Regulation of Cyclic AMP Phosphodiesterase Activity by Particulate Protein Tyrosine Kinase and Phosphotyrosine Phosphatase Activities Sensitive to Sodium Orthovanadate

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Sodium orthovanadate (vanadate) stimulated cAMP phosphodiesterase (PDE) and protein tyrosine kinase (PTK) activities and inhibited the phosphotyrosine phosphatase (PTPase) activity in the particulate of isolated rat fat pads. Okadaic acid never showed any increase in the PDE activity up to 1 μM. Amiloride inhibited in part both stimulations of PDE and PTK activities by vanadate. The particulate PTK activity had an optimal divalent ion requirement of 15 mM Mg^{2+} + 2 mM Mn^{2+} in the assay medium and was not inhibited by 1 mM β-ethylmaleimide, suggesting it to be a different type from the insulin receptor and cytosolic PTK activities. The PDE, PTK, and PTPase active fractions were separated from the solubilized particulate fraction on a DEAE-Sepharose column. PDE activity was increased by the addition of the PTK active fraction. A further increase was observed by using the PTK active fraction pretreated with 1 mM vanadate. In contrast, the addition of PTPase active fraction decreased the PDE activity. This decrease disappeared by using the PTPase active fraction pretreated with 1 mM vanadate. These results suggest that the PDE activity is in part regulated through a process involving the particulate PTK and PTPase activities sensitive to vanadate.

Key words vanadate; cAMP phosphodiesterase; protein tyrosine kinase; phosphotyrosine phosphatase; fat pad particulate

Many different phosphodiesterases (PDEs) selectively hydrolyze cyclic nucleotides to regulate cellular responses.1) One type, the cGMP-inhibited low K_M cAMP PDE in the particulate fraction is stimulated by the incubation of rat adipocytes with insulin.2) Degerman et al. reported on the regulation of the PDE activity in which insulin, as well as catecholamine, induced serine phosphorylation of the PDE molecule under conditions that stimulated the activity, via the activation of an unidentified protein serine kinase.3) Our previous report showed that the incubation of rat fat pads with sodium orthovanadate (vanadate) stimulated insulin-sensitive PDE activity through the activation of protein kinase-mediated processes.4) Further details, however, are still unclear. In a cell-free system, the addition of soluble fractions from insulin-treated rat adipocytes or liver to the particulate fraction of adipocytes stimulated PDE activity in the presence of ATP, suggesting that the soluble fractions contain a protein kinase or substance phosphorylated to a catalytically active form.5) It is, still, unclear whether the particulate protein tyrosine kinase (PTK) and protein tyrosine phosphatase activities are involved in the regulation of PDE activity, though there is a report that the insulin treatment of hepatocytes shows stimulatory phosphorylation of tyrosyl residue(s) of cAMP PDE of the plasma membrane fraction and an increase in enzymatic activity.6) Vanadate is reported to be a potent inhibitor of protein tyrosine phosphatase and to exert the stimulatory phosphorylation of insulin receptors.7) Thus, vanadate appears to affect a process involving the phosphorylation--dephosphorylation of tyrosyl residues of proteins.

In this paper, we show that the PDE activity is in part regulated through mechanisms involving particulate PTK and phosphotyrosine phosphatase (PTPase) activities sensitive to vanadate.

MATERIALS AND METHODS

Materials The sources of chemicals used in this work were as follows: Vanadate (Na_3 VO_4), okadaic acid, and ATP from Wako Pure Chemical Industries (Osaka, Japan); biochanin A and poly (Glu_4, Tyr_1) copolymer from Sigma Chemical Co. (St. Louis, MO, U.S.A.); cAMP from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan); O-phosphotyrosine from Boehringer-Mannheim Biochemicals (Mannheim, Germany); Bio-Rad Protein Assay kit from Nippon Bio-Rad Laboratories, K.K. (Tokyo, Japan); DEAE-Sepharose from Pharmacia LKB Biotechnology (Uppsala, Sweden); [2,8-^3^H]cAMP (1.158 TBq/mmol) from New England Nuclear (Boston, MA, U.S.A.); [7-^3^P]ATP (167 TBq/mmol) from ICN Biomedicals, Inc. (Irvine, CA, U.S.A.). All other chemicals used were of analytical grade.

