Vanadium ions stimulate DNA synthesis in Swiss mouse 3T3 and 3T6 cells
(orthovanadate/ vanadyl sulfate/insulin/taxol/Na+/K+ ATPase)

JEFFREY BINGHAM SMITH
Department of Pharmacology, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294
Communicated by Leon A. Heppel, June 24, 1983

ABSTRACT  Vanadyl sulfate and sodium orthovanadate in the concentration range between 5 and 50 μM are shown to be mitogenic for quiescent cultures of Swiss mouse 3T3 and 3T6 cells. The compounds caused a striking shift in the dose–response for the effect of serum on [3H]thymidine incorporation and DNA synthesis. In the absence of serum the effect of vanadium was greatly potentiated by insulin. Vanadium ions produced no more than addtive increases in [3H]thymidine incorporation when combined with epidermal growth factor, cholera toxin, or phorbol 12-myristate 13-acetate. Both vanadium compounds stimulated ouabain-inhibitable 86Rb+ uptake, indicating that the vanadium ions increase, rather than inhibit, Na+/K+ pump activity in the intact cell. Neither vanadium compound had any effect on cellular cAMP under a variety of different conditions. The mitogenic effect of the vanadium compounds was similar to that of cholodcholine. Taxol, which stabilizes cytoplasmic microtubules, prevented the stimulation of DNA synthesis by vanadium.

Quiescent cultures of mammalian cells can be induced to synthesize DNA and proliferate by certain combinations of growth-stimulating compounds (1–3). Several animal cell lines have been continuously passaged for many generations in a serum-free medium supplemented with an optimal combination of growth-promoting hormones (2). In fibroblastic Swiss mouse 3T3 cells; a variety of different agents have been identified as potent comitogens: epidermal growth factor (EGF) (4); insulin-like growth factors (5); vaspressin (6); the tumor promoter phorbol 12-myristate 13-acetate (PMA) (7, 8); platelet-derived growth factor (PDGF) (9); choleasa toxin (10, 11); sarcoma-derived growth factor (12); and antitubulin agents (13–16).

Some of these agents alter distinctly different cellular processes, whereas others appear to have a common mechanism of action. For example, choleasa toxin increases CAM (11), and EGF and PDGF modulate photophosphorylase formation via a protein kinase (17, 18). Studies with cholodcholine and other antitubulin compounds suggest that the depolymerization of cytoplasmic microtubules enhances the mitogenic response to various growth factors (13–16). Vaspressin and PMA seem to share a common mechanism (19), perhaps involving an increase in the permeability of the cell to ions (20, 21). A greater permeability to Na ions appears to be at least partially responsible for the increase in Na+/K+ pump activity (22), which occurs shortly after mitogen addition in a variety of different cell types (23–28).

We are using vanadium compounds to help in the identification of specific biochemical events involved in the stimulation of DNA synthesis and proliferation in quiescent cells in vitro. Vanadium is an essential nutrient for higher animals (30), but no specific physiologic role of vanadium has been identified. In the rat, a dietary deficiency of vanadium produces a general retardation of growth, and only about 100 parts per billion (2 nM vanadium is needed for normal growth (30). McKeelth et al. (31) reported that about 5 nM vanadate has an optimal effect on the clonal growth of human diploid fibroblasts. Here we describe the effects of 5–50 μM vanadon on DNA synthesis and potassium transport in quiescent fibroblasts.

MATERIALS AND METHODS
Cell Culture. Two lines of fibroblastic Swiss mouse cells (32), 3T3 and 3T6, were grown in Dulbecco’s modified Eagle’s medium (DME medium) containing 10% (vol/vol) fetal bovine serum in a humidified atmosphere of 5% CO2/95% air as described (33). The 3T3 cells were obtained from Andrea M. Mastro (Department of Biochemistry and Biophysics, The Pennsylvania State University, University Park, PA). Nontransformed Swiss 3T3 cells (American Type Culture Collection) were plated at about 1 x 10^4 cells per 35-mm culture dish (Falcon). They grew until a confluent monolayer was formed at about 3 x 10^4 cells per cm^2. They became quiescent about 1 week after plating. Transformed Swiss 3T6 cells (American Type Culture Collection) were plated at about 1 x 10^5 cells per 35-mm culture dish in 0.5% fetal bovine serum. One week later they were washed twice with DME medium and left in serum-free medium for 24 hr before being used for thymidine incorporation.

