Vanadium Activates or Inhibits Receptor and Non-receptor Protein Tyrosine Kinases in Cell-free Experiments, Depending on Its Oxidation State

POSSIBLE ROLE OF ENDogenous VANADIUM IN CONTROLLING CELLULAR PROTEIN TYROSINE KINASE ACTIVITY*

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Gerard Elberg, Jinping Li, and Yoram Shechter
From the Department of Hormone Research, Weizmann Institute of Science, Rehovot 76100, Israel

We have shown that vanadium mimics several insulin effects in rat adipocytes, via a staurosporine sensitive cytosolic protein tyrosine kinase (CytPTK; Shisheva, A., and Shechter, Y. (1993) J. Biol. Chem. 268, 6463). Here we demonstrate that vanadium effects on protein tyrosine kinases are preserved after cell disintegration. Vanadium inhibits or activates protein tyrosine kinases depending on its oxidation state and the tyrosine kinase studied. Vanadyl (4+) but not vanadate (5+) inhibits receptor tyrosine kinases such as the insulin receptor (IC50 value = 23 ± 4 μM) and the insulin-like growth factor-I receptor (IC50 = 19 ± 3 μM). Inhibition is non-competitive with respect to ATP, Mn2+, or substrate concentrations. Preincubation of adipocytes with vanadyl (0.4 mM), and staurosporine (which arrests the cytosolic enzyme) substantially inhibited insulin-stimulated lipogenesis. Vanadyl is readily oxidized to vanadate by hydrogen peroxide.

In contrast, CytPTKs were poorly inhibited by vanadyl and vanadate stimulated several CytPTKs 2-6-fold. CytPTK derived from rat adipocytes, liver, and brain were activated, and CytPTK from Nb lymphoma cells was not affected. CytPTK extracted from insulin-responsive tissues are more sensitive to vanadate activation (ED50 = 3 ± 0.7 μM), whereas the brain enzyme is less sensitive (ED50 = 27 ± 3 μM). Tungstate, molybdate, and phenylarsine oxide also stimulate CytPTK, suggesting that the vanadate effect is secondary to inhibiting protein phosphotyrosine phosphatases.

This study supports a working hypothesis implicating the intracellular vanadyl pool in modulating CytPTK activity. Any physiological conditions converting vanadyl to vanadate (i.e. H2O2 production) will activate CytPTK and consequently CytPTK-dependent bioeffects.

There is a growing interest in vanadium in the field of insulin action and diabetes, since vanadium mimics most or all of the actions of insulin in various insulin-responsive cells and tissues (reviewed in Ref. 2). Vanadium therapy normalizes blood glucose levels in streptozotocin-treated diabetic rats and alleviates many of the aberrations associated with hyperglycemia (3-5). More importantly, oral administration of vanadium lowers blood glucose levels in the experimental animals representing Type II diabetes and severe insulin resistance (6-9). In several instances, this treatment dramatically increases tissue responsiveness to insulin in insulin-resistant rodents (i.e. Ref. 9). We have recently found that, in addition to the insulin receptor tyrosine kinase, rat adipocytes contain another protein tyrosine kinase (10) which is a water-soluble cytosolic protein (CytPTK) of an estimated mass of 53 kDa. The activity of CytPTK is supported by Co2+ rather than by Mn2+. Further studies revealed a 3-5-fold increase in the specific activity of CytPTK in intact adipocytes preincubated with vanadate. Of the several tyrosine kinase blockers tested, staurosporine was the most potent inhibitor of the soluble kinase (IC50 = 3 ± 0.2 μM), but a poor inhibitor of the InsRTK (IC50 = 8 μM). In intact cells, staurosporine inhibited vanadate-induced stimulation of lipogenesis and glucose oxidation, but only had a marginal effect on the insulin-stimulated bioeffects. Overall, it was concluded that vanadate mediates its effects on glucose metabolism via this cytosolic tyrosine kinase by an alternative non-insulin-dependent pathway (1, 10). This conclusion was in agreement with several studies indicating that vanadate per se (as opposed to pervanadate) does not activate the insulin receptor in intact cellular systems (9, 11-13).

