Insulin-Mimetic Vanadyl(IV) Complexes as Evaluated by Both Glucose-Uptake and Inhibition of Free Fatty Acids (FFA)-Release in Isolated Rat Adipocytes

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We have recently proposed the existence of some potent vanadyl complexes with blood glucose-lowering activity in experimental diabetic animals based on the results of an *in vitro* FFA (free fatty acids)-release assay in isolated rat adipocytes treated with epinephrine and evidence of an *in vivo* blood glucose lowering effect in experimental diabetic animals. However, the FFA assay depends indirectly on the glucose-uptake of vanadyl complexes in adipocytes. It is therefore necessary to develop a more reliable *in vitro* glucose-uptake assay, in place of the glucose uptake method using radioactive compounds such as ^{14}C-glucose, to identify insulin-mimetic vanadyl complexes. In the present study, we proposed a combined *in vitro* assay by using the conventional glucose oxidase method for glucose-uptake and FFA assay in isolated rat adipocytes. Insulin, vanadyl sulfate ($VOSO_4$), bis(picolinato)vanadyl ($VO(pa)_2$), and bis(6-methylpicolinato)vanadyl ($VO(6mpa)_2$) complexes exhibited concentration-dependent uptake of (+)-D-glucose and inhibition of FFA release in the adipocytes treated with epinephrine. Vanadyl complexes were found to accelerate glucose-uptake at lower concentrations than $VOSO_4$. *In vitro* high insulin-mimetic activity of $VO(pa)_2$ and $VO(6mpa)_2$ were thus indicated by both glucose-uptake and FFA-release, with the insulin-mimetic activity of $VO(6mpa)_2$ being higher than that of $VO(pa)_2$, as suggested by the partition coefficient (0.330 for $VO(pa)_2$ and 0.595 for $VO(6mpa)_2$). The proposed assay provides a more reliable method than each single method for the evaluation of *in vitro* insulin-mimetic activity of compounds.

Key words vanadyl compound; glucose-uptake assay; free fatty acids (FFA)-release assay; insulin-mimetic effect; adipocyte

The number of patients suffering from diabetes mellitus (DM) is increasing throughout the world, as it becomes the most significant disease of the 21st century.[1—4] However, no agents other than insulin have been developed for the treatment of either type 1 DM or serious type 2 DM. As such, there is an urgent need for developing new types of therapeutic agents for the treatment of diabetes.

Since the discovery of the insulin-mimetic effects of vanadate(V) compounds in adipocytes in 1980,[5,6] the effects of vanadium compounds have attracted many researchers. Among the several oxidation states of vanadium from II to V, this metal ion in living systems is considered to exist exclusively as vanadyl(IV) cation (VO^{2+}) and a small amount of vanadate(V) anion (VO_4^{3-}).[7] In experimental diabetic animals, both vanadyl and vanadate ions have been found to have an insulin-mimetic effect on glucose metabolism.[8—14] Interestingly, both compounds have been proposed to partially improve human DM.[15—24] However, the vanadyl ion is less toxic than the vanadate ion, as judged by the LD_{50} values in several animals,[25,26], and most vanadium in normal rats treated with vanadate exists in the vanadyl form.[7,27] Based on these findings, vanadyl(IV) complexes with low molecular weight organic ligands have recently been prepared to provide more effective insulin-mimetic compounds than vanadyl sulfate ($VOSO_4$). In recent decades, some vanadyl complexes with several types of organic ligands have been proposed by many research groups for clinical use in humans.[28—34]

To evaluate the insulin-mimetic vanadyl complexes, glucose-uptake in cells or tissues has been monitored, which requires radioisotope (RI) reagents.[5,6,27] In 1995, we proposed a new *in vitro* assay, based on the inhibition of FFA (free fatty acids)-release from isolated rat adipocytes treated with epinephrine (adrenalin), which is simple and convenient compared with the use of RI reagents.[35] By this *in vitro* assay, we have evaluated some insulin-mimetic activities of vanadyl complexes with different chemical structures and coordination modes such as $VO(O_4)$,[36] $VO(N_2O_2)$,[37—43] $VO(S_2N_2)$,[36] $VO(S_2O_2)$,[44,45] $VO(N_3O)$,[39] and $VO(N_4)$.[28] Our results indicate that the complexes with a strong ability to inhibit FFA-release from adipocytes lowered the high blood glucose levels in type 1 and 2 diabetic animals more effectively than $VOSO_4$. However, this method evaluates the glucose-uptake in the cells indirectly. We therefore attempted to develop a more reliable method than the FFA-release assay to evaluate the insulin-mimetic activity of vanadyl complexes.