Preparation of Fat Pads and Particulate Fraction Epididymal adipose tissues were quickly removed from Wistar rats (male, weighing 200—220 g) and cut into small pieces (30—40 mg) with scissors to prepare fat pads. The particulate was prepared from the homogenate of fat pads by centrifugation at 105000 × g, finally, as described previously.2,4)

Separation of PDE, PTK, and PTPase Activities The particulate (10—15 mg in protein) was solubilized in 1 ml of 0.5% Triton X-100 containing 5 mM 2-mercaptoethanol, 1 mM benzamidine, and 0.1 mM p-amidinophenylmethane-sulfonyl fluoride at 4 °C for 1 h and centrifuged at 13000 × g for 10 min. The supernatant was applied to a DEAE-Sepharose column to obtain separately PDE, PTK, and PDPase activities sensitive to vanadate.

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PTPase active fractions. Protein concentration was calculated from the absorbance at 280 nm or determined by the method using a Bio-Rad Protein Assay kit.

**Determination of Enzymatic Activities** The PDE activity was determined by a modification of the method of Kono et al., using 33 mM Tris–HCl buffer, pH 7.4, containing 250 mM [3H]cAMP and 4 mM MgCl₂, in a total volume of 0.25 ml. The activity was expressed in terms of picomoles cAMP hydrolyzed per min per mg protein. Determination of the PTK activity was carried out by the method using 100 μg of poly (Glu₄₅, Tyr₃₅) copolymer and 1 or 10 μM [γ-⁵²P]ATP in 60 μl of 20 mM HEPES buffer, pH 7.4, containing 0.2% Triton X-100, 15 mM MgCl₂, and 2 mM MnCl₂. The activity was expressed in terms of picomoles ³²P incorporated into the substrate (100 μg) per min per mg protein. The PTPase activity was determined by the method using 10 mM of O-phosphotyrosine in 0.1 ml of 0.1 mM sodium acetate, pH 6.0, containing 1 mM EDTA. Pi released was determined according to the method of Ames. The activity was expressed in terms of nanomoles Pi released per min per mg protein.

Results are expressed as the means ± S.E. of four observations. Similar results were obtained with two or three separate experiments.

**RESULTS**

**Effect of Vanadate on PDE, PTK, and PTPase Activities** When the particulate was incubated with vanadate (1 mM) over 60-min periods, a rapid increase in the PDE activity was observed (Fig. 1). The vanadate-stimulated increase reached a maximum with a 10-min incubation period, and was maintained at a steady-state level up to 30 min. The activities of both PDE and PTK in the particulate were increased with vanadate over a concentration range of 10⁻²—1 mM (Figs. 2, 3). Okadaic acid never increased the PDE activity up to 1 μM (Fig. 2). Conversely, the particulate PTPase activity was decreased with an increase in vanadate concentration and was negligible at 10⁻² mM or more (Fig. 4). Thus, the particulate contained PDE, PTK, and PTPase activities sensitive to vanadate.

**Effects of Amiloride, Divalent Ions, and N-Ethylmaleimide on PDE and PTK Activities** Figure 5 shows the

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**Fig. 1. Time Course of Stimulatory Effect of Vanadate on PDE Activity in Particulate**

The particulate (25 μg protein) was incubated with vanadate (1 mM, ⋄) or the buffer alone (○) at 30°C for the indicated periods.

**Fig. 2. Dose–Response Curves for Actions of Vanadate and Okadaic Acid on PDE Activity in Particulate**

Incubations of the particulate (25 μg protein) were carried out for 15 min at the indicated concentrations of vanadate (●) or okadaic acid (○).