Thymidine Incorporation. The incorporation of [3H]thymidine into thichloroacetic acid-insoluble material was carried out as previously described (19). The cultures were washed twice with DME medium and incubated at 37°C for 24 (3T6) or 40 (3T3) hr with growth factors and [3H]thymidine. Then the cultures were washed twice with saline at 4°C, twice with 5% trichloroacetic acid at 4°C, and twice with absolute ethanol. The acid-insoluble radioactive material was extracted from each culture for 30 min at 37°C with 1 ml of 0.1 M NaOH, and the radioactivity in 0.8 ml of the extract was measured in 10 ml of scintillation fluid containing 33% Triton X-100 and 0.10 ml of 1 M HCl.

 Autoradiography. Labeling indices were measured after a 40-hr incubation at 37°C with [3H]thymidine (5 μCi/ml, 0.2 μM, 1 Ci = 3.7 x 10^10 Bq) as described (33). The cultures were fixed with formaldehyde/saline for 20 min at room temperature before washing as described above. Stripping film (Kodak AR.10) or photographic emulsion (Kodak NBT2 nuclear track emulsion) was applied to the cultures after coating them with chrome alum. They were incubated in the dark at 4°C for 1 week. After development with D-19 (Kodak), the cultures were stained with

 The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Vanadyl sulfate enhances the stimulation of DNA synthesis by fetal bovine serum in Swiss 3T3 cells. Quiescent cultures were washed with DME before addition of 2 ml of DME medium containing the indicated concentration (vol/vol) of fetal bovine serum (A). Vanadyl sulfate was added at 20 μM (A). A continuous labeling with [3H]thymidine (0.8 μM) at 0.14 μCi/ml was carried out for 41 hr. Giemsa stain. At least eight separate fields containing about 300 cells each were counted on each culture dish. All [3H]thymidine incorporation and nuclear labeling experiments were done on duplicate cultures in 35-mm dishes.

Materials. Sodium orthovanadate (Fisher) and vanadyl sulfate were diluted in water from 1 M solutions, which were prepared fresh daily. Vanadyl sulfate trihydrate (99.999%) was obtained from Aldrich. Cholera toxin, colchicine, and bovine pancreatic insulin (25 units/mg) were from Sigma. PMA was from Consolidated Midland (Brewster, NY). EGF was from Collaborative Research (Waltham, MA). [6-3H]Thymidine (24 Ci/mmol) was from Amersham. Taxol was obtained from John Douros (National Cancer Institute, Bethesda, MD).

RESULTS

Effect of Vanadyl Sulfate on Serum-Stimulated [3H]Thymidine Incorporation. Vanadyl sulfate produced a striking shift in the dose–response for the effect of serum on [3H]thymidine incorporation by cultures of 3T3 cells arrested in the G1/G0 phase of the cell cycle (Fig. 1). VOSO₄ caused a synergistic stimulation of [3H]thymidine incorporation in the presence of 1–4% fetal bovine serum. Because neither bovine serum albumin nor conalbumin had any significant effect on the stimulation of [3H]thymidine incorporation by vanadyl sulfate, it appeared that a growth factor in fetal bovine serum was responsible for the synergism between VOSO₄ and fetal bovine serum.

Synergism Between Vanadium Compounds and Insulin. The addition of either sodium orthovanadate or vanadyl sulfate by itself to quiescent cultures of Swiss 3T3 or 3T6 cells slightly increased [3H]thymidine incorporation (Fig. 2). The maximal stimulation of thymidine incorporation occurred between 25 and 50 μM vanadium (Fig. 2). Each vanadium compound by itself maximally increased [3H]thymidine incorporation by only about 5% as much as fetal bovine serum (Fig. 2). Higher concentrations of vanadium (above 50 μM) caused cell detachment (data not shown). Swiss 3T6 cells have been passaged several times in DME medium containing 53 μM Na₃VO₄ and 10% fetal bovine serum without any toxic effect being observed. Neither vanadium compound produced any detectable precipitate in the culture medium, nor was the mitogenic effect of VOSO₄ diminished by filtration.

Both vanadium compounds interacted synergistically with insulin in serum-free DME medium to induce [3H]thymidine incorporation (Figs. 2 and 3). Fig. 3 shows that vanadyl sulfate and insulin synergistically activated DNA synthesis in 3T6 cells as measured by the autoradiographic labeling of nuclei. In Swiss 3T3 cells the combination of VOSO₄ and insulin or low amounts of serum synergistically increased DNA synthesis (Table 1). In all cases the bulk amounts of [3H]thymidine incorporated into trichloroacetic acid-precipitable material correlated well with the number of stimulated cells observed by autoradiography. Therefore it is concluded that the enhancement of [3H]thymidine incorporation by the vanadium ions reflects a change in the number of cells entering the S phase of the cycle rather than a change in the amount of thymidine transported or incorporated per stimulated cell.