We propose herein the usefulness of simultaneous evaluations of both FFA-release and glucose-uptake based on a conventional glucose oxidase method in isolated rat adipocytes in identifying insulin-mimetic compounds.

Experimental

Materials Vanadyl sulfate ($VOSO_4 \cdot 2.8H_2O$, VS) was purchased from Wako Pure Chemicals (Osaka). The purity of $VOSO_4 \cdot 2.8H_2O$ was determined by chelatometry using an indicator, Cu-Pan (Cu-1-(2-pyridylazo-2-naphthol)) (Dojindo, Kumamoto). Bis(picolinato)vanadyl ($VO(pa)_2$), and bis(6-methylpicolinato)vanadyl ($VO(6mpa)_2$) complexes were prepared as described.[37,38] Collagenase (Type II), bovine serum albumin (BSA; fraction V), and (±)-epinephrine hydrochloride (adrenaline) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Insulin was purchased from Novo Nordisk Pharma. (Tokyo). (+)-D-Glucose was purchased from Nacalai Tesque, Inc. (Kyoto). Other reagents were of the highest purity commercially available.

Animals Male Wistar rats (7—8 weeks old) weighing 200—250 g were obtained from Shimizu Experimental Material Co. (Kyoto). Animals were maintained in a 12-h light/dark cycle in our central animal facility, and were allowed free access to solid food (MF, Oriental Yeast Co. Tokyo) and tap water. Animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and were per-

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formed according to the Guidelines for Animal Experimentation of KPU.

Cell Isolation and Measurements of Free Fatty Acids and Glucose Concentrations Epididymal fat pads, excised from male Wistar rats (7—8 weeks) anesthetized with ether, were cut into the appropriate pieces and incubated in type II collagenase in Krebs Ringer Bicarbonate (KRB) buffer (120 mM NaCl, 1.27 mM $CaCl_2$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 1.2 mM KH_2PO_4 and 24 mM $NaHCO_3$: pH 7.4) containing 2% BSA at 37 °C with gentle shaking at 100 cycles/min for 1 h. At the end of the incubation, the prepared cells were filtered through sterilized cotton gauze and washed three times with KRB buffer. The cells were counted in a hemacytometer after trypan blue staining. Isolated rat adipocytes cells (1.0—2.0×10^6 cells/ml) were preincubated at 37 °C for 30 min with various concentrations of insulin or vanadyl compounds in KRB buffer containing 5.0 mM glucose. Epinephrine (adrenaline) (10 μM) was then added to the reaction mixtures, and the resulting solutions were incubated at 37 °C for 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 h. The reactions were stopped by soaking in ice water, and the resulting mixtures were centrifuged at 3000 rpm for 10 min at 4 °C. For the outer solution of cells, glucose concentrations were measured by a Fuji Dry Chem (Fuji Medical Co., Tokyo),[46] and FFA levels were determined with an FFA kit (NEFA C-test Wako, Wako Pure Chemicals).[35]

Inhibitory Activity of FFA-Release from Isolated Rat Adipocytes Treated with Epinephrine (FFA-Release Assay) The inhibitory activity of the compounds on the FFA-release from isolated rat adipocytes treated with epinephrine (adrenalin) was evaluated with respect to the apparent IC_{50} values, the 50% inhibitory concentration of the compound in the FFA-release with a 3-h incubation. To standardize the IC_{50} values of the compound, that of $VOSO_4$ was expressed as 1.00 mM.

