

Vanadate Stimulates System A Amino Acid Transport Activity in Skeletal Muscle

EVIDENCE FOR THE INVOLVEMENT OF INTRACELLULAR pH AS A MEDIATOR OF VANADATE ACTION*

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Sodium orthovanadate caused a 2-fold stimulation of system A transport activity in soleus muscle, as assessed by the uptake of the nonmetabolizable analog 2-(methylamino)isobutyric acid (MeAIB). The effect of vanadate on system A was rapid, concentration-dependent and was characterized by an increased V_{\max} without modification of K_m for MeAIB. Under these conditions, vanadate also activated 3-O-methylglucose uptake and lactate production. The effects of vanadate on muscle metabolism showed a complex interaction with the effects of insulin. Thus, the stimulatory effects of vanadate and insulin on MeAIB and 3-O-methylglucose uptake were not additive; however, the effects of insulin and vanadate on lactate production were additive. In spite of the lack of additivity, insulin- and vanadate-induced stimulation of system A differed in their sensitivity to gramicidin D, being the vanadate effect more susceptible to inhibition by gramicidin D than the insulin effect.

System A transport activity shows a dependence on pH, and recent results suggest the presence of critical histidine residues on the A carrier that may be responsible for its pH dependence (Bertran, J., Roca, A., Pola, E., Testar, X., Zorzano, A. & Palacín, M. (1991) *J. Biol. Chem.* 266, 798–802). In this regard, a rise in extracellular pH led to a substantial activation of system A. Furthermore, lowering of muscle intracellular pH induced by ethylisopropylamiloride (EIPA), a specific inhibitor of sodium/proton exchange activity, led to inhibition of system A. This suggests that critical histidine residues are present in an intracellular localization on the A carrier. Furthermore, the rate of muscle glycolysis was also altered in response to a rise in extracellular pH or to EIPA treatment.

Regarding the mechanisms involved in vanadate action, vanadate treatment in the incubated soleus muscle did not cause any significant stimulation of tyrosine kinase activity after partial purification of muscle insulin receptors. On the other hand, vanadate but not

insulin caused a substantial increase in muscle intracellular pH as assessed by 5,5'-dimethylloxazolidine-2,4-dione equilibrium. This effect of vanadate on intracellular pH was not due to activation of the sodium/proton exchanger, since it was not blocked by EIPA. Based on these findings, we suggest that alkalization of muscle intracellular pH might mediate the effects of vanadate on system A and on glycolysis.

The system A carrier is a plasma membrane-bound activity which translocates short polar, straight chain amino acids, including the nonmetabolizable analog 2-(methylamino)isobutyric acid. Transport by system A is sodium-dependent and its activity is greatly reduced at lowered extracellular pH, as assessed in isolated cells or in plasma membrane vesicles (1–4). In this connection, system A transport activity is sensitive to histidine-modifying reagents such as diethyl pyrocarbonate (5). Furthermore, diethyl pyrocarbonate-mediated system A carrier modification shows a clear pH dependence, suggesting that the modified residue/s are involved in the pH dependence of system A carrier activity (5).

System A transport activity is subjected to hormonal regulation, transinhibition, and adaptive regulation in a variety of cell types (6–8). In skeletal muscle, system A transport activity is stimulated in response to amino acid starvation, *i.e.* adaptive regulation (9, 10), by a mechanism that requires protein synthesis and unaltered microtubular function (11). On the other hand, system A transport activity is rapidly activated in skeletal muscle by insulin (12) or acute exercise (13). In skeletal muscle, the effect of insulin on system A transport activity is characterized by its independence of protein synthesis (14, 15), microtubular function (11), and the sodium electrochemical gradient (14, 16).

Vanadate is an agent which mimics many of the insulin effects in insulin-sensitive tissues (17). In connection with muscle, vanadate has been reported to stimulate the rates of hexose uptake, glycogen synthesis and glycolysis (18, 19), processes that are also activated in response to insulin. However, not all insulin effects are mimicked in skeletal muscle in response to vanadate, as for muscle protein synthesis and protein degradation (18). In addition, chronic administration of vanadate to rats augments muscle insulin sensitivity (20) and, under some conditions, chronic vanadate normalizes muscle glycogen levels and glycogen synthase activity in diabetic rats (21). The mechanisms by which vanadate exerts these acute and chronic effects in skeletal muscle are unknown; however, based on observations in mouse diaphragm

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"*in vivo*," vanadate actions do not seem to be related to activation of insulin receptor kinase (22).

In this communication we demonstrate that vanadate and modification of intracellular pH markedly alters system A transport activity as well as glycolysis in skeletal muscle. In addition, we show that vanadate does not stimulate tyrosine kinase activity of insulin receptors, but it increases muscle intracellular pH. Our data indicate that raising of muscle intracellular pH might mediate the effects of vanadate on system A and on glycolytic rate.

EXPERIMENTAL PROCEDURES

Materials—Porcine monocomponent insulin was a gift from T. L. Jeatran, Eli Lilly & Co. Ethylisopropylamiloride (EIPA)¹ was kindly donated by Dr. Jürg Biber (University of Zurich). [¹²⁵I-Tyr¹⁴]Moniodoinsulin, [1-¹⁴C]2-(methylamino)isobutyric acid, [³H]mannitol, [¹⁴C]-3-*O*-methylglucose, and [1-¹⁴C]5,5'-dimethylloxazolidine-2,4-dione (DMO) were obtained from Du Pont-New England Nuclear. [^γ-³²P]ATP was prepared from [³²P]orthophosphate (Du Pont-New England Nuclear) using a Gamma-prep kit from Promega Biotech. Wheat germ agglutinin (WGA) bound to agarose was obtained from Vector. Bovine serum albumin (fraction V albumin, fatty acid free), sodium orthovanadate, gramicidin D, copolymer of Glu/Tyr (4:1), and most commonly used chemicals were from Sigma.