There are many unanswered questions with regard to how vanadate mimics the actions of insulin. It is not clear whether vanadate (5+ oxidation state) or vanadyl (4+ oxidation state), is the active species that mimics the hormone. Another issue is whether or not the insulin-like effects of vanadium are secondary to the effects of vanadate in inhibiting cellular protein phosphotyrosine phosphatases (PTPases). At least vanadate (5+ oxidation state) has been shown in several studies, to exert inhibitory actions on some members of this family of enzymes (14-16). However, it is now clear that vanadate will only slightly inhibit or have no effect on many of the mammalian PTPases tested (16, 17). Other vanadate-sensitive phosphoryl-olizes are alkaline phosphatase, Na+,K+ ATPase, Ca++/Mg++-ATPase, and glyceraldehyde-3-phosphate dehydrogenase (reviewed in Refs. 18 and 19). These effects, however, may not occur at all in intact cellular systems, since exogenously added vanadate, after permeating into the cell interior, is efficiently

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† Incumbent of the C. H. Hollenberg Chair in Metabolic and Diabetes Research, established by the Friends and Associates of Dr. C. H. Hollenberg of Toronto, Canada. To whom correspondence should be addressed.
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§ This abbreviations used are: CytPTK, cytosolic protein tyrosine kinase; InsRTK, insulin receptor tyrosine kinase; poly(L-glutamic acid) or poly(Glu,Tyr), random copolymer containing l-glutamic acid and l-tyrosine at 4:1 molar ratio; IGF-I, insulin-like growth factor I; WGA, wheat germ agglutinin; PTPase, protein phosphotyrosine phosphatase.

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reduced to vanadyl ions (20). Effects of vanadyl in attenuating enzymatic activities have not been extensively documented. The exceptions are ribonuclease and alkaline phosphatase, both of which were shown to be inhibited by vanadyl even more effectively as compared to vanadate (21, 22).

In this study, we demonstrate for the first time the inhibitory or stimulatory effects of vanadyl and vanadate on receptor and non-receptor protein tyrosine kinases in cell-free experiments. The implications of this study to the insulin-like effects of vanadyl are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**

Poly(Glu,Tyr) was purchased from Sigma. Monoclonal antibodies to phosphotyrosine were obtained from Monoclonal (Rehovot, Israel), and rabbit polyclonal antibodies to phosphotyrosine were purchased from Zymed. Protein A conjugated with horseradish peroxidase and enhanced chemiluminescence Western blot detection system were purchased from Amersham Corp. β-<sup>3</sup>H<sub>T</sub>C<sub>14</sub>glucose was purchased from Du Pont. NEN, γ<sup>-</sup>P<sub>32</sub>ATP was purchased from Amersham (Buckinghamshire, United Kingdom), and collagenase type I (134 units/mg) was obtained from Worthington Biochemicals. Porcine insulin was purchased from Eli Lilly Co. Krebs-Ringer-bicarbonate (KRB) buffer, pH 7.4, contained 110 mM NaCl, 25 mM NaHCO<sub>3</sub>, 5 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 1.3 mM MgSO<sub>4</sub>.

**Procedures**

Rat adipocytes were prepared from fat pads of male Wistar rats (100–200 g) by collagenase digestion, according to the method of Rodbell (23).

**Assay of Lipogenesis**—Assay of lipogenesis was measured by the incorporation of β-<sup>3</sup>H<sub>T</sub>C<sub>14</sub>glucose into lipids as described in detail in Ref. 1.

**Partially Purified Insulin and IGF-I Receptors**—Partially purified insulin and IGF-I receptors were prepared from rat liver or rat ovary membranes following homogenization, solubilization with Triton X-100, and affinity purification on a WGA-agarose column (24). The ovaries for the IGFI receptor preparations were excised from 25-day-old female rats, 48 h after receiving 10 units of pregnant mare’s serum gonadotropin.

**Preparation of High Speed Supernatant Fractions from Various Tissues**—Rat adipocytes, rat liver, rat brain or rat cultured fibroblastoma cells were homogenized (in the absence of detergents) with a Polytron homogenizer in 25 mM HEPES, pH 7.4, containing 0.25 mM sucrose. The cell homogenates were then centrifuged at 12,000 × g for 20 min, and the supernatant was further centrifuged at 40,000 × g for 60 min. The supernatant fractions were stored at −137 °C until used (1).

