increase in the phosphorylation was observed with 100 μM vanadate, suggesting that stimulation of PDE3 activity by vanadate is independent of a signaling pathway via the insulin receptor. The PKA activity in hepatocytes treated with 100 μM vanadate reached the maximum at a 15-min incubation and returned to the control level after 45 min (Fig. 4). A partial inhibition of the effect of vanadate on PKA activity was observed with propranolol or genistein (Table 3). The results suggest that processes utilizing the β-adrenergic receptors and receptor tyrosine kinases of growth factors are involved in the mechanism of the vanadate action. The MAPK activity was increased by vanadate over a 60-min incubation period, while a transient increase by insulin returned to the control level after 30 min (Fig. 5). The effect of vanadate was markedly inhibited by H-89 or PD98059, suggesting that it is mediated by PKA and MAPKK activations upstream of MAPK (Table 4). On the other hand, a significant increase in the Akt activity was observed with insulin but not vanadate (Fig. 6). The insulin-induced increase in the Akt activity was markedly inhibited by wortmannin, suggesting that the Akt activation via PI3K is involved in the insulin action (Table 5).

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**Table 1. Effects of Inhibitors on Vanadate or Insulin-Induced Stimulation of PDE Activity in Hepatocytes**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Vanadate activity (%)</th>
<th>Insulin activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol (μM)</td>
<td>0: 190±9</td>
<td>150±5</td>
</tr>
<tr>
<td>Genistein (μg/ml)</td>
<td>0: 186±6</td>
<td>147±8</td>
</tr>
<tr>
<td>H-89 (μM)</td>
<td>0: 194±8</td>
<td>149±9</td>
</tr>
<tr>
<td>Wortmannin (mM)</td>
<td>0: 195±9</td>
<td>149±6</td>
</tr>
<tr>
<td>PD98059 (μM)</td>
<td>0: 189±6</td>
<td>149±5</td>
</tr>
</tbody>
</table>

Hepatocytes were preincubated with inhibitors at the indicated concentrations for 15 min and then incubated with 100 μM vanadate or 10 nM insulin for 30 min. The particulate fraction contained mainly PDE3 sensitive to vanadate and insulin.

**Fig. 3. Effects of PDE Inhibitors on Particulate PDE Activity**

The particulate fraction (500 μg protein/ml) was preincubated with PDE inhibitors at the indicated concentrations in 0.1 ml of the PDE assay buffer for 15 min and then assayed for PDE activity. Results are expressed as a percentage of each group without inhibitors (control).

**Fig. 4. Effect of Vanadate on PKA Activity of Hepatocytes**

Hepatocytes were incubated with 100 μM vanadate for up to 60 min. Significant differences compared with the control at each time point: *p<0.05 and **p<0.01.

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**Table 2. Effect of Vanadate or Insulin on Phosphorylation of IRS-1**

<table>
<thead>
<tr>
<th></th>
<th>Phosphorylation of IRS-1 (%)</th>
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<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Vanadate (100 μM)</td>
<td>100±5</td>
</tr>
<tr>
<td>Insulin (10 nM)</td>
<td>103±8</td>
</tr>
</tbody>
</table>

Hepatocytes (2×10⁶ cells) were incubated with or without 100 μM vanadate or 10 nM insulin for 10 min in 5 ml of RPMI 1640 and lysed in a solubilizing buffer at 4°C for 30 min. After immunoblotting with an anti-phosphotyrosine IgG, band intensities were measured. Significant differences compared with each initial group: *p<0.05.
Table 3. Effect of Propranolol or Genistein on Stimulation of PKA Activity by Vanadate

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>PKA activity (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Propranolol ((\mu\text{M}))</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>101±4</td>
</tr>
<tr>
<td>Genistein ((\mu\text{g/ml}))</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>98±5</td>
</tr>
<tr>
<td>10</td>
<td>100±2</td>
</tr>
</tbody>
</table>

Hepatocytes were preincubated with inhibitors in KRBGA for 15 min at the indicated concentrations and further incubated with or without 100 \(\mu\text{M}\) vanadate for 30 min for assay PKA activity. Significant differences compared with each vanadate-treated group without inhibitors: *\(p<0.05\).