Glucose-Uptake Activity in Isolated Rat Adipocytes Treated with Epinephrine (Glucose-Uptake Assay) The glucose-uptake enhancing ability of the vanadyl compounds was evaluated based on decreases in the glucose concentrations in the medium, as calculated by the following equation:

$$\text{glucose-uptake level} = C_{\text{control}} - C_{\text{compound}}\ (\mu\text{mol/ml})$$

where C_{control} was the glucose concentration in the medium which contained cells without compounds such as insulin and vanadyl after the incubation time, and C_{compound} was the residual glucose concentration in the medium which contained cells treated with these compounds after the incubation time. The glucose-uptake ability of the compounds was evaluated with the apparent EC_{50} values, the 50% enhancing concentration of the compound with respect to the maximal glucose-uptake concentration in glucose-uptake during a 3-h incubation. To standardize the EC_{50} values of the compound, that of $VOSO_4$ was expressed as 1.00 mM.

Statistical Analyses All experimental results are expressed as the mean values$\pm$standard deviations (S.D.). Statistical analysis was performed by analysis of variance (ANOVA) at a 1 or 5% significance level of the difference.

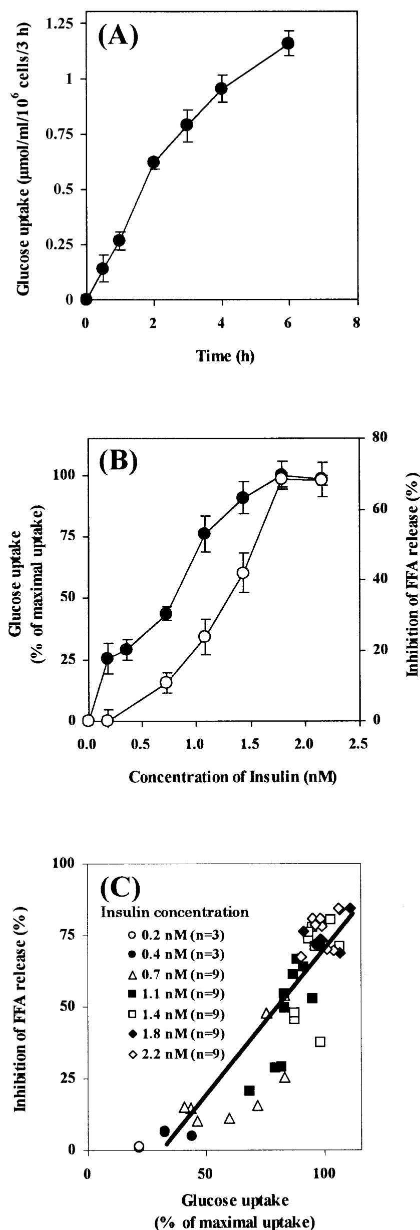

Fig. 1. Incubation Time-Dependent Glucose-Uptake Levels in Isolated Rat Adipocytes Treated with 10 μM Epinephrine in the Presence of 1.07 nM Insulin [A], Insulin Concentration-Dependent Glucose-Uptake (●), and Inhibition of FFA-Release (○) in Isolated Rat Adipocytes Treated with 10 μM Epinephrine for a 3-h Incubation [B], and the Relationship between Glucose-Uptake (%) and the Inhibition of FFA-Release (%) for 3-h Incubation of Adipocytes with 0.2—2.2 nM Insulin ($n=51$, $r=0.92$) [C]

Results

Glucose-Uptake Enhancement and FFA-Release Inhibition by Insulin and $VOSO_4$ in Isolated Rat Adipocytes Treated with Epinephrine Because FFA-release from adipocytes was significantly stimulated by epinephrine,[28,29,35] the correlation between the inhibitory activity of FFA-release and the glucose-uptake ability of vanadyl compounds was examined in the presence of 10 μM epinephrine.

To confirm whether glucose-uptake enhancement by vanadyl compounds can be observed, glucose-uptake by insulin was first examined at various incubation times or insulin concentrations. Insulin (1.07 nM$=$150 μU/ml) stimulated the glucose-uptake, as determined by a decrease in glucose concentration in the medium, almost linearly for a 6-h incubation period (Fig. 1A). From this result, the incubation period was fixed at 3 h, similarly to the FFA-release assay.[35] During the 3-h incubation period, insulin induced a concentration-dependent increase in the glucose-uptake at 0—2.2 nM (0—300 μU/ml) (Fig. 1B). Maximal glucose-uptake was observed at 1.8 nM insulin, the apparent EC_{50} value of insulin being estimated as 0.71 ± 0.05 nM. In the same system, inhibition of FFA-release was observed with increases in the glucose-uptake.[35] To confirm whether the glucose-uptake correlates with the inhibition of FFA-release by insulin, stimulation (%) of the maximal glucose-uptake by insulin was plotted against the inhibitory activity of FFA-release (%) (Fig. 1C). A good correlation between these two parameters ($r=0.92$) was found, indicating that the glucose-uptake by insulin was closely related to the inhibition of FFA-release.