**Tyrosine Kinase Activity Measurements**—Tyrosine kinase activity measurements for the insulin and IGF-I receptors and for the cytosolic tyrosine kinases, were performed essentially as described in detail in Ref. 1. The final concentrations of Co<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, ATP, and poly(Glu,Tyr) were specified in the text for each protein tyrosine kinase examined. Phosphotyrosine content in poly(Glu,Tyr) was quantitated by radioimmunoassay according to Ref. 25.

**Auto phosphorylation of InsRTK or IGF-I Receptor Tyrosine Kinase in Vitro**—Wheat germ agglutinin purified InsRTK or IGF-I receptor tyrosine kinase (5 μg of protein) were incubated for 20 min at 22 °C with the indicated concentrations of vanadate or vanadyl in a final volume of 60 μl containing 25 mM HEPES, pH 7.4, 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub> and 100 μM ATP with or without 0.2 μM insulin or IGF-I. Samples were then subjected to 7.5% SDS-polyacrylamide gel electrophoresis (25).

**Western Blot Analyses**—Western blot analyses were carried out by transferring proteins to nitrocellulose membranes (29). These were sequentially treated with polyclonal antibodies to phosphotyrosine and with protein A-horseradish peroxidase conjugate, and finally subjected to enhanced chemiluminescence Western blot detection system as described in detail in the manufacturers’ instructions.

**Protein Concentration**—Protein concentration was determined by the method of Bradford (29). All the assays were performed either in duplicate or triplicate. Each figure or table is the result of a representative experiment performed three to five times.

**RESULTS**

**Inhibition of InsRTK-dependent Poly(Glu,Tyr) Phosphorylation by Vanadyl**—Addition of increasing concentrations of vanadyl to WGA-purified insulin inhibited InsRTK-catalyzed phosphorylation of poly(Glu,Tyr) in a concentration-dependent manner (Fig. 1). Half-maximal inhibition was obtained at 23 ± 4 μM, and full inhibition was evident at 90 ± 7 μM. Vanadate (5+ oxidation state) did not inhibit InsRTK-catalyzed poly(Glu,Tyr) phosphorylation at any concentration examined (Fig. 1). Thus, this inhibitory effect seems to be specific to vanadyl (4+ oxidation state) and does not result from vanadyl oxidation to vanadate that may occur, to a small extent, at neutral pH values (19). Similarly, pervanadate (peroxides of vanadate), an agent demonstrated to inhibit PTPases, therefore activating protein tyrosine kinases in intact cells (11), had no effect on the activity of InsRTK in cell-free experiments (Fig. 1).

Fig. 2 summarizes the experiments performed in order to...
WGA-purified InsRTK (5 ng of protein) was incubated with the indicated concentrations of vanadyl at 22 °C for 15 min in a mixture containing 25 mM Hepes (pH 7.4), 0.1% Triton X-100, 5 mM MnCl₂ and 5 mM MgCl₂, either in the absence or presence of insulin. Autophosphorylation was carried out for 20 min by adding ATP (100 µM) and terminated by sample buffer. The proteins were then resolved in 7.5% SDS-polyacrylamide gel electrophoresis.

Stimulated autophosphorylation was observed that vanadyl is almost equipotent in inhibiting InsRTK by vanadyl. However, we have further observed that vanadyl is almost equipotent in inhibiting poly(Glu,Tyr) phosphorylation, when added subsequent to receptor autophosphorylation and its activation (Table I). This fact again emphasizes the uniqueness of the vanadyl-dependent inhibition. It is also noteworthy that VO²⁺ inhibits autophosphorylation more effectively as it inhibits substrate phosphorylation. This is not expected kinetically if the inhibitor is competitive with the substrate, the metal ion, or ATP.

