Vanadate Effect on the Na,K-ATPase and the Na-K Pump in In Vitro-Grown Rat Vascular Smooth Muscle Cells

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SUMMARY. The impact of vanadate on the Na,K-ATPase system in the vascular smooth muscle cell is poorly understood. The present study describes the kinetics of the effect of vanadate on Na,K-ATPase and the Na-K pump in in vitro grown rat VSMC's. Vanadate interaction with the Na,K-ATPase system in vascular smooth muscle cells was examined by observing its influence on ouabain-sensitive adenosine triphosphate hydrolysis in disrupted cells rendered permeable by osmotic shock, and the uptake of rubidium by intact cells. The I50 for vanadate inhibition of ouabain-sensitive hydrolysis of adenosine triphosphate occurred at vanadate concentrations of 10^{-6} to 10^{-7} M. This inhibition was potassium dependent. The maximal inhibitory effect of vanadate occurred at potassium concentrations of 10-20 mEq/liter. Sodium exerted a moderate antagonistic influence on vanadate inhibition of ouabain-sensitive adenosine triphosphate hydrolysis. Rubidium uptake by vascular smooth muscle cells was not altered within 120 minutes when 10^{-3} M vanadate was added to the medium containing intact vascular smooth muscle cells. Yet, vanadium concentrations in the vascular smooth muscle cells within this incubation period reached levels 1.48-fold higher than the extracellular vanadate concentrations of 10^{-5} M. These observations indicate that vanadate is a potent inhibitor of the VSMC Na,K-ATPase in disrupted vascular smooth muscle cells. However, in intact vascular smooth muscle cells vanadium gaining access into the vascular smooth muscle cell's interior does not inhibit the Na-K pump, probably because of its binding to intracellular proteins and/or conversion from the vanadate to the vanadyl ion. (Circ Res 53:186-191, 1983)

VANADATE produces vascular contraction and vasoconstriction in laboratory animals (Inciarte et al., 1980; Rapp, 1981; Jackson, 1912). It has been shown that inhibition of vascular Na,K-ATPase could result in vascular contraction (Webb and Bohr, 1978; Webb et al., 1981). Thus, one possible mechanism for vanadate-induced contraction of vascular tissue is inhibition of the vascular smooth muscle cell (VSMC) Na,K-ATPase. However, the concentration of vanadate required for the induction of vascular contraction in vitro is approximately 100 to 1000-fold greater than the concentration known to inhibit Na,K-ATPase in homogenates or subcellular fractions from a variety of tissues (Rapp, 1981). The low activity of the ouabain-sensitive Na,K-ATPase in vascular tissues has made it difficult to accurately and directly measure this enzyme in the VSMC. Furthermore, homogenates and subcellular fractions of vascular tissue contain non-VSMC elements. Therefore, the kinetics of the effect of vanadate on the VSMC have been poorly defined. Tissue culture preparations of VSMC's maintain the experimental conditions that avoid some of the problems inherent in naturally occurring vascular tissue. We have recently developed techniques enabling us to characterize the Na,K-ATPase in in vitro preparations of mammalian VSMC's (Aviv et al., 1983). The lack of definitive knowledge regarding the effect of vanadate on the VSMC Na,K-ATPase, coupled with our new techniques for direct measurements of the activity of this enzyme in the VSMC, prompted us to study the effect of vanadate and related ions on the Na,K-ATPase system in in vitro preparations of VSMC's. Vanadate impact on this system in VSMC's was examined by observing its effect on (1) the rate of ouabain-sensitive hydrolysis of ATP, and (2) the rate of rubidium (Rb) uptake by intact cells. The latter variable represents the activity of the Na-K pump, whereas the former modality is a function of the enzymatic correlate of the Na-K pump, the Na,K-ATPase.

Methods

Sodium metavanadate and sodium orthovanadate were obtained from Fisher Scientific and from Sigma Chemical Company, respectively. Sodium niobate and sodium metatantalate were purchased from Thiokol-Ventron Division. Two types of ATP compounds were used initially: Sigma ATP (catalogue #A-5394) and Boehringer Mannheim ATP (catalogue #519987). All other reagents used in the present experiments were commercially available ACS grade. Radioisotopes used in these experiments in-
The VSMC's originated from the carotid arteries of male adult Sprague-Dawley rats. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco 320-1885) plus 292 µg/ml of l-glutamine with antibiotics (50 µg penicillin/ml, 150 µg streptomycin/ml and 150 µg neomycin/ml), and 17% heat-inactivated fetal calf serum (FCS). The method for growing the cells, their enrichment, and techniques used to assess the purity of the VSMC preparations have been described (Aviv et al., 1983).

The assay of the Na,K-ATPase in the VSMC's was performed as follows. Aliquots consisting of 0.8–1.0 × 10⁶ cells were inoculated into each well of Costar 24-well flasks. DMEM plus 17% FCS without antibiotics was used to grow the cells in the wells. Enzymatic assays were performed 48 hours after the cell inoculation when the cell number in each well was approximately 2.5–3 × 10⁶.