Similarly, glucose-uptake by vanadyl compounds was examined at various vanadyl concentrations and incubation periods. $VOSO_4$ at 0.5 mM induced enhancement of glucose-uptake almost linearly for a 6-h incubation, similar to insulin (Fig. 2A). When the incubation time was fixed at 3 h, $VOSO_4$ induced a concentration-dependent increase in the glucose-

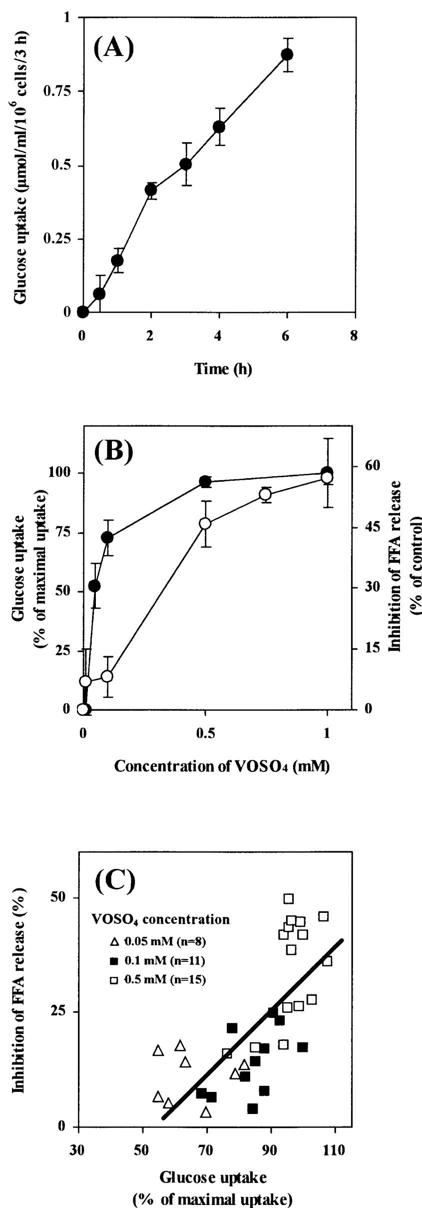

Fig. 2. Incubation Time-Dependent Glucose-Uptake Levels in Isolated Rat Adipocytes Treated with 10 μM Epinephrine in the Presence of 0.5 mM $VOSO_4$ [A], $VOSO_4$ Concentration-Dependent Glucose-Uptake Levels (●), and the Inhibition of FFA-Release (○) in Isolated Rat Adipocytes Treated with 10 μM Epinephrine for a 3-h Incubation [B], and the Relationship between Glucose-Uptake (%) and the Inhibition of FFA-Release (%) in the Presence of 0.05—0.5 mM $VOSO_4$ during a 3-h Incubation ($n=34$, $r=0.70$) [C]

uptake at 0.01—1.0 mM, with the maximal glucose uptake being observed at 0.5—1.0 mM (Fig 2B). However, no glucose-uptake by $VOSO_4$ was observed at lower concentrations than 0.01 mM. Thus, a correlation between the glucose-uptake and the inhibition of FFA-release by $VOSO_4$ was found in the concentration range of 0.05—0.5 mM ($r=0.70$) (Fig. 2C), because the FFA-release was not inhibited by $VOSO_4$ at lower concentration than 0.05 mM and the saturation of glucose-uptake by $VOSO_4$ was observed at the lower concentrations than those of the inhibition of FFA-release.

Based on these results, the glucose concentration added to the medium was fixed at 5 mM, which is within physiological range in the blood of normal rats or mice, and the incubation time used to determine the glucose-uptake ability of vanadyl complexes was fixed at 3 h.