Table 4. Effect of H-89 or PD98059 on Vanadate-Induced Stimulation of MAPK Activity

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration ((\mu\text{M}))</th>
<th>MAPK activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>H-89</td>
<td>0 0.1 1 10</td>
<td>100</td>
</tr>
<tr>
<td>PD98059</td>
<td>0 1 2 10 50</td>
<td>100</td>
</tr>
</tbody>
</table>

Hepatocytes were preincubated with inhibitors in KRBGA for 15 min at the indicated concentrations and further incubated with or without 100 \(\mu\text{M}\) vanadate for 30 min for assay of MAPK activity. Significant differences compared with each vanadate-treated group without inhibitors: *\(p<0.05\) and **\(p<0.01\).

Table 5. Effect of Wortmannin on Insulin-Induced Stimulation of Akt Activity

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration ((\text{nM}))</th>
<th>Akt activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Wortmannin</td>
<td>0 20 100 500</td>
<td>100</td>
</tr>
</tbody>
</table>

Hepatocytes were preincubated with wortmannin in KRBGA for 15 min at the indicated concentrations and further incubated with or without 10 \(\text{nM}\) insulin for 30 min for assay of Akt activity. Significant differences compared with the insulin-treated group without inhibitor: *\(p<0.05\) and **\(p<0.01\).

Purification of PDE3, MAPK, and Akt

The highest PDE3 activity, 140 pmol/min/mg, was found in fraction nos 49 and 50 which were eluted with a column buffer containing 0.4 \(\text{M}\) NaCl from a DEAE-Sephasel column (Fig. 7A). Neither MAPK nor Akt activity was found in the PDE3 fractions. Fraction no. 28, which was eluted from an anti-MAPK IgG column, contained the highest MAPK activity, 325 pmol/min/mg (Fig. 7B). The highest Akt activity, 19 pmol/min/mg, was found in fraction no. 29, which was eluted from an anti-Akt IgG column (Fig. 7C). No PDE3 activity was detected in the MAPK and Akt fractions.

Effect of MAPK or Akt on Isolated PDE3 Activity

When a partially purified PDE3 was incubated with the

MAPK or Akt in the presence of 1 \(\text{mM}\) ATP, MAPK as well as Akt increased the PDE3 activity in an added dose-dependent manner (Fig. 8). These results show that MAPK also stimulates the PDE3 activity upstream of PDE3.

Immunoblot Analysis of Phosphorylated MAPKs

The phosphorylation of MAPK was assessed by immunoblotting with antibodies which specifically detect either the p42 or p44 isoform of MAPK. The cytosolic fraction contained a major protein of 44 kDa and a minor one of 42 kDa, which are the p42 and p44 isoforms of MAPK, respectively (Figs. 9A and B). A 15 or 30-min treatment of hepatocytes with vanadate resulted in a retardation of p44 MAPK mobility, probably due to the increased phosphorylation. Almost half of the phosphorylated p44 MAPK returned to the basal level in the presence of PD98059, suggesting that the increased phosphorylation of p44 MAPK is induced via MAPKK.

Effect of Insulin or Vanadate on MKP Activity

When hepatocytes were incubated with 100 \(\text{\(\mu\text{M}\)}\) vanadate or 10 \(\text{\(\text{nM}\)}\) insulin for up to 60 min, a significant increase in the cytosolic MKP activity was observed with insulin at 15 and 30-min
incubations (Fig. 10). Vanadate did not increase the cytosolic MKP activity. A 15-min incubation of an activated MAPK (Sigma Chemical) with the cytosolic fraction containing the increased MKP activity resulted in a significant decrease in the MAPK activity (data not shown). A rapid activation of MKP may lead to the inactivation of MAPK by dephosphorylation.

Fig. 7. Purification of PDE3, MAPK, and Akt
A: The supernatant of solubilized particulate fraction was applied to a DEAE-Sepha-
cel column and eluted stepwise with 0.2—0.4 M NaCl in the column buffer. B: The cytosolic fraction was applied to an anti-MAPK IgG column and eluted with the column buffer, pH 11, containing 0.1 M Na2CO3 buffer. C: The cytosolic fraction was applied to an anti-Akt IgG column and eluted with the column buffer, pH 3, containing 0.1 M glycine buffer.