The medium was aspirated from each well and the cell layers were washed twice with 150 mM Tris-HCl (pH 7.4). The VSMC's permeability then was increased by the addition of distilled water to the cells (0.25 ml per each well) and by placing the flasks in dry ice for 30 minutes. Thereafter, the cells were thawed at 37°C for a period of 10 minutes. The substrate solution, and—when appropriate—different concentrations of vanadate, niobate, and tantalate, were then added into each well. The final standard substrate solution (1.25 ml) in each well consisted of the following in mM: NaCl, 100; KCl, 10; MgCl₂, 5; EGTA, 1; imidazole-HCl, 100; ATP, 3 (pH 7.4). To inhibit Na,K-ATPase activity, KCl was omitted and ouabain (1 mM) was added. Variable concentrations of ATP, Na⁺, and K⁺ were used for the enzyme kinetic analyses. Blank wells containing cells treated before incubation with 30% trichloroacetic acid (TCA) were used for measurement of the nonenzymatic hydrolysis of ATP. The reaction in all other wells was terminated after incubation at 37°C for 30 minutes by placing the flasks on ice and by the addition of 250 µl ice-cold 30% TCA. The inorganic phosphate (Pᵢ) generated was measured by the Fiske and Subbarow (1925) method. Protein was determined by the Lowry method (Lowry et al., 1951), and the cell number was counted in a Coulter counter (model ZBII). The activity of the Na,K-ATPase was determined by subtracting the activity of the wells containing ouabain without potassium from wells containing potassium and no ouabain. The specific activity of the ATPase was expressed per unit cellular protein, per unit cell, or per unit cellular water volume.

The uptake of vanadate by the VSMC's was measured in the following manner. Cells in each well were washed twice with 150 mM Tris-HCl. DMEM containing 10⁻⁵ m vanadate with 0.25–0.50 µCi/ml of ⁴¹vanadate was added. The radioisotope was received from Amersham in the form of ⁴¹V/nanodyl chloride. Before its use, the ⁴¹V/nanodyl ion was converted to vanadate by its incubation at 37°C (pH 7.4) in the DMEM at 5% CO₂-air for 3 hours. This is a modification of a method described previously (Cantley et al., 1978b). During the uptake experiments, the cells were incubated for various time intervals and then rapidly washed four times with a solution of 150 mM NaCl with/without 2.5 mM noradrenalin. The cells were subsequently subjected to either of the following treatments: extraction with 5% TCA for 1 hour, or trypsinization with 0.01% trypsin in phosphate buffer plus 10⁻⁴ m EDTA. Aliquots of the various preparations were counted in a γ-counter.

In experiments examining the uptake of vanadium, we also measured the intracellular water volume. This parameter was determined using the intracellular distribution of 3-O-methyl-D-glucose as an indicator of the intracellular water space. This method was described for the measurement of intracellular water volume in tissue cultures of hepatic parenchymal cells (Kletzein et al., 1975) and in vitro grown VSMC's (Brock and Smith, 1982). Briefly, the VSMC's were incubated at 37°C for time intervals of 2–20 minutes in a phosphate buffer (pH 7.4) containing either 2 or 10 mM 3-O-methyl-D-glucose with 1.2 µCi/ml of 3-O-methyl-[¹⁴C]-D-glucose. Equilibrium distribution of 3-O-methyl-D-glucose was reached within 10 minutes of incubation. The cells then were washed four times with ice-cold phosphate buffer containing 1 mM phloretin, and were either trypsinized as described earlier or extracted with 5% TCA. Aliquots of the samples were counted in a liquid scintillator using Aquasol-2 as the scintillation fluid. There were no differences between the activities of the trypsinized samples and samples extracted with TCA.

The uptake of vanadium and its accumulation in the VSMC's were expressed per unit cellular protein, per unit cell, or per unit cellular water volume.

Vanadium in the two ATP preparations was measured by flameless atomic absorption spectrophotometry using a Perkin-Elmer model 503 Atomic Absorption spectrometer equipped with an HGA-2100 heated graphite atomizer and a deuterium-lamp background corrector (Higashino et al., 1983).

Results

ATP preparations from a variety of sources may be contaminated with vanadium. The aforementioned preparations of ATP by Sigma and Boehringer Mannheim are considerably vanadium free (less than 1,000 ppb according to the claims of their manufacturers). However, concentrations of vanadium at levels of less than 1,000 ppb of the ATP may still exert an inhibitory effect on Na,K-ATPase. Our measurements of vanadium in the Boehringer Mannheim ATP and Sigma ATP yielded results of 55 ppb and 75 ppb, respectively. Double reciprocal analyses of the effect of ATP on the specific activity of Na,K-ATPase (not shown) indicated that the Vₚₕ and apparent Kₘ obtained using the two preparations of ATP were essentially identical. In the re-
maining experiments, the Sigma ATP was used to assess the effect of vanadate and related compounds on the VSMC Na,K-ATPase.