Inhibition of InsRTK Autophosphorylation by Vanadyl—Fig. 3 demonstrates that vanadyl inhibits InsRTK autophosphorylation in a concentration-dependent manner. Fifty percent inhibition was obtained at 60–80 µM and maximal inhibition at 100 µM, namely at somewhat higher concentrations in which vanadyl inhibited receptor-catalyzed poly(Glu,Tyr) phosphorylation (Fig. 1). Inhibition of autophosphorylation per se is sufficient to explain the inhibitory action of VO²⁺ on poly(Glu,Tyr) phosphorylation as autophosphorylation precedes and activates the kinase (30, 31). However, we have further observed that vanadyl is almost equipotent in inhibiting poly(Glu,Tyr) phosphorylation, when added subsequent to receptor autophosphorylation and its activation (Table I). This fact again emphasizes the uniqueness of the vanadyl-dependent inhibition. It is also noteworthy that VO²⁺ inhibits autophosphorylation more effectively as it inhibits substrate phosphorylation. This is not expected kinetically if the inhibitor is competitive with the substrate, again stressing the non-competitive nature of vanadyl in inhibiting InsRTK (see above).

Inhibition of IGF-I Receptor Tyrosine Kinase by Vanadyl—
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Fig. 6. Inhibition of insulin-stimulated lipogenesis in rat adipocytes by vanadyl, in the presence of staurosporine. Rat adipocytes (3 x 10^6 cells/vial) were preincubated with or without staurosporine (final concentration 0.3 μM, 20 min, 37 °C) followed by a 20-min incubation in the presence or the absence of 0.4 mM vanadyl as specified in each column in the figure. The cells were then supplemented with [U-^14C]glucose (final concentration 0.16 mM) and insulin (17 μM). Lipogenesis was carried out for 60 min at 37 °C.

Vanadyl blocked IGF-I receptor catalyzed poly(Glu,Tyr) phosphorylation (Fig. 4), as well as the ligand-dependent receptor autophosphorylation (Fig. 5). The concentration dependent inhibitions in both cases were strikingly similar to that observed with the InsRTK. IC₅₀ values amounted to 19 ± 3 μM for the substrate-catalyzed and 60–80 μM for the autophosphorylating event. Thus, the sites of vanadyl-binding to both enzymes seem to have a considerable degree of homology within the three-dimensional structure of those related receptors (32).

Inhibition of Insulin-stimulated Lipogenesis in Rat Adipocytes by Vanadyl—We wished to determine whether the inhibitory effect of vanadyl upon InsRTK activity can also be produced in the intact cell system. Vanadyl, however, stimulates lipogenesis in an insulin-independent manner, via the staurosporine-sensitive CytPTK (1). To avoid this, the effect of vanadyl was carried out in the presence of staurosporine. Fig. 6 demonstrates that preincubating adipocytes with staurosporine (0.3 μM) and vanadyl (0.4 mM) nearly fully inhibited insulin's potency to stimulate lipogenesis. Thus, the inhibitory effect of vanadyl can also be produced in the intact cell, in the presence of staurosporine.

Hydrogen Peroxide Reverses the Inhibitory Effect of Vanadyl on InsRTK-catalyzed Poly(Glu,Tyr) Phosphorylation—The inclusion of stoichiometric amount of H₂O₂ to vanadyl in the InsRTK-catalyzed poly(Glu,Tyr) phosphorylation assay reversed the inhibitory effect of vanadyl on the InsRTK. H₂O₂ reverses the inhibitory effect of vanadyl in inhibiting InsRTK-catalyzed poly(Glu,Tyr) phosphorylation. Assay was performed under the conditions specified in Fig. 1, in the presence of the indicated concentrations of H₂O₂, vanadyl, or both at a molar ratio of 1:1 or 2:1, as specified in the figure.

Fig. 7. Hydrogen peroxide reverses the effect of vanadyl in inhibiting InsRTK-catalyzed poly(Glu,Tyr) phosphorylation. The concentration dependent inhibitions in both cases were strikingly similar to that observed with the InsRTK. IC₅₀ values amounted to 19 ± 3 μM for the substrate-catalyzed and 60–80 μM for the autophosphorylating event. Thus, the sites of vanadyl-binding to both enzymes seem to have a considerable degree of homology within the three-dimensional structure of those related receptors (32).