Animals and Dissection Procedures—Male Wistar rats (60–70 g), obtained from our own colony were used. The rats were fed on Purina Laboratory chow ad libitum. Animals were housed in animal quarters maintained at 22 °C with a 12-h light/12-h dark cycle. The dissection and isolation of the soleus muscle was carried out under anesthesia with pentobarbital (5–7 mg/100 g body weight, intraperitoneally) as described previously (23). The isolated soleus muscle was fixed to a stainless-steel clip in order to maintain the muscle under slight tension (approximating the resting length) during the incubation. Such muscles (20–30 mg weight) are able to maintain normal ATP and creatine phosphate concentrations during a 3-h incubation.

Incubations—Soleus muscles were incubated in a shaking incubator at 37 °C for 3 h in 3 ml of Krebs-Henseleit buffer, pH 7.4, containing 5 mM glucose, 0.10% bovine serum albumin, and 20 mM Hepes. After addition of the muscles to the vials, they were stoppered and placed in a Dubnoff metabolic shaker set at 37 °C and a shaking rate of 60 cycles/min. Vials were gassed with 95% O₂ and 5% CO₂ throughout the incubation period. The incubation medium was kept for no longer than 90 min, and during prolonged incubations it was renewed every 90 min. At different times, sodium orthovanadate and insulin as well as several drugs such as gramicidin D or EIPA were added to the incubation medium (see details in figure legends). Experimental series were performed by comparing biological activity of one muscle to the contralateral one from the same rat (paired muscles).

Measurement of Amino Acid and Glucose Uptake and Lactate Production by Muscle—Amino acid uptake by system A was measured in soleus muscles using the nonmetabolizable amino acid analog 2-(methylamino)isobutyric acid (MeAIB). Following incubation with sodium orthovanadate and the above-mentioned agents, muscles were transferred to vials with 1.5 ml of Krebs-Henseleit buffer, pH 7.4, containing 5 mM glucose, 0.10% bovine serum albumin, 20 mM Hepes, and 0.1 mM [1-¹⁴C]2-(methylamino)isobutyric acid (800 μCi/mmol), 1 mM [³H]mannitol (330 μCi/mmol), and the different modulators at the same concentrations as for the preceding incubation period. The vials were stoppered and incubated at 37 °C in a shaking incubator for 30 min. The uptake of MeAIB is linear with time for at least 30 min (11, 14). The gas phase in the vials was 95% O₂ and 5% CO₂. In experiments designed to measure 3-*O*-methylglucose uptake, muscles were incubated in Krebs-Henseleit buffer containing 2 mM pyruvate instead of glucose, and for the last 30 min of incubation, the medium contained 0.1 mM [¹⁴C]3-*O*-methylglucose (800 μCi/mmol) and 1 mM [³H]mannitol (330 μCi/mmol). Following incubation, muscles were rapidly washed in saline and frozen in liquid nitrogen. Muscles were then digested in 0.25 ml of 0.5 M Protosol tissue solubilizer (Du Pont), and radioactivity of muscle digests and aliquots of the incubation media was counted. The amount of each isotope present in the

samples was determined, and this information was used to calculate the extracellular space. The extracellular space of soleus muscles, estimated after 30 min of [³H]mannitol addition, was 0.314 ± 0.002 ml/g and was not modified by treatment with vanadate, EIPA, high extracellular pH, or gramicidin D (data not shown). Intracellular concentration of ¹⁴C-amino acid analog or [¹⁴C]3-*O*-methylglucose was calculated by subtracting its amount in the extracellular space from the total label found in tissue, as previously reported (24). It should be mentioned that more than 85% of total MeAIB uptake by soleus muscle was attributable to activity of a Na⁺-dependent transport system (system A), in keeping with previous observations in the incubated epitrochlearis muscle (25). Thus, whereas total MeAIB uptake (in the presence of sodium) was 35.8 ± 2.1 nmol/g per 30 min, MeAIB uptake in the presence of choline chloride (sodium-free medium) was 5.5 ± 0.1 nmol/g per 30 min. Lactate release to the incubation media was measured for the last 30 min of incubation as in Ref. 26. Student's *t* tests for paired and unpaired data were used for statistical analysis.

Determination of Intracellular pH—Intracellular pH was measured in incubated soleus muscle in the absence or presence of vanadate, insulin, gramicidin D, or EIPA by using DMO (27). Following an initial incubation period for 90 min, muscles were transferred to vials with 3 ml of Krebs-Henseleit buffer, pH 7.4, containing 5 mM glucose, 0.10% bovine serum albumin, 20 mM Hepes, 1 mM [1-¹⁴C]DMO (800 μCi/mmol), 1 mM [³H]mannitol (330 μCi/mmol), and the different modulators at the same concentrations as for the preceding incubation period. The vials were stoppered and incubated at 37 °C in a shaking incubator for 90 min. Following incubation, muscles were digested in Protosol tissue solubilizer, and radioactivity of muscle digests and aliquots of the incubation media was counted. Extracellular space was calculated, and hence the distribution ratio [DMO]_i/[DMO]_o was obtained. Intracellular pH (pH_i) was calculated from the following equation (27).

$$\text{pH}_i = \text{pK}_a + \log \frac{[\text{DMO}]_i}{[\text{DMO}]_o} \times (1 + 10^{\text{pH}_o - \text{pK}_a}) - 1$$

pK_a was taken to be 6.13 (27).