Fig. 8. Stimulation of PDE3 Activity by MAPK or Akt
A partially purified PDE3 (1.5 μg protein) was incubated with MAPK or Akt at the indicated amounts at 30 °C for 15 min and assayed for PDE activity. Significant differences compared with the control group without MAPK or Akt: *p<0.05 and **p<0.01.

Fig. 9. Immunoblot of phosphorylation of MAPKs
Hepatocytes were preincubated with 50 μM PD98059 (A) or without the inhibitor (B) for 15 min and further incubated with or without 100 μM vanadate for 15 or 30 min. The cytosolic fraction was subjected to SDS-PAGE and then immunoblotting using anti-MAPK antibody. The control group was incubated for 0 (a), 15 (b), and 30 min (c). The vanadate-treated group was incubated for 15 (d) and 30 min (e). Approximate molecular masses are indicated on the left.

Fig. 10. Time-Dependent Effect of Insulin or Vanadate on MKP Activity in Hepatocytes
Hepatocytes were incubated with 10 nM insulin or 100 μM vanadate or in KRBGA alone for up to 60 min. The cytosolic fraction was assayed for MKP activity. Basal MKP activity was 93 nmol/min/mg protein. Significant differences compared with the control at each time point: *p<0.05 and **p<0.01.
DISCUSSION

Two PDE3 isoforms, PDE3A and PDE3B, are known to be products of different but related genes.\(^46,47\) PDE3A is highly expressed in cardiac muscle, vascular and visceral smooth muscles, and platelets where it regulates contractility and aggregation, while PDE3B is abundant in adipocytes, hepatocytes, spermatocytes, and renal collecting duct epithelium being involved in hormonal regulation of lipolysis and glycogenolysis.\(^48\) The liver dense vesicle and peripheral plasma membrane PDEs have been reported to be PDE3 and PDE4, respectively, both of which are sensitive to insulin.\(^47,49\) The dense vesicle PDE3 is associated with the particular fraction similar to a classical marker enzyme for endoplasmic reticulum.\(^28\) It is also strongly inhibited by cGMP or milrinone.\(^47,50\) The particulate fraction used throughout this investigation appears to mainly contain PDE3, probably type B, which is stimulated by insulin and vanadate, as suggested by the potent inhibition by milrinone but not rolipram or Ro 20-1724. In adipocytes, the insulin-induced activation of PDE3B was due to Akt-mediated phosphorylation of the enzyme at serine-273 in a PI3K-dependent manner.\(^51,52\) Akt, also termed protein kinase B, is a serine/threonine kinase and mediator of cell survival and anti-apoptotic signaling.\(^53—55\) It is also strongly inhibited by cAMP or milrinone.\(^47,50\) The particulate fraction used throughout this investigation appears to mainly contain PDE3, probably type B, which is stimulated by insulin and vanadate, as suggested by the potent inhibition by milrinone but not rolipram or Ro 20-1724. In adipocytes, the insulin-induced activation of PDE3B was due to Akt-mediated phosphorylation of the enzyme at serine-273 in a PI3K-dependent manner.\(^51,52\) Akt, also termed protein kinase B, is a serine/threonine kinase and mediator of cell survival and anti-apoptotic signaling.\(^53—55\)

