Evidence for the Distinct Vanadyl(+4)-Dependent Activating System for Manifesting Insulin-Like Effects†

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ABSTRACT: Both exogenously added vanadate (oxidation state +5) and vanadyl (oxidation state +4) mimic the rapid responses of insulin through *alternative* signaling pathways, not involving insulin receptor activation [reviewed in Shechter *et al*. (1995) *Mol. Cell. Biochem. 153*, 39−47]. Vanadium exhibits complex chemistry, fluctuating between vanadate(+5) and vanadyl(+4), according to the prevailing conditions. Using several experimental approaches, we report here on a distinct vanadate(+5)-independent, vanadyl(+4)-dependent activating pathway. The key components of this pathway are membrane protein phosphotyrosine phosphatases (PTPases) and a cytosolic (nonreceptor) protein-tyrosine kinase (CytPTK). We further suggest that vanadate(+5) is not reduced rapidly to vanadyl(+4) inside the cell, and entered vanadyl sulfate(+4) is capable of undergoing spontaneous oxidation to vanadate(+5) *in vivo*. Finally, we show that the promotion and full expression of a downstream bioeffect such as lipogenesis requires both activation of CytPTK and prolonged stability of vanadyl(+4) against oxidation.

Exogenous vanadyl(+4) and vanadate(+5) ions both mimic the biological actions of insulin [Shechter & Karlish, 1980; Degani *et al.*, 1981; Shechter & Ron, 1986; reviewed by Shechter (1990)]. In rat adipocytes, similar concentrations activate hexose uptake, stimulate glucose metabolism, and inhibit lipolysis (Shechter & Karlish, 1980; Degani *et al.*, 1981; Shechter & Ron, 1986; Shechter, 1996; unpublished results), but the effect of $VOSO_4$(+4)[1] is only 25−35% of that of vanadate or insulin (Shechter & Karlish, 1980; Shechter *et al*., 1992). This quantitative difference has in the past been attributed to the lower solubility, or stability, of $VOSO_4$(+4) at neutral pH values and/or to a difference in permeation into cell interior. The question is whether vanadate(+5), vanadyl(+4), or both are the active species mimicking insulin. The issue is further complicated, since exogenous vanadyl(+4) can be oxidized to vanadate at neutral pH values (Macara, 1980; Macara *et al.*, 1980), while vanadate can be reduced intracellularly to vanadyl(+4) in several cell types, including rat adipocytes (Cantley *et al*., 1979; Macara *et al*., 1980; Degani *et al*., 1981; Willsky *et al*., 1984). The notion that this intracellular reduction is nonenzymatically mediated by glutathione (Cantley & Aisen, 1979; Macara *et al*., 1980; Willsky *et al*., 1984; Crans, 1995) is now challenged with our recent findings in the cell-free system, which demonstrate that GSH is an ineffectual reductant of vanadate [Li *et al*. (1995) and this study]. The possibility that vanadyl(+4) is capable of being oxidized to vanadate inside the cell has not been highly favored, because the established dogma assumes that vanadate(+5) cannot exist intracellularly in cells containing millimolar concentrations of glutathione.

We have, therefore, attempted to systematically explore this problem, using different approaches, some of which are based on quite recent developments: (a) utilizing the newly discovered vanadate-activatable cytosolic protein-tyrosine kinase (CytPTK) that participates in several vanadate effects in intact adipocytes, such as activation of lipogenesis (Shisheva & Shechter, 1993); (b) establishing a procedure to enable activation of this CytPTK (by vanadate and related compounds) in a cell-free experimental system in which there is no vanadium interconversion (Elberg *et al*., 1994); and (c) substituting for $VOSO_4$ with an organic vanadyl(+4)-compound such as $VO(acac)_2$ which has greater hydrolytic and redox stability.

Cytosolic PTK (CytPTK) is an adipose, water soluble, nonreceptor protein, tyrosine kinase with an estimated MW of 53 kDa (Shisheva & Shechter, 1992b, 1993). Cytosolic protein-tyrosine kinases were identified in several mammalian tissues [reviewed by Srivastava (1990)]. Unlike several other rat tissue extracts, the 40000*g* supernatant of rat adipose cells appears to predominantly contain a single $PolyGlu_4Tyr$ phosphorylating species (Elberg *et al*., 1995).