Preparation of Insulin Receptors—Soleus muscles were incubated for 3 h in 3 ml of Krebs-Henseleit buffer, pH 7.4, containing 5 mM glucose, 0.10% bovine serum albumin, and 20 mM Hepes. For the last 90 min, muscles were incubated in the absence or presence of 8 mM orthovanadate. At the end of the incubation period, muscles were frozen in liquid nitrogen. Pools from 10–15 muscles (approximately 250–300 mg of tissue) were homogenized and solubilized in 1% Triton X-100 as described (28) in the presence of inhibitors of proteases and phosphatases. The solubilized homogenate was centrifuged at 150,000 × *g* for 90 min at 4 °C. The 150,000 × *g* supernatant (1.8 ml) was recycled for 30 min (approximately five to seven times) through a column containing 0.2 ml of WGA bound to agarose at 4 °C. The resin was washed with buffer (20 ml) containing 25 mM Hepes, 0.1% Triton X-100, pH 7.4. Receptors were eluted from the WGA column with 2 ml of buffer containing 25 mM Hepes, 0.1% Triton X-100 and 0.3 M *N*-acetyl-D-glucosamine, pH 7.4. Insulin receptors were eluted in the first 400 μl of elution buffer. Insulin binding was measured by incubating 20 μl of WGA eluate in 30 mM Hepes buffer containing 0.1% bovine serum albumin, 100 units/ml bacitracin (pH 7.6, 1 h, 22 °C, 200 μl) and 20,000 cpm [¹²⁵I-Tyr¹⁴]moniodoinsulin (≈60 pM) (40). Nonspecific binding was estimated as [¹²⁵I]-insulin bound in the presence of 1 μM insulin (5–10% of total binding). Binding data were expressed per microgram of protein with the latter measured using the method of Bradford (29).

Assay of Tyrosine Receptor Kinase Activity—Phosphorylation of an exogenous substrate was carried out with receptor preparations which were preincubated for 1 h in 30 mM Hepes buffer, pH 7.6, containing 50 mM Mg acetate, 4 mM MnCl₂, and varying concentrations of insulin. The receptor kinase activity was initially activated by the addition of 50 μM [^γ-³²P]ATP (5–10 μCi) for 10 min. The reaction was initiated by the addition of the exogenous substrate (copolymer of Glu/Tyr, 4:1, 0.25 mg/ml). The reaction was stopped after 30 min by applying samples to filter paper squares (Whatmann 3 MM), which were immediately washed in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate. Papers were washed, dried, and counted as described (30).

RESULTS

Vanadate Stimulates System A and Glucose Metabolism in Skeletal Muscle—The effect of sodium orthovanadate on sys-

¹The abbreviations used are: EIPA, ethylisopropylamiloride; DMO, 5,5'-dimethylloxazolidine-2,4-dione; WGA, wheat germ agglutinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MeAIB, 2-(methylamino)isobutyric acid.

tem A amino acid transport activity was assessed in the incubated soleus muscle preparation. We traced, in parallel, a previously reported effect of vanadate, *i.e.* its stimulatory action on muscle lactate production (18). Vanadate (8 mM) caused a near 2-fold increase in the rate of lactate production in soleus muscle, which was already maximal at 30 min after vanadate addition (Fig. 1A). Vanadate also stimulated system A transport activity as determined by the uptake of MeAIB (80% increase) (Fig. 1A). This effect of vanadate was time-dependent so the time taken for 50% effectiveness of vanadate action was approximately 30 min, and maximal effect of vanadate was found at 1 h (Fig. 1A).

These effects of vanadate were dependent upon its concentration in the incubation medium (Fig. 1B). Thus, the half-maximal effect of vanadate on muscle lactate production was already obtained at approximately 0.5 mM (Fig. 1B). In contrast, the dose-response curve of vanadate on MeAIB uptake was clearly shifted to the right, and two components were apparent. Thus, low vanadate concentrations (up to 1–2 mM) caused a 25% stimulation of MeAIB uptake (Fig. 1B). At higher vanadate concentrations, the stimulation exerted on MeAIB uptake was characterized by a markedly gentler slope, and a plateau phase was not reached even at 12 mM vanadate (Fig. 1B).

Kinetic analysis of the stimulatory effect of vanadate on MeAIB uptake (Fig. 2) indicated that it was characterized by an increased V_{\max} (291.7 ± 24.1 and 511.8 ± 38.2 nmol of MeAIB/g per 30 min in the absence and in the presence of vanadate, respectively), without modifications of K_m for MeAIB (0.85 ± 0.14 and 0.92 ± 0.09 mM in the absence and

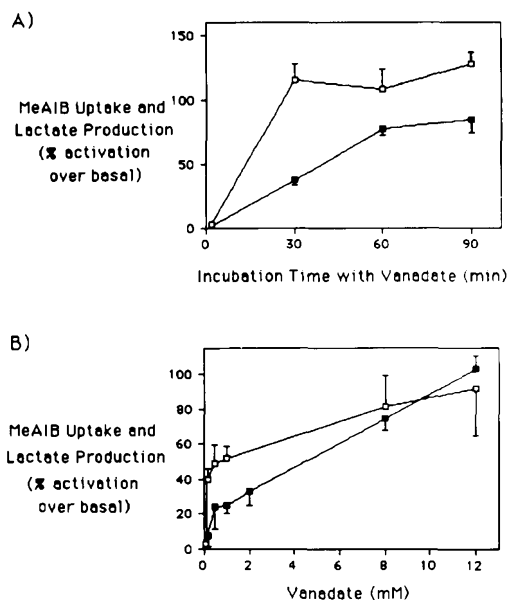


FIG. 1. Time course and concentration dependence of vanadate-induced activation of MeAIB uptake and lactate production by soleus muscle. Results are means \pm S.E. for five to 11 observations per group. MeAIB uptake (\blacksquare) and lactate production (\square) were determined during the last 30 min of incubation. Basal MeAIB uptake and lactate production were 35.8 ± 1.7 nmol/g per 30 min and 10.9 ± 0.7 μ mol/g per h, respectively. A, soleus muscles were incubated for 180 min in the absence or in the presence of 8 mM vanadate added during the last 30, 60, or 90 min of the incubation time. When vanadate was present during the last 30 min, refers to vanadate added only during the MeAIB uptake period, whereas vanadate for 60 or 90 min refers to vanadate added 30 or 60 min before the MeAIB uptake period. B, soleus muscles were incubated for 180 min with different concentrations of vanadate (ranging 0–12 mM) added during the last 90 min of the incubation period.