Evaluation of Insulin-Mimetic Activities of Vanadyl(IV) Complexes To estimate the insulin-mimetic activity of vanadyl complexes, we first examined the FFA-releasing assay, which has been developed in our laboratory,[35] and many data have been accumulated.[28,29] The concentration-dependent inhibitory effects of insulin and vanadyl compounds on FFA-release from isolated rat adipocytes treated with epinephrine are shown in Fig. 3, from which the IC_{50} values of the compounds were calculated (Table 1). The IC_{50} value of insulin (3.37±0.02 nM) was found to be significantly lower than that of $VOSO_4$ (1.00±0.02 mM), and those of $VO(pa)_2$ (0.73±0.07 mM) and $VO(6mpa)_2$ (0.60±0.03 mM) complexes were lower than that of $VOSO_4$. These results indicate that the insulin-mimetic activities evaluated by FFA-release assay are in the following order: Insulin$\gg$ $VO(6mpa)_2$>$VO(pa)_2$>$VOSO_4$.

Next, the insulin-mimetic abilities of vanadyl complexes were estimated using the glucose-uptake assay. As shown in Fig. 4, the concentration-dependent enhancing effects of glucose-uptake by insulin as well as vanadyl compounds were observed at lower concentrations than those in the FFA-release assay, and both EC_{50} values and maximal glucose-uptake ability were obtained from these data, as shown in Table 1. The EC_{50} value of insulin (2.27±0.27 nM) was found to be significantly lower than those of vanadyl compounds, and those of vanadyl complex were lower than those of $VOSO_4$ ($VO(pa)_2$=0.61±0.03 mM and $VO(6mpa)_2$=0.71±0.05 mM), indicating that the glucose-uptake abilities of vanadyl complexes are higher than that of $VOSO_4$. The maximal glucose-uptake ability of $VO(6mpa)_2$ complex was found to be higher than those of other vanadyl compounds. These results indicate that the insulin-mimetic activities, as estimated by the glucose-uptake assay, are in the following order: Insulin$\gg$ $VO(6mpa)_2$>$VO(pa)_2$>$VOSO_4$.

Discussion

When epinephrine binds to the β-receptor of adipocytes, the adenylate cyclase is activated to transform ATP to cyclic adenosine 3′,5′-monophosphate (cAMP), which in turn activates some protein kinases. The lipases are then activated to hydrolyze triglycerides to low molecular weight free fatty acids, which will be release outside of the cells.[47] In the system, when insulin is added, this hormone binds to the α-subunit of the insulin receptor and catalyzes the auto-phosphorylation, which in turn stimulates the tyrosine kinase in the β-subunit insulin receptor, and insulin receptor tyrosine kinase then phosphorylates IRS (insulin receptor substrate). After these reactions, the signal information is conveyed to downstream locations such as phosphatidyl inositol-3 kinase (PI3-K), and the glucose transporter-4 (GLUT-4) is then translocated to the cell membrane. Consequently, glucose-uptake is related to the inhibition of FFA-release from isolated rat adipocytes treated with epinephrine.[28,29,48]

Vanadium compounds are considered to activate IRS,[49] PI3-K,[50] GLUT-4,[51,52] cyclic nucleotide phosphodiesterases (PDEs),[53] and protein tyrosine phosphatases (PTPs),[54,55] analogous with the action of insulin. While, vanadyl ions inhibit FFA-release from adipocytes treated with epinephrine.[35] Based on observation of FFA-release inhibition by the

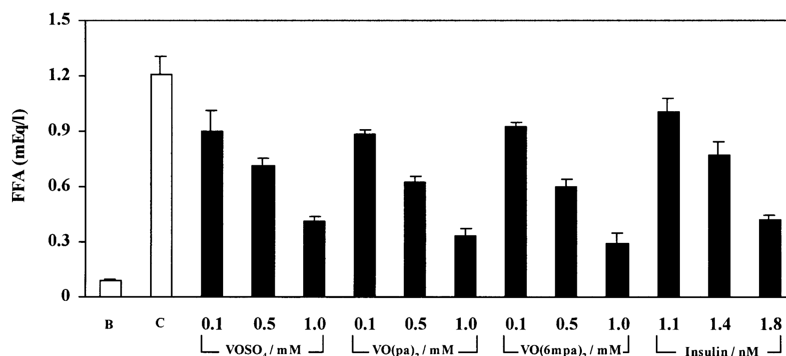

Fig. 3. Inhibitory Effects of Insulin and Vanadyl Compounds on FFA-Release from Isolated Rat Adipocytes Treated with 10 μM Epinephrine