Our results with hepatocytes showed that 100 \(\mu M\) vanadate did not induce the activation of Akt although insulin activated Akt in a PI3K-dependent manner. A partially purified PDE3 activity was increased by addition of MAPK or Akt to the reaction mixture, suggesting MAPK as well as Akt acts upstream of PDE3. IRS-1 proteins undergo tyrosine phosphorylation immediately after insulin stimulation and mediate insulin receptor tyrosine kinase signaling such as binding of PI3K and the associated activation of PDE3B.\(^56,57\) Treatment of CHO cells over-expressing the human insulin receptor with vanadate caused increased phosphorylation of the p42 and p44 isoforms of MAPK but not the insulin receptor \(\beta\)-subunit or IRS-1 even at 1 \(\mu M\).\(^58\) Treatment of hepatocytes with vanadate also stimulated both PDE3 and MAPK activities without the increased phosphorylation of IRS-1. Thus, vanadate differed from insulin with regard to the phosphorylation cascade of PDE3. The vanadate-induced activations of PDE3 and MAPK were inhibited by either H-89 or PD98059 but not wortmannin. These results suggest that vanadate stimulates the MAPK activity via a PKA-dependent process leading to the PDE3 activation. MAPKs, also termed extracellular signal-regulated kinases, are serine/threonine kinases and found to be activated in response to insulin and growth factors which promote cell growth and differentiation.\(^59\) Phosphorylation of some proteins including MAPK causes a retardation of their mobility in SDS-PAGE gels.\(^60\) Therefore, the appearance of slower migration forms of phosphorylated MAPK seems to represent the activation of enzyme. Treatment of preadipocytes with agents which increase the intracellular level of cAMP induced phosphorylation/activation of the p42 and p44 isoforms of MAPK via MAPKK.\(^60\) The phosphorylation/activation of p42 MAPK was inhibited by PD 098059 or H-89, suggesting that PKA causes the activation of a MAPK pathway upstream of MAPKK. In hepatocytes in which the p44 isoform of MAPK is mainly detected, vanadate induced a retardation of p44 MAPK mobility, probably due to the increased phosphorylation. In the presence of PD98059, almost half of the phosphorylated p44 MAPK returned to the basal level, suggesting that vanadate stimulates the phosphorylation of p44 MAPK via MAPKK. The adenyl cyclase activity is stimulated by either extracellular or intracellular stimuli to produce cAMP leading to the PKA activation. Our previous report showed that the vanadate-induced decrease in intracellular leptin of isolated mouse fat pads was prevented by propranolol or bupranolol, a \(\beta\)-adrenergic receptor antagonist.\(^61\) suggesting that a process utilizing the \(\beta\)-adrenergic receptor is involved in mechanisms of the action of vanadate.\(^12\) On the other hand, vanadate stimulated a tyrosine kinase-dependent enhancement of adenyl cyclase function in human and rat aortic vascular smooth muscle cells, human lymphocytes, and human aortic endothelial cells.\(^62\) The activation of adenyl cyclase isoform 6 expressed in HEK 293 cells also was mediated by the receptor tyrosine kinase activation with growth factors and vanadate.\(^63\) In response to vanadate, an increase in serine, but not tyrosine, phosphorylation of adenyl cyclase isoform 6 was observed. The increased serine phosphorylation was suggested to be due to the activation of a serine/threonine kinase, raf-1 kinase, sensitive to receptor tyrosine kinase. Thus, the tyrosine kinase-mediated pathway also may be seen as a regulator of adenyl cyclase function. The vanadate-induced stimulation of PKA activity in hepatocytes was inhibited in part by propranolol or genistein, suggesting that vanadate may exert its actions via dual signaling pathways of the \(\beta\)-adrenergic receptors and receptor tyrosine kinases of growth factors. MAPKs are inactivated via dephosphorylation of either the threonine or tyrosine residue or both by MKPs.\(^64\) In vascular smooth muscle cells, insulin results in a small transient effect on MAPK activity and a rapid return to the basal levels within 30 min.\(^65\) A rapid decrease in the insulin-stimulated MAPK activity seemed to be mediated in part by the induction of MKP-1 via the NO/cGMP signaling pathway.\(^66,67\) Treatment of hepatocytes with insulin also resulted in increased MKP activity in the cytosolic fraction. The incubation of activated MAPK with the fraction containing the increased MKP activity led to a decrease in the MAPK activity by dephosphorylation. Vanadate did not affect the MKP activity although it is well known as a potent inhibitor of protein tyrosine phosphatases.\(^68\) This may be a reason why the stimulation of PDE3 by insulin is independent of a MAPK pathway. Thus, vanadate appears to be a useful biological tool for studying the regulation of PDEs through a receptor signaling pathway.

In conclusion, the results of this study indicate that vanadate stimulates hepatic PDE3 activity by activating mainly p44 MAPK via a PKA-dependent process, and that it differs from insulin with regard to the phosphorylation cascade of PDE3.

REFERENCES