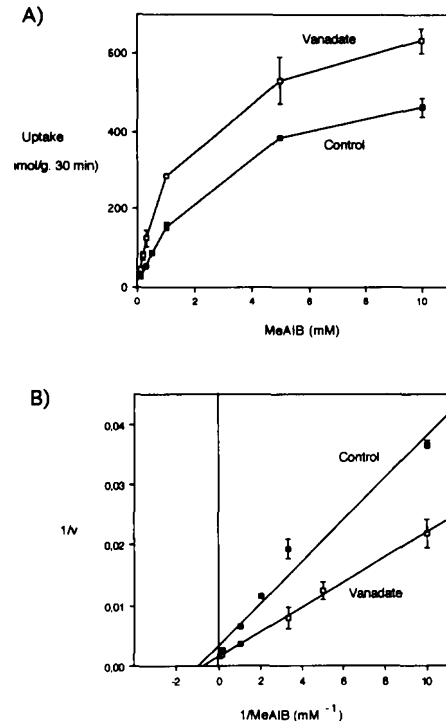


FIG. 2. Effect of vanadate on the kinetic analysis of MeAIB uptake by soleus muscle. Muscles were incubated as described under "Experimental Procedures" for 180 min, in the absence or presence of 8 mM vanadate for the last 90 min of the incubation period. Results are mean \pm S.E. of four observations per group. Uptake was measured at different concentrations of MeAIB (mM) for 30 min (A). A Lineweaver-Burk plot is presented in B. Statistical analysis of double-inverse representation demonstrated that linear regression was significantly different in control ($r = 0.969$; $y = 0.00350 + 0.00347x$) as compared with vanadate-treated group ($r = 0.998$; $y = 0.00148 + 0.00206x$) at $p < 0.05$.

in the presence of vanadate, respectively).

In order to provide a first insight into the mechanisms by which vanadate stimulates skeletal muscle activity, we investigated the interaction of insulin and vanadate on MeAIB uptake, lactate production, and glucose transport. Incubation in the presence of vanadate (8 mM, 90 min) or with insulin (100 nM, 60 min) stimulated MeAIB uptake by soleus muscle to the same extent (Fig. 3A). However, the combined effects of insulin and vanadate did not cause any additional significant stimulatory effect on MeAIB uptake (Fig. 3A). The interaction between insulin and vanadate on 3-O-methylglucose uptake followed the same pattern as MeAIB uptake. Thus, either agent acting separately stimulated uptake to approximately the same extent, but no additive effects were detected (Fig. 3B). On the contrary, insulin and vanadate stimulated lactate production in such a way that their effects were fully additive (Fig. 3C).

The complex interaction of insulin and vanadate on biological effects in skeletal muscle suggested that at least some effects of vanadate might be a consequence of the triggering of events other than those involved in insulin action. Therefore, we attempted to investigate whether it was possible to differentiate the effects of vanadate and insulin on MeAIB uptake. To that end, we investigated the effect of gramicidin D, an ionophore known to abolish membrane potential (31). In keeping with its effects on membrane potential, exposure for only the last 30 min of incubation to gramicidin D caused a 29–33% reduction in basal and insulin-stimulated MeAIB uptake by muscle (Table I), in agreement with previous observations performed in the incubated rat extensor digitorum

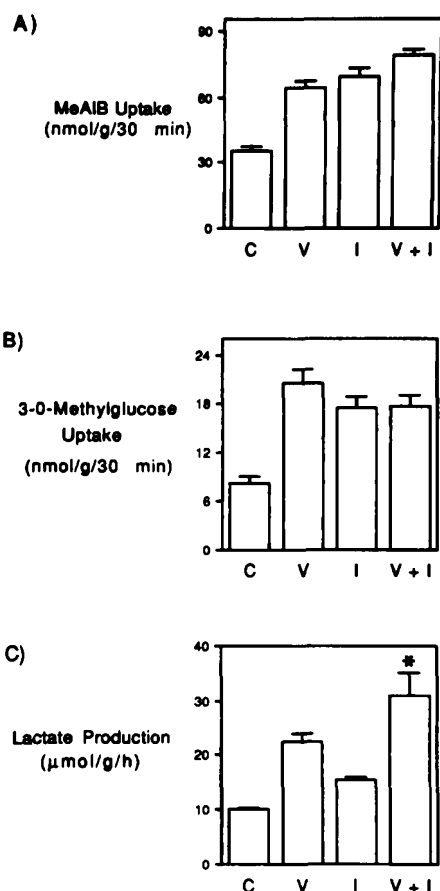


FIG. 3. Interaction of vanadate and insulin on MeAIB and 3-O-methylglucose uptake and lactate production in soleus muscle. Results are means \pm S.E. for 5 to 12 observations per group. Soleus muscles were incubated for 180 min in the absence or presence of insulin (100 nM, last 60 min of incubation period) or vanadate (8 mM, last 90 min of incubation period). MeAIB uptake and lactate production were determined during the last 30 min of incubation. Under all conditions, insulin and vanadate significantly stimulated MeAIB uptake, 3-O-methylglucose uptake, and lactate production at $p < 0.05$. *, a significant difference between vanadate + insulin (V + I) group compared to the vanadate (V) group or insulin (I) group ($p < 0.05$) (unpaired t test).