EXPERIMENTAL PROCEDURES

Materials

D-[2-^{3}H]Glucose was purchased from New England Nuclear (Boston, MA). Collagenase type I (134 u/mg) was obtained

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[1] Abbreviations: CytPTK, cytosolic protein-tyrosine kinase; PTK, protein-tyrosine kinase; GSH, reduced glutathione; $VO(acac)_2$, vanadyl acetylacetonate; PNPP, *P*-nitrophenylphosphate; InsRTK, insulin receptor tyrosine kinase; $PolyGlu_4Tyr$, poly[(GluNa:Tyr)4:1]; KRB, Krebs−Ringer bicarbonate; DTT, dithiothreitol; βMeSH, β-mercaptoethanol; PTPase, protein phosphotyrosine phosphatase; $VOSO_4$, vanadyl sulfate, $NaVO_3$, sodium metavanadate.

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from Worthington Biochemicals (Freehold, NJ). Porcine insulin was obtained from Eli Lilly Co. (Indianapolis, IN). Sodium metavanadate ($NaVO_3$) was from BDH Chemicals Ltd. Vanadium(+4) acetylacetonate [$VO(acac)_2$] was purchased from Aldrich Chemical Co. (Milwaukee, WI). $PolyGlu_4Tyr$ was purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antibodies to phosphotyrosine were obtained from Mono-Yeda (Rehovot, Israel). All other chemicals used here were of analytical grade. Krebs−Ringer bicarbonate (KRB) buffer at pH 7.4 contained 110 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.2 mM, KH_2PO_4, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$.

Procedures

Rat adipocytes were prepared from fat pads of 100−200 g male Wistar rats according to Rodbell (1964), and stimulation of lipogenesis (the incorporation of D-[2-^{3}H]-glucose into lipids) was performed according to Moody *et al.* (1974) with no modifications.

High-speed supernatant fraction as a source for CytPTK was prepared as follows. Fresh adipocytes were washed three times with KRB buffer at pH 7.4 containing 0.7% BSA and then twice more with 50 mM Hepes buffer at pH 7.4 containing 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 5 μg/mL aprotinin. The cells (about 10 mL of packed adipocytes) were homogenized with hand Teflon homogenizer, the fat was removed, and the cell homogenate was centrifuged at 40000*g* for 60 min. This fraction was used immediately in the next stage.

Cell-Free CytPTK Activating System. Unless otherwise indicated, the assay included (in 60 μL of 50 mM Hepes buffer at pH 7.4) the enzyme source (0.5−5 μg of protein from rat adipocytic high-speed supernatant fraction), 15 mM $MgSO_4$, 1 mM $CO(OOCCH_3)_2$, and 100 μM ATP and was performed in the absence or presence of the indicated concentrations of vanadate, $VOSO_4$ or $VO(acac)_2$. Following preincubation (20 min at 22 °C), $PolyGlu_4Tyr$ (0.5 mg/mL) was added; the reaction was allowed to proceed for 40 min at 22 °C and was terminated by the addition of EDTA (40 mM).

Phosphotyrosine content in $PolyGlu_4Tyr$ was quantitated by a radioimmunoassay procedure, using specific monoclonal antibodies to phosphotyrosine (final dilution of 1:100000) and [^{125}I]BSA−phosphotyrosine conjugate (Shisheva *et al.*, 1991).

Reduction of vanadate(+5) to vanadyl(+4) was determined spectroscopically at 765 nm by the appearance of the characteristic "blue" absorbance of the vanadyl(+4) ion following reduction. Samples were acidified to 0.2 M HCl prior to determining absorbance intensity, using an absorption coefficient of 14 ± 0.1 (Gupta & Chatterjee, 1978).