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alone had no effect on InsRTK activity (Fig. 7). This indicates that vanadyl is readily oxidized by H$_2$O$_2$ to vanadate at neutral pH values, in a stoichiometric fashion and at low concentrations of the reactants. In experiments not shown here, vanadyl oxidation to vanadate were also confirmed by H$_2$O$_2$ consumption following its mixing with vanadyl and by the disappearance of the typical vanadyl's electron spin resonance (ESR) spectrum upon adding H$_2$O$_2$. Competition experiments (not shown here) seem to indicate that vanadyl is several times more sensitive to H$_2$O$_2$ oxidation, as compared to several endogenous reducing agents found in mammalian cytosols, including reduced glutathione.²

Effects of Vanadyl and Vanadate on Non-receptor Cytosolic Tyrosine Kinases—In contrast to the receptor protein tyrosine kinases, vanadyl inhibited CytPTK activity at high concentrations only (50-fold higher concentration, Fig. 8). Moreover, at least one, namely the brain cytosolic tyrosine kinase, was significantly stimulated by vanadyl (Fig. 8). Vanadate (which had no effect at all on the receptor protein tyrosine kinases; Figs. 1 and 4) stimulated three of the four CytPTKs studied, namely liver, fat, and brain CytPTKs. Liver and fat CytPTK were stimulated about 2-4-fold, whereas brain CytPTK was stimulated 4-7-fold in the various experiments. ED$_{50}$ values amounted to $3 \pm 0.7$ for the adipocytic and liver CytPTK and $27 \pm 3 \mu M$ for the brain CytPTK, respectively. CytPTK derived from Nb$_2$-lymphoma cells was not activated by vanadate (Fig. 8). It is of interest to note that CytPTKs derived from fat and liver, classical insulin-responsive tissues, were activated by lower concentrations of vanadate (i.e. ED$_{50}$ values of $3 \pm 0.4 \mu M$). Brain CytPTK was activated by significantly higher concentrations of vanadate (ED$_{50}$ = $27 \pm 3 \mu M$), whereas lymphoma CytPTK was not activated by vanadate. The possibility that fat, liver, and muscle typically possess a vanadate-sensitive system is currently being studied.

It was our further intention to determine whether vanadate activated the CytPTK due to direct VO$_2^-$ binding to the enzymes. The alternative was tyrosyl phosphorylation and activation of the kinases resulting from inhibition of PTPases. To approach this issue, the activating effects of fluorde, tungstate molybdate, and phenylarsine oxide were analyzed. Several types of PTPases were reported to be inhibited by those agents (17, 34, 35). The results have been summarized in Fig. 9. Fluoride did not activate brain- and adipocyte-derived CytPTK; however, molybdate, tungstate, and phenylarsine oxide stimulated the enzyme to the same extent as did vanadate, at concentrations of 10–100 µM (Fig. 9). Thus, vanadate activation of CytPTKs seems to be secondary, resulting from inhibition of PTPases, whereas vanadyl inhibition of receptor tyrosine kinases is likely to occur by direct vanadyl effect on the enzymes.

In control experiments, vanadate, up to a concentration of 10 mM did not inhibit at all dephosphorylation of phosphorylated poly(Glu,Tyr) induced by the 40,000 x g supernatants of the various tissues (not shown). This seems to indicate that the activations observed here are not related to vanadate's effects in blocking poly(Glu,Tyr) dephosphorylation. It also seems to indicate that vanadate's inhibition of PTPases is more effective and specific when the substrate for the PTPase is an autophosphorylated-protein tyrosine kinase.

Since tungstate and molybdate resembled vanadate in stimulating rat adipocytic CytPTK, in the cell-free experiment they were expected (if permeable to cell interiors) to stimulate glucose metabolism as well. Indeed, molybdate and tungstate (but not fluoride) significantly activated lipogenesis in intact rat adipocytes. Their stimulating effects could be blocked by staurosporine.²

### DISCUSSION

Vanadium mimics most of the biological actions of insulin in various target tissues, and endogenous cellular tyrosine kinase activity is believed to be an essential early component in activating insulin-like bioeffects such as glucose and fat metabolism (36). With our recent discovery of the vanadium-dependent (insulin-independent) CytPTK, the steps linking vanadium pretreatment to activated CytPTK and enhanced rates of glucose metabolism remain to be determined. Due to the overall complexity of the intact mammalian cell, such a task is sometimes possible only if the effect is preserved following cell disintegration. This can be achieved if the stimulus and the intracellular target are proximal to each other.