The duration of the exposure to VOSO₄ influenced the response to quiescent 3T3 cells to low serum and insulin. Delaying the time of addition of vanadyl sulfate by 3 hr from the time of insulin or serum addition significantly reduced the stimulatory effect of VOSO₄ on thymidine incorporation. When vanadyl sulfate was added 17 hr after fetal bovine serum or insulin, the amount of [3H]thymidine incorporated in a 42-hr
continuous labeling was only slightly increased. The optimal stimulation was produced when vanadyl sulfate and insulin were added together at the start of the experiment. No stimulation of \(^{3}H\)thymidine incorporation was observed when VO\(_{4}\) was removed after 2 hr. Incubating insulin with either of the vanadium compounds had no effect on the capacity of the insulin to stimulate thymidine incorporation. These results indicate that the vanadium compounds help to activate the quiescent cell directly rather than by modifying insulin chemically.

**Effect of Vanadium Ions on the Stimulation of \(^{3}H\)Thymidine Incorporation by PMA, Cholera Toxin, and EGF.** In contrast to the synergism between insulin and both vanadium compounds, other growth factors did not potentiate the action of vanadium ions on the induction of DNA synthesis in quiescent 3T3 or 3T6 cells. Only additive increases in DNA synthesis were observed when vanadyl ions were combined with EGF, cholera toxin, or PMA, a phorbol ester tumor promoter (data not shown). A synergistic increase in DNA synthesis was observed with insulin and EGF, insulin and cholera toxin, and insulin and PMA, as reported by others (7, 8, 11, 19).

**Vanadium Ions and Colchicine Have Similar Effects on \(^{3}H\)Thymidine Incorporation.** Colchicine and other antitubulin agents markedly enhance the stimulation of DNA synthesis by polypeptide growth factors in quiescent 3T3 cells (14). Vanadate or colchicine by itself slightly increased \(^{3}H\)thymidine incorporation (Table 2). Both compounds had a strongly synergistic interaction with insulin but not EGF (Table 2). The combination of vanadate plus colchicine only slightly increased thymidine incorporation in the presence of insulin (Table 2). Taxol, which stabilizes microtubules (15), almost completely prevented the stimulation of DNA synthesis by vanadyl sulfate (Fig. 4) and vanadate (data not shown). Taxol partially inhibited \(^{3}H\)thymidine incorporation in the presence of insulin and colchicine or insulin and vanadyl sulfate.

**Vanadium Ions Increase 86Rb\(^{+}\) Uptake.** Orthovanadate inhibits the ATPase activity of the Na\(^{+}\)/K\(^{+}\) pump and other transport ATPases in vitro (34, 35). Surprisingly, neither sodium orthovanadate nor vanadyl sulfate had any inhibitory effect on 86Rb\(^{+}\) uptake in quiescent 3T3 cells (Table 3). In fact both vanadium compounds, either alone or in combination with insulin, increased the rate of ouabain-sensitive 86Rb\(^{+}\) uptake (Table 3). The fact that the vanadium compounds either had no effect on or slightly increased total cell K\(^{+}\) provides additional evidence that the Na\(^{+}\)/K\(^{+}\) pump in the intact fibroblast was not inhibited by vanadium (Table 3).

**DISCUSSION**

The stimulation of DNA synthesis by vanadyl ions appears to be distinct from the nonspecific and transient effects that certain other metal ions have on DNA synthesis. Rubin (37, 38) showed that Hg\(^{2+}\), Cd\(^{2+}\), and Zn\(^{2+}\) stimulate \(^{3}H\)thymidine incorporation by chicken embryo fibroblasts. However, these metal ions had a very narrow dose–response optimum, such that the concentration of the metal that increased DNA synthesis was just below the level that grossly altered cell mor-

### Table 1. Effect of vanadyl sulfate on the autoradiographic labeling of nuclei in quiescent 3T3 cells

<table>
<thead>
<tr>
<th>Labeled nuclei, %</th>
<th>No</th>
<th>40 (\mu)M</th>
<th>40 (\mu)M VO(_{4})</th>
<th>40 (\mu)M VO(_{4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DME medium only</td>
<td>1</td>
<td>9</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>Insulin</td>
<td>25</td>
<td>50</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>EGF</td>
<td>9</td>
<td>23</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>Insulin + EGF</td>
<td>42</td>
<td>63</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>2% fetal bovine</td>
<td>20</td>
<td>65</td>
<td>65</td>
<td>65</td>
</tr>
<tr>
<td>10% fetal bovine</td>
<td>76</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
</tbody>
</table>

Quiescent cultures of Swiss 3T3 cells were washed twice with DME medium before the indicated additions were made. Labeling with \(^{3}H\)thymidine (5.0 \(\mu\)Ci/ml, 0.2 \(\mu\)M) was carried out for 40 hr. Insulin and EGF were present as indicated at 1 \(\mu\)g/ml and 1.2 ng/ml, respectively.