**Fig. 3. Effect of Vanadate on PTK Activity in Particulate**

The particulate (10 μg protein) was incubated with vanadate at the indicated concentrations and 25°C for 5 min.

**Fig. 4. Effect of Vanadate on PTPase Activity in Particulate**

The particulate (10 μg protein) was incubated with vanadate at the indicated concentrations and 37°C for 15 min.
Fig. 5. Inhibitory Effect of Amiloride on Stimulation of PDE and PTK Activities by Vanadate

The particulate protein was pretreated with amiloride at the indicated concentrations and 30°C for 15 min in the assay medium for the PDE activity and further incubated with vanadate (1 mM) for 15 min. Results are shown as percentage of the control (C).

<table>
<thead>
<tr>
<th>Divalent ions</th>
<th>PTK activity (pmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−Vanadate</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺ (15 mM)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Mn²⁺ (2 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Co²⁺ (2 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺ (15 mM) + Mn²⁺ (2 mM)</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>Mg²⁺ (15 mM) + Co²⁺ (2 mM)</td>
<td>2.7 ± 0.3</td>
</tr>
</tbody>
</table>

The particulates (10 µg protein) were incubated in the media containing indicated divalent ions with or without vanadate (1 mM) at 25°C for 5 min.

The inhibitory effect of amiloride on the stimulation of PDE and PTK activities by 1 mM vanadate. The vanadate-stimulated PDE activity was still retained at 50% of the original activity level at a 2 mM concentration of amiloride, showing complete inhibition of the vanadate-stimulated PTK activity. Table I shows the results of the requirements of divalent ions for PTK activity in the assay medium in which the particulate was incubated with or without 1 mM vanadate. The PTK activity was markedly increased with 15 mM Mg²⁺ + 2 mM Mn²⁺ in either medium, with or without vanadate. N-Ethylmaleimide never showed any inhibition of the PTK activity, even at 1 mM concentration.

**Separation of PDE, PTPase, and PTK Activities on a DEAE-Sepacel Column**

We next attempted to separate PTPase, PTK, and PDE activities. The supernatant of solubilized particulate (12.9 mg protein) was applied to a DEAE-Sepacel column (Fig. 6). The PDE activity was eluted with a buffer containing 0.4 M NaCl as the last peak (No. 35) of four protein peaks (Fig. 6a). Of several peaks of PTK and PTPase active fractions, active peaks Nos. 24 and 27 were used as fractions containing PTK and PTPase activities, respectively, due to their relatively low con-
Effects of PTPase and PTK Active Fractions on PDE Activity. PDE activity was distinctly increased by the addition of the PTK active fraction (Fig. 7). A further increase in the PDE activity was found by pretreatment of the PTK active fraction with 1 mM vanadate. A marked decrease in the PDE activity, on the other hand, was observed by the addition of the PTPase active fraction to the PDE one. This decrease completely disappeared by pretreatment of the PTPase active fraction with 1 mM vanadate (Fig. 8). These results suggest that the particulate PDE activity is in part regulated by a process involving particulate PTPase and PTK activities sensitive to vanadate.

DISCUSSION

Stimulation of PDE activity in the particulate by vanadate was found to be due to an increase in the PTK activity and a decrease in the PTPase activity in the particulate. PTKs, which were relatively newly discovered in several normal tissues and cells, would be divided into two types: 1) The receptor-type includes the cytoplasmic domains of the receptors for insulin and various growth factors and is characterized by having an extracellular ligand binding ability and transmembrane domains. The non-receptor-type, on the other hand, is the oncogene product present in particulates as well as in cytosolic fractions, and lacks ligand binding or transmembrane domains. Recently, a novel cytosolic PTK, a non-receptor-type, was found in rat adipocytes and showed an optimal divalent ion requirement of $15 \text{ mM Mg}^{2+} + 2 \text{ mM Co}^{2+}$ in the assay medium. 2) In contrast, the optimal divalent ion requirement for the particulate PTK activity was $\text{Mn}^{2+}$ rather than $\text{Co}^{2+}$, in addition to $\text{Mg}^{2+}$. N-Ethylmaleimide (1 mM) inactivated the insulin receptor PTK activity but did not inactivate the particulate PTK activity. Thus, the particulate PTK appears to have some properties different from PTKs in the cytosol and insulin receptor, though the three PTK activities were stimulated by vanadate to various extents.