TABLE I

Effect of gramicidin D on vanadate- and insulin-stimulated MeAIB uptake by soleus muscle

Results are means \pm S.E. for four to six observations per group. Soleus muscles were incubated for 180 min. When indicated, insulin (100 nM) was present for the last 60 min of incubation, vanadate (8 mM) for the last 90 min of incubation, and gramicidin D (25 μ g/ml) was added for the last 30 min of the experiment. Gramicidin D was dissolved in 60% ethanol, so the final ethanol concentration in the incubation medium was 1%; therefore, the control groups also contained 1% ethanol in the medium.

	Basal	Vanadate	Basal	Insulin
	nmol MeAIB \cdot g $^{-1}$ \cdot 30 min $^{-1}$			
Control	30.6 \pm 3.0	60.2 \pm 3.5 ^a	36.6 \pm 2.6	65.9 \pm 6.6 ^a
Gramicidin D	21.8 \pm 2.0 ^b	28.3 \pm 2.2 ^{a,b}	22.9 \pm 1.5 ^b	43.9 \pm 5.3 ^{a,b}

^a Value significantly different from that of the basal (no insulin or vanadate) group ($p < 0.05$) (paired t test).

^b Indicates a significant difference between control and gramicidin D-treated groups, at $p < 0.05$ (unpaired t test).

longus muscle (14). However, gramicidin D caused a 53% reduction in the vanadate-stimulated MeAIB uptake (Table I). Thus, the vanadate effect is more susceptible to inhibition

by gramicidin D than the insulin-stimulated or basal transport activity of system A.

Effect of Vanadate on Muscle Insulin Receptor—To provide information on the mechanisms by which vanadate stimulates skeletal muscle metabolism, we investigated whether exposure to vanadate caused stimulation of the tyrosine kinase activity of insulin receptors. Prior reports on this issue are controversial, and whereas some authors indicated a vanadate-induced activation of the receptor kinase activity (32–34), others did not find any stimulation (22, 35). To this end, insulin receptors from control and 90-min vanadate-treated soleus muscles were partially purified by WGA affinity chromatography. No difference in the yield of glycoproteins was detected between the two groups (0.40 ± 0.02 and 0.39 ± 0.16 μ g/mg of muscle in control and vanadate-treated muscles, respectively, obtained in four separate experiments). However, treatment with vanadate led to a decrease in insulin binding compared to control receptors (the means \pm S.E. of three separate experiments were 0.15 ± 0.002 and 0.11 ± 0.005 fmol/ μ g of protein eluted from the column in control and vanadate-treated muscles, respectively, being the differences statistically significant at $p < 0.05$).

The kinase activity of the insulin receptor in control and vanadate-treated soleus muscle was characterized by using an exogenous substrate. The dose/response relationship between insulin and 32 P incorporation into a copolymer of Glu/Tyr, in the presence of purified insulin receptor, is presented in Fig. 4. Insulin stimulated the exogenous kinase activity of the insulin receptor from control muscle as previously shown (28, 30). Thus, 1 nM insulin caused an approximately half-maximal stimulation of the rate of exogenous substrate phosphorylation, and at 10 nM insulin stimulation was almost maximal (Fig. 4). Supramaximal insulin caused a 2-fold increase in exogenous kinase activity from control insulin receptors. Insulin receptors partially purified from vanadate-treated muscles exhibited a similar ability to phosphorylate

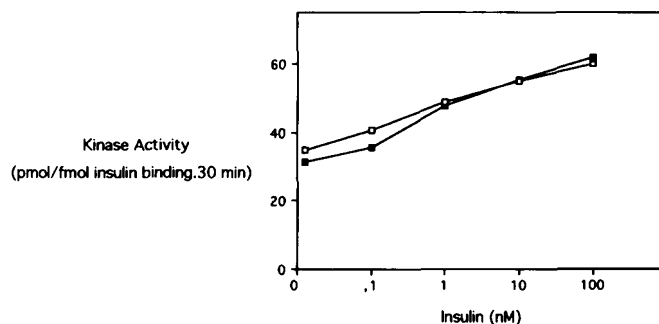


FIG. 4. Effect of vanadate on exogenous kinase activity of insulin receptors from muscle. Insulin receptors from soleus muscles treated (□) or not (■) with 8 mM vanadate for 90 min were partially purified as described under "Experimental Procedures." Each preparation was obtained by pooling muscles from 10–12 rats. WGA eluates (10 μ l) were incubated at 22 $^{\circ}$ C for 1 h in 30 mM Hepes buffer, pH 7.6, containing 50 mM magnesium acetate, 4 mM manganese chloride, and various concentrations of insulin. [γ - 32 P]ATP (50 μ M) was added and samples were incubated for an additional 10 min. The substrate (copolymer of Glu/Tyr, 4:1; 0.25 mg/ml) was then added and allowed to react for 30 min. The reaction was stopped by applying samples to filter paper squares and soaking in 10% trichloroacetic acid/10 mM sodium pyrophosphate. Papers were washed, dried, and counted by Cerenkov radiation. All values have been corrected by nonspecific association of 32 P with the paper, which was estimated by incubating samples in the absence of receptor addition. Each data point is the mean of triplicate estimations, and the results shown are representative of four different experiments with independent receptor preparations. Standard errors ranged between 3 and 7% of mean values.

the exogenous substrate in the absence as well as in the presence of insulin as compared with the control group, when data were expressed per fmol of insulin binding (Fig. 4).