### Table 2. Effects of vanadate and colchicine on the stimulation of DNA synthesis by EGF and insulin in Swiss 3T3 cells

<table>
<thead>
<tr>
<th>[^{3}H]Thymidine incorporation, (\text{cpm} \times 10^{-5}) per culture</th>
<th>No addition</th>
<th>Na(<em>{2})VO(</em>{4})</th>
<th>Colchicine</th>
<th>Na(<em>{2})VO(</em>{4}) + colchicine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Additions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DME medium only</td>
<td>5.8</td>
<td>36.3</td>
<td>24.4</td>
<td>24.9</td>
</tr>
<tr>
<td>10% fetal bovine serum</td>
<td>733.4</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Insulin</td>
<td>58.6</td>
<td>260.1</td>
<td>390.8</td>
<td>116.3</td>
</tr>
<tr>
<td>EGF</td>
<td>6.8</td>
<td>27.1</td>
<td>14.7</td>
<td>37.6</td>
</tr>
<tr>
<td>EGF + insulin</td>
<td>158.0</td>
<td>295.3</td>
<td>353.4</td>
<td>173.4</td>
</tr>
</tbody>
</table>

Quiescent 3T3 cells were labeled continuously for 40 hr with \(^{3}H\)thymidine (1.0 \(\mu\)M) at 1 \(\mu\)Ci/ml. Insulin, sodium orthovanadate, and colchicine were present as indicated at 1 \(\mu\)g/ml, 50 \(\mu\)M, and 4 \(\mu\)M, respectively. ND, not done.
phology (38). The morphology of the 3T6 cells, as observed by phase-contrast microscopy, was not significantly affected by a 24-hr incubation with mitogenic levels of vanadium or repeated passaging in DME medium containing 33 μM Na3VO4 and 10% fetal bovine serum. If vanadium were stimulating DNA synthesis by some nonspecific mechanism, then it seems unlikely that there would be a specific synergistic interaction between vanadium and a single growth-stimulating factor, namely insulin, as reported here.

The most thoroughly studied effect of vanadate is its potent inhibitory action on the purified Na+/K+ ATPase (34). Vanadate ions inhibit other ATPases (reviewed in ref. 35). ribonuclease (39), and other enzymes involved in phosphate transfer such as alkaline phosphatase (40) and phosphofructokinase (41). In this way the vanadium compounds may increase cell Na+ by inhibiting the Na+/K+ pump and increase cell Ca2+ by inhibiting the Ca2+ pump. Vanadate enters erythrocytes (42), adipocytes (43), and cultured heart cells (44). However, vanadate is rapidly reduced to the vanadyl ion intracellularly (45–48), and the vanadyl ion is a poor inhibitor of the Na+/K+ ATPase (45–48). Moreover, after the treatment of 3T3 cells with vanadate or vanadyl ions, ouabain-sensitive 86Rb+ uptake was found to be increased (Table 3). Vanadate also failed to inhibit 86Rb+ uptake by adipocytes (43) and cultured heart cells (44). Vanadium ions were recently shown to hyperpolarize neuroblastoma–glioma hybrid cells by 20–30 mV (49). Although vanadium ions influence some transmembrane ion movements in intact mammalian cells, vanadate apparently does not decrease Na+/K+ pump activity in intact mammalian cells other than erythrocytes (42).

Although added VO3− is just as effective as added Na3VO4 in stimulating DNA synthesis (Fig. 2), it is difficult to prevent the vanadyl ion from being oxidized to vanadate extracellularly. The addition of 0.5 mM glutathione to the culture medium did not diminish the stimulation of [3H]thymidine incorporation by VO3− and insulin (unpublished data). It is unclear whether vanadium acts intracellularly or extracellularly or if the vanadyl or vanadate ion is the species responsible for the stimulation of DNA synthesis.