Here, we have described direct attenuating effects of vanadium on protein tyrosine kinases in the cell-free experiments. Moreover, discriminating effects were seen of either vanadate or vanadyl toward receptor or non-receptor protein tyrosine kinases. Vanadyl (but not vanadate) inhibited receptor tyrosine kinase activity is believed to be an essential early component in insulin-like bioeffects such as glucose and fat metabolism (36). With our recent discovery of the vanadium-dependent (insulin-independent) CytPTK, the steps linking vanadium pretreatment to activated CytPTK and enhanced rates of glucose metabolism remain to be determined. Due to the overall complexity of the intact mammalian cell, such a task is sometimes possible only if the effect is preserved following cell disintegration. This can be achieved if the stimulus and the intracellular target are proximal to each other.

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² G. Elberg, J. Li, and Y. Shechter, manuscript in preparation.
kinases non-competitively, whereas the non-receptor protein tyrosine kinases were not inhibited by vanadyl and stimulated by vanadate. These results together with those of the several previous studies, support the contention that the activating effects of added vanadium on glucose and fat metabolism are indeed mediated via protein tyrosine kinases.

It should be noted, at this point, that the coincidence of finding a substance (such as vanadyl), that is present endogenously in cells and inhibits InsRITK at low concentrations in an ATP and substrate independent manner is unique and may have a physiological significance. This is particularly validated by the fact that vanadyl could also block insulin-stimulating effects in cells, pretreated with staurosporine (Fig. 6). We are especially intrigued by the effectiveness of vanadyl in inhibiting autophosphorylation. This observation is an exception among many InsRITK blockers studies, including those competing with ATP (37) or substrate binding (38, 39). Both types are more effective (about 10-fold or more) in blocking phosphorylation of exogenous substrate, rather than inhibiting autophosphorylation (37–39). The latter is an intramolecular transphosphorylation within the insulin receptor heterotetramer (40). Therefore, inhibitors must compete against high local substrate or ATP concentrations.

Perhaps of more significant interest are our cell-free observations demonstrating that (a) vanadyl (5+) is the species that activates CytPTK, (b) it does so at relatively low concentrations in insulin-responsive tissues, and (c) vanadyl is readily converted to vanadate by hydrogen peroxide.

A feasible working hypothesis, currently being studied in our laboratory, is illustrated schematically in Fig. 10. According to this hypothesis, the intracellular vanadium pool may have an essential role in modulating those CytPTKs not controlled by external stimuli. Under resting conditions, the bulk of the intracellular vanadium is in the form of vanadyl (4+) (Refs. 18 and 19), which, as was shown here, exerts no effect on CytPTK activity, keeping the latter in basal resting state. Any physiological conditions, however, that activate NADPH oxidase and lead to the formation of H$_2$O$_2$ are expected to oxidize a fraction of the endogenous vanadyl pool to vanadate. This, in turn, will inhibit those vanadate-sensitive PTPases and correspondingly increase the steady states of phosphorylation and activation of CytPTK (Fig. 10). Most protein tyrosine kinases studied, including the rat adipocytic CytPTK (1, 10), seem to be activated as a result of autophosphorylation on tyrosine moieties (41, 42). Although it should be emphasized that a gap exists between the vanadate concentrations activating adipocytic CytPTK in cell-free experiments (i.e. $E_{D_0} = 3.0$ $\mu M$) and the documented intracellular vanadium levels (i.e. 0.1–1.0 $\mu M$; Refs. 18 and 19).

We assume, however, that activation of a minute fraction of the total CytPTK activity in the cell may suffice to give the desired level of phosphorylation and amplification required for further stimulating glucose and fat metabolism. This may especially be valid, as rat adipocytic cytosol contains an extremely high poly(Glu$_n$Tyr) phosphorylating capacity, which exceeds that of the plasma-membrane fraction by more than 1 order of magnitude (1, 10).

This working hypothesis explains the activating effects of added vanadate on CytPTK activity in intact cells, as well as in cell-free experiments. That vanadyl can be converted to vanadate via an NADPH-oxidative pathway has been previously demonstrated (48), and a link between vanadate, NADPH, and activation of tyrosine phosphorylation in cells was frequently observed (44–47).

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