Effect of Modification of Muscle Intracellular pH on System A Transport Activity and Lactate Production—Previous observations performed on isolated cells or plasma membrane vesicles have substantiated that system A transport activity is pH-sensitive, so a rise from pH 7.0 to 7.6 leads to an increase in its activity (1–4). In this regard, this effect is somewhat analogous to that displayed by phosphofructokinase 1, an enzyme which plays a regulatory role in glycolysis, and which can be activated by an increase in pH (36). Furthermore, we have recently reported the presence of critical histidine residues in the hepatic A carrier that may be responsible for its pH dependence (5). In an initial set of experiments, we investigated the effect of increasing the pH of the extracellular medium on MeAIB uptake by soleus muscle. Previous observations, in the incubated mouse soleus muscle, indicate that intracellular pH increases approximately 0.38 for a 1-unit increase in extracellular pH (37). The increase in the pH of the extracellular medium from 7.4 to 8.6 caused a 76% increase in MeAIB uptake by muscle (Table IIA). Further increase to 8.9 in the pH of the extracellular medium caused an additional stimulation of muscle MeAIB uptake (Table IIA). Similarly, a substantial stimulation in the rate of muscle lactate production was detected in response to an increase in the extracellular pH (Table IIB). The effect of vanadate and extracellular pH on MeAIB uptake or lactate production were never additive (Table II).

To explore whether modifications of pH restricted to the intracellular compartment might be sufficient to alter system A transport, we investigated the effect of EIPA, a specific blocker of the sodium/proton exchange (38), on intracellular pH, lactate production, and MeAIB uptake by the incubated soleus muscle. To determine intracellular pH, we used labeled DMO as a probe, which provides good estimations of muscle pH, when extracellular pH is neutral (pH_o of 7.4) (27). In the basal state, intracellular muscle pH ranged between 7.04 and 7.11 (Tables III and IV), in agreement with previous obser-

TABLE III

Effect of EIPA treatment on intracellular pH, MeAIB uptake, and lactate production by soleus muscle

Results are means \pm S.E. for five to six observations per group. Soleus muscles were incubated for 180 min in the absence or presence of EIPA (10 μ M, last 120 min of incubation period). Intracellular pH was determined by using [¹⁴C]DMO as a probe. MeAIB uptake and lactate production were determined during the last 30 min of incubation.

	Intracellular pH	MeAIB uptake nmol MeAIB \cdot g ⁻¹ \cdot 30 min ⁻¹	Lactate production μ mol lactate \cdot g ⁻¹ \cdot h ⁻¹
Control	7.03 \pm 0.04	33.5 \pm 2.4	7.4 \pm 0.2
EIPA	6.89 \pm 0.05 ^a	21.5 \pm 2.5 ^a	5.9 \pm 0.6 ^a

^a Value significantly different from that of the control (no EIPA) group ($p < 0.05$) (paired t test).

TABLE IV

Effect of vanadate and insulin on intracellular pH in soleus muscle

Results are means \pm S.E. for five to six observations per group. Soleus muscle was incubated for 180 min in the absence or presence of insulin (100 nM, 60 min) or vanadate (8 mM, 90 min). Intracellular pH was determined by using [¹⁴C]DMO as a probe.

	Intracellular pH
Basal	7.03 \pm 0.03
Insulin	7.04 \pm 0.01
Basal	7.11 \pm 0.02
Vanadate	7.22 \pm 0.05 ^a

^a Value significantly different from that of the basal group ($p < 0.05$) (paired t test).

variations using DMO equilibration, ¹H NMR, or application of enzyme equilibria (39–41). As expected, treatment of soleus muscle with 10 μ M EIPA caused a substantial decrease in intracellular muscle pH (0.14-unit decrease, equivalent to a 38% increase in intracellular [H⁺]) (Table III). Furthermore, EIPA reduced muscle MeAIB uptake by 36% (Table III). This effect of EIPA on MeAIB uptake was not consequence of a direct effect of EIPA on system A transport activity and, in fact, concentrations of EIPA as high as 20 μ M did not alter system A in plasma membrane vesicles isolated from rat liver after incubation for 10 min at room temperature (data not shown). Incubation of rat liver plasma membrane vesicles for 10 min in the presence of 20 μ M EIPA was enough to detect an almost total inhibition of system ASC transport activity (data not shown). EIPA also caused a significant decrease (21%) in lactate production by soleus muscle (Table III). In keeping with the view that changes in the intracellular pH lead to alterations in the activity of system A, we found that gramicidin D (25 μ g/ml, 30 min), an ionophore that markedly reduces system A in muscle (Table I), caused a large decrease in muscle intracellular pH (7.04 \pm 0.01 and 6.93 \pm 0.02 in control and gramicidin D-treated muscles, respectively, being the differences statistically significant at $p < 0.05$). Therefore, at least part of the inhibitory effect of gramicidin D on system A might be due to its action on intracellular pH.

Effect of Vanadate and Insulin on Muscle Intracellular pH—Vanadate has been shown to cause modifications of intracellular pH in A431 cells (42). Based on that, we next searched for a possible effect of vanadate on intracellular muscle pH. In the basal state, intracellular muscle pH ranged between 7.04 and 7.11 (Table IV). Under these conditions, supramaximal concentrations of insulin did not cause any modification of intracellular pH (Table IV). In contrast, treatment for 90 min with 8 mM vanadate caused a marked and significant increase in muscle intracellular pH (0.11-unit increase) (Table IV). Next, we studied whether the effects of vanadate were due to activation of the sodium/proton exchanger. To this end, the activity of the sodium/proton exchange was inhibited

TABLE II

Effect of extracellular pH and vanadate on MeAIB uptake and lactate production by soleus muscle

Results are means \pm S.E. for three to six observations per group with the exception of MeAIB uptake data corresponding to pH 8.9 under control conditions which were performed in duplicate (individual values are shown in parenthesis). Soleus muscles were incubated for 180 min in incubation media at different pH and in the absence or presence of vanadate (8 mM, last 90 min of incubation). MeAIB uptake and lactate production were determined during the last 30 min of incubation.