Oxidation of $VOSO_4$ or $VO(acac)_2$ to Vanadate. Fresh solutions of $VOSO_4$ (3 mM in H_2O) were made 0.1 M Hepes (pH 7.4), prior to the experiment. Fractions (0.4 mL each) were incubated at 37 °C in the absence or presence of GSH (10 mM). At different time points, aliquots were acidified and the absorbance at 765 nm was recorded. Essentially, the same procedure was applied for $VO(acac)_2$ except that the decrease in absorbance was monitored at 737 nm, with no acidification. $VO(acac)_2$ can be prepared at concentrations of 3−10 mM in H_2O to give pH values of 7.0−7.2. It has an absorbance maximum at 737 nm with a molar extinction $\epsilon_{737\ nm}$ of 44 ± 3, at neutral and slightly alkaline pH values (unpublished results).

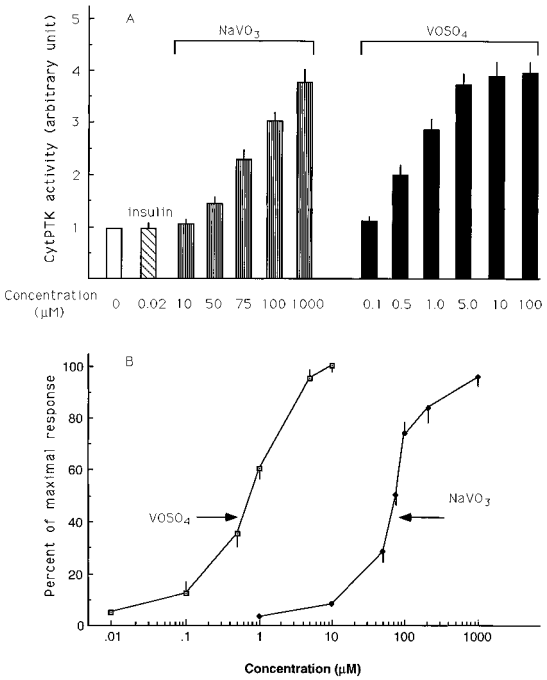

FIGURE 1: Activation of CytPTK by vanadate(+5) and $VOSO_4$-(+4) in intact rat adipocytes. Freshly prepared adipocytes (5×10^6 cells/mL) were preincubated for 20 min at 37 °C with the indicated concentrations of $NaVO_3$ and $VOSO_4$. The cells were then washed and disrupted by a freezing/thawing procedure, and aliquots from the 40000*g* supernatant fraction (0.5−5 μg protein) were analyzed for their protein tyrosine kinase activity, in an assay containing $MgSO_4$ (15 mM), Co^{2+} (1 mM), ATP (0.1 mM), and $PolyGlu_4Tyr$ (0.5 mg/mL) and the indicated concentrations of $NaVO_3$, $VOSO_4$, or insulin [Experimental Procedures and Shisheva and Shechter (1992b, 1993) and Elberg *et al.* (1994)].

Protein concentration was determined according to Bradford (1976). All the assays were performed in duplicate or triplicate. The data in the figures and table are presented as the means ± SE from at least three separate experiments.

RESULTS

Vanadyl Sulfate(+4) Is a Potent Activator of CytPTK in Intact Rat Adipocytes. Figure 1 shows that $VOSO_4$ strongly activated CytPTK in intact rat adipocytes, reaching a maximal increase of (4.2 ± 0.4)-fold (Figure 1A), with an ED_{50} at 0.8 ± 0.05 μM. Vanadate activated CytPTK to the same extent; however, its ED_{50} was 85 ± 5 μM. Therefore, $VOSO_4$ is about 100 times more potent than vanadate in activating CytPTK in the intact cell.