Blank: the cells only, Control: the cells plus 10 μM epinephrine. Insulin: the cells were treated with 10 μM epinephrine plus 1.1, 1.4, and 1.8 nM insulin. Vanadyl compounds: the cells were treated with 10 μM epinephrine plus 0.1, 0.5, and 1.0 mM of each compound. In each system, adipocytes were treated with the samples in numerical order for 30 min and then incubated with 10 μM epinephrine for 3 h at 37 °C. Each column is expressed as the mean value $\pm$ S.D. for 3 experiments.

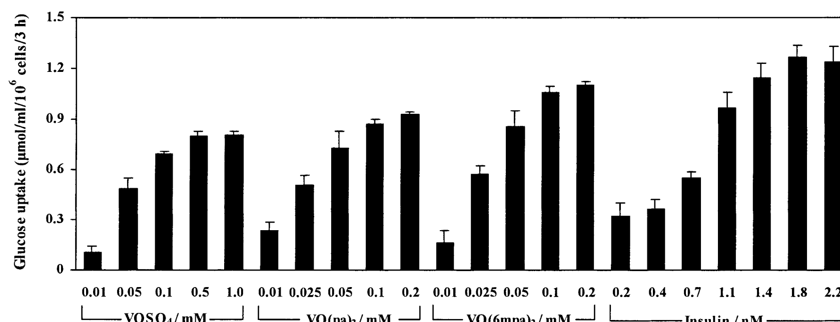

Fig. 4. Insulin and Vanadyl Concentration-Dependent Glucose-Uptake Enhancement by Insulin and Vanadyl Compounds in Isolated Rat Adipocytes Treated with Epinephrine

$VOSO_4$: the cells were treated with 10 μM epinephrine plus 0.01, 0.05, 0.1, 0.5, and 1.0 mM $VOSO_4$. Vanadyl complex ($VO(pa)_2$, and $VO(6mpa)_2$): the cells were treated with 10 μM epinephrine plus 0.01, 0.025, 0.05, 0.1, and 0.2 mM vanadyl complex. Insulin: the cells were treated with 10 μM epinephrine plus 0.2, 0.4, 0.7, 1.1, 1.4, 1.8, and 2.2 nM insulin. In each system, adipocytes were treated with the samples in numerical order for 30 min and then incubated with 10 μM epinephrine for 3 h at 37 °C. Each column is expressed as the mean value $\pm$ S.D. for 3 experiments.

Table 1. Standardized Insulin-Mimetic Activities of Insulin and Vanadyl(IV) Compounds

Compound	FFA-release assay	Glucose-uptake activity	
	IC_{50} value (mM)	EC_{50} value (mM)	Maximal uptake activity (% of $VOSO_4$)
$VOSO_4$	1.00 ± 0.02	1.00 ± 0.14	100 ± 2
$VO(pa)_2$	$0.73 \pm 0.07^{a)}$	$0.61 \pm 0.03^{c)}$	$114 \pm 3^{c)}$
$VO(6mpa)_2$	$0.60 \pm 0.03^{a,b)}$	$0.71 \pm 0.05^{c)}$	$133 \pm 6^{c,d)}$
Insulin	$3.37 \pm 0.02\ (\times 10^{-6})^{a,b)}$	$2.27 \pm 0.27\ (\times 10^{-5})^{c,d)}$	$141 \pm 8^{c,d)}$

Data are expressed as means $\pm$ S.D. for 3 experiments. *a*) Significance at $p<0.05$ *vs.* $VOSO_4$ in FFA-release assay. *b*) Significance at $p<0.05$ *vs.* $VO(pa)_2$ in FFA-release assay. *c*) Significance at $p<0.05$ *vs.* $VOSO_4$ in glucose-uptake assay. *d*) Significance at $p<0.05$ *vs.* $VO(pa)_2$ in glucose-uptake assay.

vanadyl ion, we have indirectly evaluated the *in vitro* glucose-uptake ability of vanadyl complexes.[28,29,35]

A determination method for glucose-uptake based on an enzymatic reaction and that does not require radio-labeled compounds such as ^{14}C-glucose was proposed in 1989[56] and has been used widely for many experiments. We applied this method to determining glucose concentrations in the outer solution of isolated adipocytes. The addition of physiological concentrations of insulin induced a concentration-dependent glucose-uptake in the isolated rat adipocytes treated with epinephrine during a 3-h incubation (Fig. 1). The strong correlation between glucose-uptake and the inhibition of FFA-release indicated that additional insulin induces a concentration-dependent inhibition of FFA-release through glucose-uptake in the adipocytes.