A. MeAIB uptake		
pH _o	Basal	Vanadate
	nmol MeAIB \cdot g ⁻¹ \cdot 30 min ⁻¹	
7.4	33.3 \pm 2.7	64.1 \pm 4.7 ^b
8.6	58.7 \pm 1.3 ^a	
8.9	86.8 (84.7, 88.8)	62.5 \pm 4.8
B. Lactate production		
pH _o	Basal	Vanadate
	μ mol lactate \cdot g ⁻¹ \cdot h ⁻¹	
7.4	10.1 \pm 0.6	20.8 \pm 1.4 ^b
8.6	15.8 \pm 1.7 ^a	
8.9	23.8 \pm 1.9 ^a	23.7 \pm 1.1

^a Value significantly different from that of the pH 7.4 group ($p < 0.05$) (unpaired t test).

^b Value significantly different from that of the basal (no vanadate) group ($p < 0.05$) (paired t test).

with EIPA. Under basal state, the presence of 10 μ M EIPA for 120 min caused a substantial decrease (0.12-unit decrease) in muscle intracellular pH (Table V). However, EIPA did not block the effect of vanadate on muscle intracellular pH, so whereas in the absence of EIPA, vanadate increased pH by 0.11 units, in the presence of EIPA the vanadate-induced increase in pH was of 0.17 (Table V). In conclusion, vanadate causes alkalinization of muscle pH by a mechanism other than sodium/proton exchange activation. Furthermore, we found that incubation of muscles in the presence of EIPA did not prevent the effect of vanadate on MeAIB uptake (Fig. 5A) and lactate production (Fig. 5B). Under these conditions

and as expected, insulin effects on MeAIB uptake and lactate production by soleus muscle were not blocked in the presence of EIPA (Fig. 5).

DISCUSSION

Our study demonstrates that vanadate stimulates system A transport activity in skeletal muscle. This finding indicates that in skeletal muscle, vanadate action is not limited to glucose metabolism through activation of glucose transport, glycogen synthesis, and glycolysis (18–20), but it also enhances amino acid uptake. The stimulatory effect of vanadate on system A is rapid (time for 50% effectiveness of 30 min), dependent on vanadate concentration and characterized by enhanced V_{max} values, suggesting a stimulatory effect of vanadate on the activity of the A carriers.

Vanadate is known to inhibit the activity of P-type ATPases *in vitro* (43), but not *in vivo* (44), probably due to the fact that the internalized vanadate is rapidly reduced to vanadyl ions (44–46), which does not inhibit the Na^+ - K^+ -ATPase (45). In addition, the actual inhibition of the Na^+ - K^+ -ATPase markedly inhibits α -aminoisobutyric acid uptake by the perfused rat hindquarter (16) and MeAIB uptake by the incubated muscle (14). Therefore, vanadate-induced activation of system A found in muscle is unrelated to inhibition of Na^+ - K^+ -ATPase activity.

Previous observations have reported that system A transport activity shows a clear pH dependence, concluded after exposure of isolated cells or membrane vesicles to increasing extracellular pH (1–4). In this regard, we have demonstrated that a rise in the extracellular pH increases MeAIB uptake by the incubated muscle. Furthermore, we have also observed that EIPA-induced acidification of muscle intracellular pH (0.14-unit decrease or a 28% increase in intracellular $[\text{H}^+]$) inhibits system A transport activity in a substantial manner. We have recently reported the presence of critical histidine residues in the system A carrier that might be responsible for the pH dependence of system A transport activity (5). Based upon all these findings, we suggest that histidine residues present a cytosolic localization in the A carrier.

Vanadate is a potent inhibitor of phosphotyrosine phosphatases (47, 48), and under some conditions it has been reported to stimulate insulin receptor kinase activity (32–34), although this is not a generalized finding (22, 35). In this report, we have failed to detect vanadate-induced modifications of tyrosine receptor kinase. This agrees with the finding that vanadate does not mimic all insulin effects in skeletal muscle: for instance it does not modify rates of muscle protein synthesis or protein degradation (18). It also agrees with observations performed in isolated rat adipocytes indicating that vanadate action is mediated at a postinsulin-receptor level (49). We have found that vanadate raises muscle intracellular pH (0.1-unit increase), and we propose that this effect could be a triggering element for some vanadate actions in skeletal muscle. This is based on the following findings: (a) a decrease in muscle intracellular pH obtained after treatment with EIPA inhibits both lactate production and MeAIB uptake, (b) the alkalinization of the extracellular medium causes an enhancement of lactate production and system A transport activity, (c) there is no additive effects between vanadate and increasing extracellular pH on lactate production or MeAIB uptake, and (d) within the range of intracellular pH from 6.96 to 7.19, there is a correlation between pH and system A activity or lactate production (Fig. 5).

It has previously been reported that vanadate raises intracellular pH in A431 cells by a mechanism dependent on the activity of the Na^+ / H^+ exchanger (42). However, we have

TABLE V

Effect of EIPA on vanadate-induced pH increase in soleus muscle

Results are means \pm S.E. for four observations per group. Soleus muscles were incubated for 180 min in the absence or presence of vanadate (8 mM, 90 min) and EIPA (10 μ M, 120 min). Intracellular pH was determined by using [^{14}C]DMO as a probe.