Increasing cellular cAMP helps to induce a mitogenic response in a variety of different cultured animal cells (10, 11, 50, 51). Moreover, vanadate has been reported to activate adenylate cyclase (52–54) and increase cAMP levels in isolated tissues (55, 56). We measured cellular cAMP at various times after the addition of vanadium ions to the quiescent fibroblasts. Vanadium ions had no significant effect on cAMP levels in quiescent 3T3 and 3T6 cells either in the presence or in the absence of insulin or an inhibitor of cAMP phosphodiesterase (unpublished data). Therefore, the activation of adenylate cyclase is probably not responsible for the mitogenic effect of the vanadium ions. Nor does it appear likely that vanadium ions alter the binding of insulin to receptors on the 3T3 and 3T6 cells, because insulin binding to adipocytes was not affected by vanadate (57).

In rat adipocytes vanadate and vanadyl ions increase glucose oxidation in an insulin-mimetic fashion (43, 47, 57). Recently Hori and Oka (58) reported that vanadate enhances the stimulatory action of insulin on DNA synthesis by mouse mammary gland explants, and we have observed that vanadate and insulin synergistically stimulate [3H]thymidine incorporation by the WI-38 line of human lung fibroblasts (unpublished data) as well as DNA synthesis by quiescent 3T3 and 3T6 cells. Carpenter (59) recently reported that vanadate and EGF synergistically increased [3H]thymidine incorporation by transformed A431 cells. Because the vanadium compounds failed to interact with EGF in Swiss 3T3 or 3T6 cells, the interaction between vanadium and insulin may be a more general phenomenon (29, 43, 57, 58). In adipocytes vanadate and insulin have equivalent rather

### Table 3. Effect of vanadium ions, insulin, and serum on the rate of 86Rb+ uptake and cell K+.

<table>
<thead>
<tr>
<th>Additions</th>
<th>86Rb+ uptake, nmol/min per culture</th>
<th>Cell K+ μmol per culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Plus ouabain</td>
</tr>
<tr>
<td>DME medium only</td>
<td>3.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>VO3−</td>
<td>4.4 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Na3VO4</td>
<td>4.5 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Insulin</td>
<td>5.8 ± 0.4</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Insulin + VO3−</td>
<td>6.4 ± 0.6</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>Insulin + Na3VO4</td>
<td>6.5 ± 0.6</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>10% fetal bovine serum</td>
<td>7.9 ± 0.5</td>
<td>2.7 ± 0.2</td>
</tr>
</tbody>
</table>

Quiescent cultures (60 mm) of 3T3 cells were washed with DME medium and incubated for 6 hr at 37°C with 2 ml of DME medium and the additions indicated. The final concentrations of vanadium and insulin were 50 μM and 1 μg/ml, respectively. Then 0.1 ml of water ("Total" column) or 40 mM ouabain ("Plus ouabain" column) was added. Ten minutes later 20 μl of 86Rb+ (63,000 cpm) was added and the incubation was continued for 10 min before washing with ice-cold MgCl2 to stop the uptake and remove extracellular isotope and K+ as previously described (21, 36). Values are means ± SEM for 4–7 identically treated cultures. The addition of ouabain reduced cellular K+ to 0.12 ± 0.03 and 0.17 ± 0.03 μmol per dish in the absence and presence of fetal bovine serum, respectively. Each culture contained about 1 × 106 cells.
than synergistic effects on glucose transport and oxidation (43). The marked synergism between insulin and vanadium that we observed for the stimulation of DNA synthesis suggests that vanadium is not acting solely in an insulin-like fashion in the 3T3 and 3T6 cells.

It is possible that vanadium shares a mechanism of action with the antithybin agents. Vanadate and colchicine had similar effects on the membrane phosphotyrosyl-protein phosphatase activity isolated from fibroblasts (61). The activation of a phosphotyrosine-specific protein kinase appears to be the initial biochemical event after the binding of EGF to its receptor on the surface of A431 cells (62). Insulin also activates a tyrosine-specific cAMP-independent protein kinase in 3T3-L1 adipocytes (63). In quiescent mouse fibroblasts we observed a marked synergism between vanadium ions and insulin, but not vanadium ions and EGF, in stimulating DNA synthesis (Figs. 2 and 3, Table 2). It seems likely that the phosphorylation of different membrane proteins is induced by EGF and insulin, because the combination of EGF and insulin synergistically stimulates DNA synthesis in quiescent 3T3 and 3T6 cells (3). The present results suggest that vanadium ions influence key events in quiescent mammalian cells that lead to the initiation of DNA synthesis.

The author thanks Paula Clawson, Sue Hughes, and Dorothy McCauley for typing the manuscript and Cindy Smith for technical assistance. This investigation was supported by U.S. Public Health Service Grant R23 CA30030.