Similarly, $VOSO_4$ induced a concentration-dependent acceleration of glucose-uptake in the adipocytes, where a strong correlation between glucose-uptake and the inhibition of FFA-release was found in the concentration range of 0.05—0.5 mM (Fig. 2). However, a correlation between glucose-uptake and the inhibition of FFA-release by $VOSO_4$ was not found at low and high concentrations. It is well known that insulin causes the translocation of GLUT-4 from intracellular sites to the cell surface,[48] which in turn increases glucose-uptake into the cells. On the other hand, the question arises as to whether the insulin-mimetic activity of vanadium compounds is mediated through the insulin receptor or other proteins in the insulin-signaling pathway. It has been pro-

posed by some that the action of vanadium compounds is independent of direct activation of the insulin receptor.[57—60] Vanadium at 1 mM has been found to stimulate the IRS-1 associated with PI3-K activity and GLUT-4 translocation without activating the insulin receptor in rats.[49] In these previous reports, it was considered that glucose-uptake into the cell by vanadium compounds was stimulated without activating the insulin receptor. Such poor evidence for the contribution of vanadium compounds to the insulin receptor suggests the concentration-dependent affinity of vanadium compounds for intracellular enzymes and substrates. Non-correlation between the glucose-uptake and the inhibition of FFA-release at low and high concentrations of $VOSO_4$ may be responsible for the different action mechanism between insulin and vanadyl compound in exhibiting insulin-mimetic effects.

On the basis of these observations, *in vitro* insulin-mimetic activities of vanadyl complexes were determined by both an FFA-release assay and a glucose-uptake assay (Table 1). The apparent inhibitory effects of FFA-release by vanadyl complexes were observed at a concentration range of 0.1 to 1.0 mM (Fig. 3). Our results indicate that $VO(6mpa)_2$ exhibits higher levels of insulin-mimetic activity than other vanadyl compounds (Table 1). In the glucose-uptake assay, the effects of vanadyl complexes were observed at concentrations ranging from 0.01—0.2 mM, being approximately one tenth of those in FFA-releasing activity. These results suggest that the glucose-uptake assay is more sensitive than the FFA-release assay. The standardized EC_{50} values of vanadyl complexes were estimated to be lower than that of $VOSO_4$ (Table 1), and the $VO(6mpa)_2$ complex was found to have the highest insulin-mimetic activity among the vanadyl compounds examined. $VO(6mpa)_2$ with a methyl group on the picolinate structure gives a higher partition coefficient ($P=0.60$) than $VO(pa)_2$ ($P=0.33$), suggesting that more $VO(6mpa)_2$ is incorporated into the cells than $VO(pa)_2$.[38] It has previously been reported that $VO(6mpa)_2$ at a lower dose is more effective than $VO(pa)_2$ in lowering blood glucose levels of the streptozotocin-induced type 1 DM rat (STZ rat).[38] The results obtained by simultaneous assay of both FFA-release and glucose-uptake are in good agreement with those of the *in vivo* experiments.

On the basis of these results, we propose a new method for evaluating the *in vitro* insulin-mimetic activity of the vanadyl complex by simultaneous glucose-uptake and inhibition of FFA-release in isolated adipocytes. The proposed method consisting of two assays can identify discriminately the action sites of the insulin-mimetic vanadyl complexes. The results of these assays will be reported in the near future.

In conclusion, we have proposed a simultaneous assay for inhibition of FFA-release and for glucose-uptake, and the new method was found to be sensitive and convenient without the use of RI reagents. This method provides an accurate evaluation technique not only for developing new potent vanadyl complexes but also studying the action mechanism of the insulin-mimetic compounds.

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