	Basal	Vanadate
Intracellular pH		
Control	7.08 \pm 0.03	7.19 \pm 0.01 ^a
EIPA	6.96 \pm 0.03 ^b	7.13 \pm 0.03 ^{a,b}

^a Value significantly different from that of the basal group ($p < 0.05$) (paired t test).

^b Value significantly different from that of the EIPA group ($p < 0.05$) (unpaired t test).

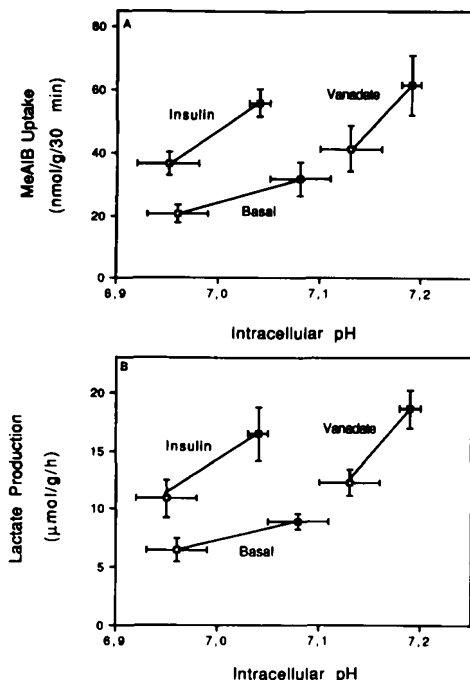


FIG. 5. Relationship between MeAIB uptake or lactate production and intracellular pH in soleus muscle. Points are means \pm S.E. for four to seven observations. Soleus muscles were incubated for 180 min in the absence or presence of insulin (100 nM, last 60 min of incubation period), vanadate (8 mM, last 90 min of incubation period), or EIPA (10 μ M, last 120 min of the incubation period). MeAIB uptake (A) and lactate production (B) were determined during the last 30 min of incubation. \blacksquare , no EIPA; \square , EIPA. Data from basal (with and without EIPA) and from vanadate-treated muscles (with and without EIPA) fitted very well to exponential functions. For MeAIB uptake data, the statistical parameters of the exponential curve was $r = 0.975$ and $y = 2.1920 \cdot 10^{-13} \cdot 10^{(2.0049x)}$. For lactate production data, the statistical parameters of the exponential curve was $r = 0.936$ and $y = 2.0086 \cdot 10^{-13} \cdot 10^{(1.9362x)}$. Lines connecting groups treated with or without EIPA were arbitrarily drawn to differentiate basal, insulin-, and vanadate-treated muscles.

found that EIPA does not block vanadate-induced alkalization in skeletal muscle and, in addition, it does not inhibit the effect of vanadate on MeAIB uptake or lactate production. Therefore, we conclude that whatever the mechanisms triggered by vanadate, the Na^+/H^+ exchange activity is not involved.

Acute exercise and electrical stimulation rapidly stimulate system A transport activity in skeletal muscle (13, 16), and this might occur in the presence of acidification of intracellular pH (40, 41, 50). That indicates (a) that pH does not mediate the exercise-induced activation of system A, and (b) that a variety of mechanisms may regulate the activity of the A carrier under *in vivo* conditions.

The previously reported effects of insulin on muscle intracellular pH are contradictory. Thus, insulin has been reported to cause cytosolic alkalization in frog sartorius muscle (39) and in cultured L_6H_9 muscle cells (51). However, no effect has been substantiated in rat skeletal muscle (52) or in avian skeletal muscle cells in culture (53). In agreement with the latter view, we failed to detect any modification of intracellular pH in rat soleus muscle, under conditions in which effects of vanadate or EIPA on intracellular pH were evident. This finding excludes pH as a regulatory element involved in signal transduction of insulin action in rat skeletal muscle.

The results of our study indicate that the mechanisms by which insulin and vanadate stimulate system A transport activity are different. This is based on two experimental findings: (a) vanadate but not insulin raises muscle intracellular pH, and this alkalization stimulates system A transport activity, and (b) the effect of vanadate is more susceptible to inhibition by gramicidin D than the insulin effect. We have observed that, at least, part of the inhibitory effect of gramicidin D on system A might be due to acidification of muscle intracellular pH. In this regard, the greater sensitivity of vanadate to gramicidin D compared to insulin, might be explained by the curvilinear relationship found between intracellular pH and MeAIB uptake, showing a steeper slope at alkaline pHs (Fig. 5).

In spite of the evidence indicating the varying mechanisms involved in insulin- and vanadate-stimulated system A transport activity, we found no additive effects of vanadate and insulin stimulating system A. If we bear in mind (a) that vanadate stimulates system A, at least to some extent, by changing muscle intracellular pH, and (b) that insulin activates system A transport activity in rat skeletal muscle by a rapid mechanism, independent of protein synthesis or Na^+ -electrochemical gradient and characterized by an increased V_{max} (14), we favor the view that insulin might enhance the intrinsic activity of A carriers in such a way that it cannot be further activated by vanadate, that is, by a rise in muscle intracellular pH.

In summary, we have demonstrated that system A in skeletal muscle can be acutely activated by vanadate treatment and by an increase in muscle intracellular pH. The mechanisms by which vanadate stimulates muscle metabolism are not a consequence of activation of tyrosine receptor kinase activity. However, vanadate causes alkalization of muscle intracellular pH and, in consequence, vanadate effects on lactate production and system A transport activity might be mediated via modification of intracellular pH. In turn, the mechanism by which a rise of intracellular pH leads to activation of system A transport activity might rely on the presence of histidine residues, recently described in A carrier (5), possibly localized in a cytosolic domain.

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