Activation of CytPTK by $VOSO_4$ in the Cell-Free System. Vanadyl sulfate(+4) had a negligible activating effect on CytPTK in a cell-free system, consisting solely of the cytosolic fraction (40000*g* supernatant; Elberg *et al.*, 1994; Figure 2A). It was no more effective when the cell-free system constituted both the cytosolic fraction and broken plasma membrane fragments (Figure 2B). However, dissolution of the broken plasma membrane fragments with Triton X-100 (1%) enabled $VOSO_4$ to activate CytPTK (Figure 2C). The maximal increase was 5.0-fold, and the ED_{50} of $VOSO_4$ was 45 ± 5 nM. In the cell-free system, vanadate activates CytPTK with an ED_{50} value of 2.0 ± 0.2 μM (Elberg *et al.*, 1994). Thus, $VOSO_4$ is a 45-fold more potent activator of CytPTK than vanadate, in the cell-free

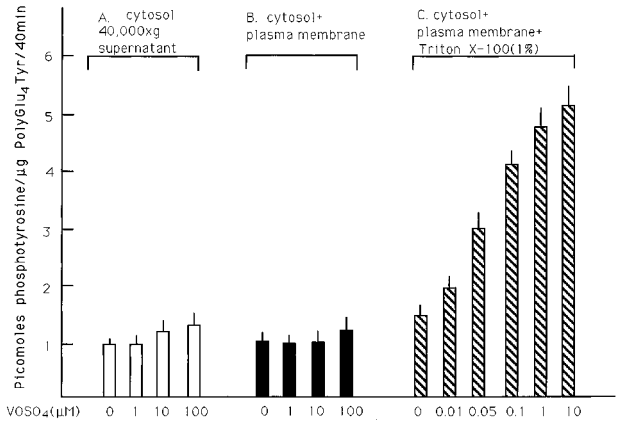

FIGURE 2: Cell-free activation of CytPTK by $VOSO_4$(+4) under different experimental conditions. The standard tyrosine kinase assay mixture (60 μL final volume in 50 mM Hepes buffer at pH 7.4) contained 0.1 mM ATP, 15 mM $MgSO_4$, 1 mM Co^{2+}, and 0.5 mg/mL $PolyGlu_4Tyr$ and was performed in the absence and presence of the indicated concentrations of $VOSO_4$: (A) cytosol (40000*g* supernatant), (B) cytosol (40000*g* supernatant) and broken plasma membrane fragments, and (C) a cytosol (40000*g* supernatant)/plasma membrane mixture in which Triton X-100 was added to a final concentration of 1%.

Table 1: Reduction of Vanadate to Vanadyl(+4) by Various Reducing Agents at pH 7.4[a]

reactant	reaction conditions	absorbance at 765 nm	conversion of vanadate to vanadyl(+4) (%)
$NaVO_3$ (5 mM) + GSH (5 mM)	1 h, 37 °C	0.002	3
$NaVO_3$ (20 mM) + GSH (20 mM)	1 h, 37 °C	0.014	5
$NaVO_3$ (20 mM) + βMeSH (20 mM)	5 min, 25 °C	0.28	100
$NaVO_3$ (20 mM) + βMeSH (10 mM)	5 min, 25 °C	0.13	46
$NaVO_3$ (20 mM) + DTT (10 mM)	5 min, 25 °C	0.27	96
$NaVO_3$ (20 mM) + DTT (5 mM)	5 min, 25 °C	0.13	46

[a] $NaVO_3$ was reduced by the specified reducing agents at pH 7.4, under the reaction conditions stated in the table. Absorbance at 765 nm was monitored prior and subsequent to the reaction. Samples were acidified to 0.1 M HCl, before absorbance intensity was measured. The amount of vanadyl(+4) formed was calculated using ϵ765nm = 14 (Gupta & Chatterjee, 1978). Neither $NaVO_3$, nor any of the reducing agents, GSH, βMeSH, and DTT, nor their oxidized forms absorb at this wavelength.

experimental system, provided that plasma membrane PTPases are solubilized by extraction with a nonionic detergent such as Triton X-100 (Figure 2C). Control experiments showed no increase of membrane PTK activity by $VOSO_4$ at concentrations lower than 10−20 μM (not shown).