Protein tyrosine phosphatases can be divided into three subfamilies, such as one receptor-like and two non-receptor-like classes. CD45, which is composed of both domains of single transmembrane and cytosol, is a typical protein tyrosine phosphatase of the receptor-like class and is present on the lymphocyte cell surface. Protein tyrosine phosphatase 1B, purified from human placenta, on the other hand, is representative of an enzyme of the non-receptor-like class, having only a cytosolic domain without a transmembrane spanning region. It is unclear whether or not the particulate PTPase is an enzyme of the non-receptor-like class. The particulate showed dephosphorylating activity toward $^{32}$P-labelled poly (Glu$_4$, Tyr$_4$) copolymer, and the activity was decreased with vanadate over concentration range similar to that of PTPase activity (data not shown). The PTPase activity assayed seems to involve protein tyrosine phosphatase activity, as reported by Leis and Kaplan. Incubation of the particulate rat adipocytes with a mixture of vanadate and reduced glutathione stimulated the insulin-sensitive PDE activity, but vanadate or reduced glutathione alone, or vanadyl-
glutathione complexes did not.\textsuperscript{14} In contrast, our results were that vanadate alone stimulated PDE activity in the particulate of rat isolated fat pads. The particulate used here has been confirmed to contain cGMP-inhibited and insulin-sensitive PDE activity.\textsuperscript{41} This discrepancy may be due to the use of fat pads as material for preparation of the particulate. The PTK activity was stimulated with an increase in vanadate concentration, even under the condition of a complete inhibition of the PTPase activity, suggesting that vanadate shows not only inhibition of the PTPase activity but also stimulation of the PTK activity. Amiloride, a PTK inhibitor,\textsuperscript{15} inhibited the stimulatory effect of vanadate on PDE activity, suggesting that PTK(s) are involved in a part of the stimulating process of PDE activity by vanadate. In contrast to vanadate, okadaic acid, a potent inhibitor of serine/threonine phosphatases of type 1 and 2\textsuperscript{a},\textsuperscript{16} was entirely negative on an increase in the particulate PDE activity, in agreement with the report of Shibata \textit{et al.}\textsuperscript{17}

In adipocytes, stimulation of the PDE activity by catecholamines and insulin was associated with phosphorylation of the PDE molecule at possibly distinct serine sites by protein kinase A and unidentified serine kinase(s), respectively.\textsuperscript{13} Further, mitogen-activated protein (MAP) kinase, which was first described as a microtubule-associated protein kinase, was isolated from the cytosol of 3T3-L1 cells and characterized as a novel serine/threonine kinase sensitive to insulin.\textsuperscript{18} In an insulin-stimulated phosphorylation cascade, MAP kinase activity was stimulated by dual phosphorylation at tyrosyl and threonyl residues of the enzyme.\textsuperscript{19} It is unclear, at present stage, whether MAP kinases are involved in the regulation of particulate PDE activity. Our results were that partially purified PDE activity was increased by the addition of the PTK active fraction and decreased by the PTPase active one. This suggests that the PDE activity changes in part by the phosphorylation–dephosphorylation of tyrosyl residues in the PDE or unidentified protein kinases which may be included in separated fractions. Further studies are required to clarify whether tyrosyl residues of the PDE are directly phosphorylated by the PTK.

In conclusion, the PDE activity appears to be in part regulated through a process involving particulate PTK and PTPase activities sensitive to vanadate.

REFERENCES