Glutathione Is an Ineffectual Reductant of Vanadate. The results summarized in Table 1 show that, at physiological temperature and pH (37 °C and pH 7.4), reduced glutathione (GSH) is an ineffectual reductant of vanadate. Incubation of $NaVO_3$ and GSH (5 or 20 mM of each reactant) for 1 h at 37 °C led to negligible reduction (3−5%) of vanadate(+5) to vanadyl(+4). Vanadate was readily reduced (within minutes) by more powerful reducing agents, such as β-mercaptoethanol and dithiothreitol. Both agents reduced vanadate to vanadyl(+4) in a stoichiometric fashion with a molar ratio of 1:1 (βMeSH) and 0.5:1 (DTT) under similar conditions (Table 1).

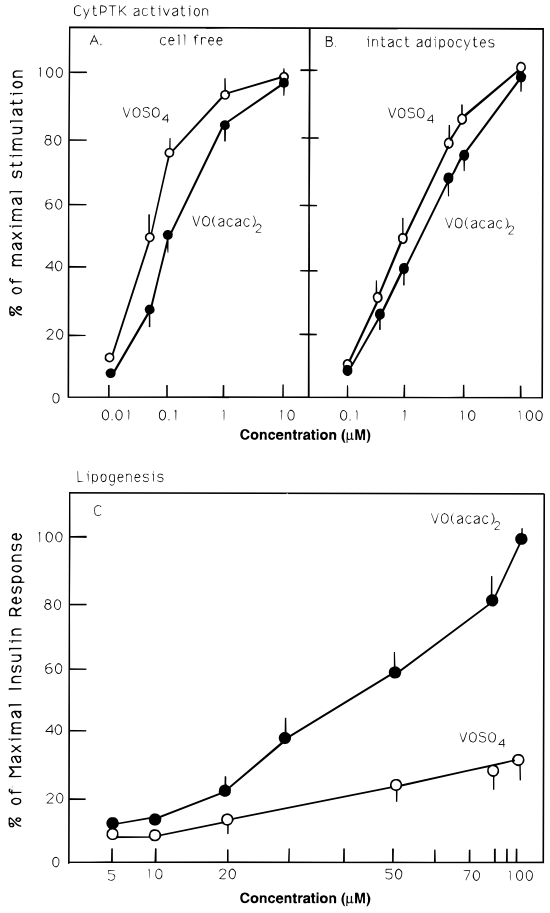

FIGURE 3: Comparison of concentrations of $VOSO_4$ and $VO(acac)_2$ to activate CytPTK and to stimulate lipogenesis: (A) activation of CytPTK performed in a cytosol/plasma membrane mixture that was made 1% Triton X-100, under the experimental conditions specified in Figure 2C, (B) concentration-dependent activation of CytPTK in intact rat adipocytes (conditions are as described in the legend to Figure 1), and (C) activation of lipogenesis by $VOSO_4$ and $VO(acac)_2$ in rat adipocytes. Lipogenesis was carried out for 1 h at 37 °C in vials containing about 1.5×10^5 cells suspended in KRB buffer, 0.2 mM D-[2-^{3}H]glucose, and the indicated concentrations of $VOSO_4$ or $VO(acac)_2$. Results are expressed as percentage of stimulation compared to the maximal response to insulin.

Vanadyl Acetylacetonate(+4) Is a More Potent Stimulator of Lipogenesis Than $VOSO_4$. In the experiments summarized in Figure 3, the potency of $VO(acac)_2$ was studied and compared to that of $VOSO_4$. $VOSO_4$ and $VO(acac)_2$ were almost equipotent and also equally efficient in activating CytPTK both in the cell-free system and in intact adipocytes (panels A and B of Figure 3), strongly suggesting no major difference in permeation of $VO(acac)_2$ and $VOSO_4$ into the cell interior. $VO(acac)_2$, however, was significantly more effective than $VOSO_4$ in activating lipogenesis (Figure 3C). The maximum increase in lipogenesis induced by $VOSO_4$ and $VO(acac)_2$ was 30 ± 4 and 95 ± 5%, respectively, of the maximal insulin response (Figure 3C). With respect to the ED_{50} values, only a modest shift to the left [for $VO(acac)_2$] was seen. The vanadyl(+4) in aqueous $VOSO_4$ and in $VO(acac)_2$ are likely to have different coordination environments. In aqueous $VOSO_4$, the vanadyl(+4) is in an octahedral topography, whereas in $VO(acac)_2$, it is likely to be in a five-coordinate position (Casteen, 1981). As both compounds are nearly equipotent in activating CytPTK, in cell-free experiments (Figure 3A), this difference in shape, by itself, is not sufficient to explain the higher potency of

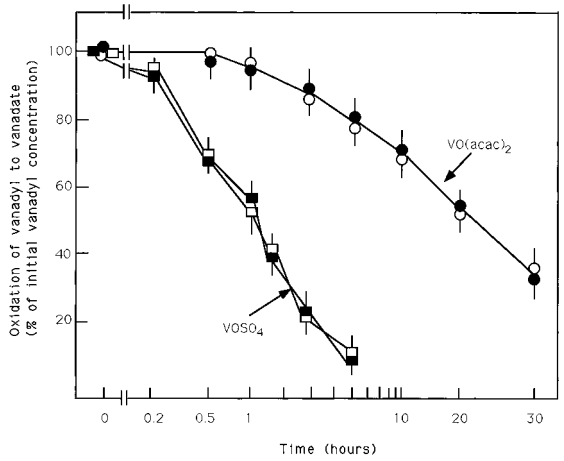

FIGURE 4: Time-dependent susceptibility of $VOSO_4$ and $VO(acac)_2$ to undergo oxidation to vanadate at pH 7.4 and 37 °C in the presence and absence of reduced glutathione. $VOSO_4$ and $VO(acac)_2$ (3 mM each) in 0.1 M Hepes at pH 7.4 were incubated in glass tubes (0.4 mL per tube) at 37 °C in the presence (●, ■) or absence (○, □) of GSH (10 mM). At the indicated time points, aliquots were acidified with HCl and the absorbance at 765 nm ($VOSO_4$) or 737 nm [$VO(acac)_2$] was monitored (Experimental Procedures).

$VO(acac)_2$ in stimulating lipogenesis. A more relevant difference to account for this is explored in Figure 4. $VO(acac)_2$ has significantly higher redox stability, compared to $VOSO_4$. Vanadyl sulfate undergoes spontaneous oxidation to vanadate with a $t_{1/2}$ of 1 ± 0.1 h at pH 7.4 and at 37 °C. Under identical experimental conditions, $VO(acac)_2$ undergoes oxidation to vanadate with a $t_{1/2}$ of 12 ± 0.5 h (Figure 4). The inclusion of GSH in the medium (10 mM) had virtually no effect on the rate of oxidation (Figure 4). Further experimental evidence, supporting vanadyl oxidation *in vivo*, was obtained by prolonged incubations of adipocytes with low concentrations of either vanadate, $VOSO_4$, or $VO(acac)_2$ prior to the biological assay (in preparation). Thus, stimulation of lipogenesis by vanadyl(+4) appears to be dependent both on activation of CytPTK and on intracellular stability against oxidation.

DISCUSSION

Vanadium salts mimic the actions of insulin, and numerous studies strongly suggest that their effects are not mediated through the insulin receptor (Green, 1986; Fantus *et al*., 1989; Mooney *et al*., 1989; Strout *et al*., 1989; Venkatesan *et al*., 1991; Shisheva & Shechter, 1992a). In fact, their signal now appears to be transduced along an entirely separate branch that is not itself involved in insulin signalling (manuscripts in preparation). Vanadium salts induce normoglycemia and improve glucose homeostasis in a variety of insulin deficient and insulin resistant diabetic rodents [Brichard *et al*., 1989, 1992; Ramanadham *et al*., 1989; Meyerovitch *et al*., 1991 reviewed by Brichard and Henquin (1995)]. So, elucidating the alternative mechanisms involved in facilitating insulin-like biological events may eventually lead to developing vanadium (or related) compounds which may assist in pathological states characterized by decreased responsiveness to insulin [reviewed by Brichard and Henquin (1995), Crans *et al*. (1995), and Shechter *et al.* (1995)]. Our findings on vanadium-activatable CytPTK (Shisheva & Shechter, 1993) and the establishment of a cell-free system (Elberg *et al*., 1994) now enable us to identify the components and the vanadium species involved under experimental conditions of no vanadium interconversion and no barrier into the cell interior.

In this study, we find that $VOSO_4$ strongly activates CytPTK in the intact cell. Its ED_{50} is 0.8 ± 0.05 μM, so it is about 100-fold more potent than vanadate (Figure 1). Next, we find that $VOSO_4$ activates CytPTK in the cell-free system with an ED_{50} value as low as 45 ± 5 nM, provided that the membrane fragments are solubilized with Triton X-100 (Figure 2). Thus, the $VOSO_4$-dependent activating system appears to include intrinsic membrane PTPases and CytPTK. At this point, it is interesting to note that a potentiating effect, following dissolution of intrinsic membrane proteins, has been demonstrated previously in other systems. For example, extracting the insulin receptor tyrosine kinase (InsRTK) with Triton X-100 is a prerequisite for the InsRTK to be activated by insulin and to phosphorylate exogenous substrates on tyrosine moieties in cell-free experimental systems [reviewed by Shechter (1987)].

The finding that vanadyl(+4) is 45−100-fold more potent than vanadate in activating CytPTK (Figures 1 and 2) suggested to us that perhaps vanadate which has entered the cell is not reduced rapidly to vanadyl(+4) by intracellular GSH as we and others have previously inferred [Cantley & Aisen, 1979; Macara, 1980; Macara *et al*., 1980; Degani *et al*., 1981; reviewed by Shechter (1990)]. Indeed, at neutral pH and 37 °C, GSH is an ineffectual reductant of vanadate [Li *et al*. (1995) and Table 1]. Thus, the (probably slow) appearance of vanadyl(+4) previously detected by electron-spin resonance spectroscopy in vanadate-treated adipocytes (Degani *et al*., 1981) is not likely to be driven by GSH in a nonenzymatic fashion. The conclusion that the intracellular reduction occurred rapidly was based on the failure of exogenously added vanadate to inhibit Na^+K^+ ATPase (Dubyak & Kleinzeller, 1980). However, vanadate has to bind to the low-affinity site of the pump (K_2 = 0.5 μM) to inhibit Na-ATPase activity (Cantley *et al*., 1978) so that a considerable amount of vanadate has to accumulate intracellularly before the pump can be inhibited.

Another (related) dogma to be discredited here was the assumption that vanadyl(+4) that has entered the cells would not be oxidized to vanadate under the known intracellular reducing atmosphere. As shown here, $VOSO_4$ is *spontaneously* oxidized to vanadate *in vitro* at physiological pH and temperature and in the presence of GSH (Figure 4). That this is likely to occur to a certain extent in the cell as well is an assumption strongly supported by the higher lipogenic potency of the redox-stable $VO(acac)_2$ compound (Figures 3 and 4). This finding also implies that both activation of CytPTK and prolonged intracellular stability of the vanadyl-(+4) compound are required for the full expression of a downstream bioeffect such as lipogenesis.

In summary, we have described here a distinct sensitive vanadyl(+4)-dependent activating system, composed of membrane PTPases and CytPTK and conjugated to the insulin-like effects of vanadium in rat adipocytes. Taken together with earlier studies, these results indicate that two other discrete insulinomimetic systems exist which do not utilize the insulin signal transmission network. One system is vanadate-dependent and vanadyl(+4)-independent, while the second system is vanadyl(+4)-dependent and vanadate-independent.

As far as we are aware, this is the first example of a detailed study identifying a sensitive, vanadyl(+4)-dependent

activating system, which is negligibly activated by vanadate. If our working hypothesis is valid in assuming that *all* the insulin-like effects of vanadium are initiated by inhibiting PTPases, it implies that the adipose cells contain distinct vanadyl(+4) sensitive and vanadate (+5) sensitive PTPases and the former is an intrinsic plasma membrane species (Figure 2). Adipose PTPase extracts are equally inhibited by vanadate and $VOSO_4$ with PNPP as the substrate (unpublished observation). More refined studies in this direction are under way.

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