The specific activity of the VSMC Na,K-ATPase was 2.25 ± 0.085 (mean ± SEM) μmol P_i per mg cell protein per hour or 0.67 ± 0.027 μmol P_i per 10^6 cells per hour. The specific activity of the Mg-ATPase (ouabain insensitive) in the VSMC's was 3.48 ± 0.137 μmol P_i per mg cell protein per hour or 0.99 ± 0.043 μmol P_i per 10^6 cells per hour. These data are derived from various experiments in which the V_max for the Na,K-ATPase was measured.

Figure 1 demonstrates the dose-response curve of the effects of orthovanadate and metavanadate on the V_max of Na,K-ATPase of the VSMC's in the presence of the standard substrate solution containing 100 mM of NaCl and 10 mM KCl. Vanadate in either form exerted a potent inhibitory effect on the enzyme. There were no distinguishable differences in the inhibitory pattern between the two vanadate species. Inhibition was essentially complete at a concentration of 10^-4 M vanadate. The apparent I_50 for vanadate inhibition of the enzyme occurred at a concentration of 10^-2 to 10^-6 M. In further studies, we used vanadate only in the ortho form because it is more readily soluble than metavanadate.

Previous studies have indicated that potassium may potentiate the inhibitory effect of vanadate on Na,K-ATPase activity. We examined this effect by altering the potassium concentrations in the substrate solutions. Figure 2 depicts the dose-response curve for vanadate inhibition at a range of potassium concentrations between 1.75-20 mEq/liter. It is apparent from this figure that an increase of potassium concentration in the substrate solution was associated with augmented inhibition of Na,K-ATPase by vanadate. Na,K-ATPase in the VSMC was more amenable to inhibition by vanadate when the sodium concentrations in the substrate solutions were low. As shown in Figure 3, sodium exerted a substantial antagonistic effect to vanadate inhibition of the enzyme.

Niobium, tantalum, and vanadium belong to the same chemical family, group VA of the periodic table. We therefore examined the effect of niobate (sodium niobate) and tantalate (sodium metatantalate) on the Na,K-ATPase in the VSMC's. Both ions at a concentration of 10^-7 to 10^-3 M demonstrated no effect on the activity of the enzyme in the VSMC's rendered permeable by osmotic shock.

Direct measurements of Na,K-ATPase in the VSMC preparations require increasing the permeability of the cells to the reactants (primarily ATP) used in the substrate solutions. We increased the permeability of the VSMC's by subjecting them to osmotic shock and rapid freezing and thawing. This procedure also entails disruption of cellular metabolism and making accessible to the cellular interior the vanadate in the vast extracellular pool. In order to examine the effect of vanadate on the intact VSMC's, we resorted to measurements of Rb uptake by the VSMC's in the presence or absence of 10^-5 M vanadate in the medium. At this concentration in the substrate solutions used to assay the ouabain-sensitive ATP hydrolysis, vanadate almost completely inhibited Na,K-ATPase. In contrast, within the incubation interval of 120 minutes at concentra-

![Figure 1. Dose-response curves depicting the effect of vanadate in two different forms, meta (○) and ortho (○○) on the activity of Na,K-ATPase in the VSMC's. Standard substrate solutions containing 100 mEq Na, 3 mM ATP, and 10 mEq K were used. Horizontal bars represent SEM. Each circle represents the mean of 5-8 pairs of wells.](http://circres.ahajournals.org/)}
FIGURE 3. The effect of sodium on vanadate inhibition of Na,K-ATPase in the VSMC's. The inhibitory effect of vanadate was examined in substrate solutions containing various concentrations of sodium with/without vanadate. Two concentrations of vanadate were used: (•) $5 \times 10^{-7}$ M and (○) $10^{-7}$ M. Percent of control activity of Na,K-ATPase refers to percent of activity at each Na concentration. Each circle represents the mean of 7-10 pairs of wells.

Expansions of $10^{-5}$ M in the medium of the intact VSMC's, vanadate had no effect on the ouabain sensitive or ouabain insensitive Rb uptake by the VSMC's (Fig. 4).

Experiments on the uptake of vanadate demonstrated that vanadium uptake by the VSMC's was quite substantial (Fig. 5). Within an incubation period of 150 minutes, vanadium concentrations per unit of cellular water volume exceeded the extracellular vanadate concentrations of $1.0 \times 10^{-5}$ M, reaching a level of $1.48 \pm 0.05 \times 10^{-5}$ M. VSMC's washed with solutions of NaCl plus 2.5 mM noradrenalin showed the same accumulation of vanadium as cells washed with NaCl without noradrenalin. As previously described for vanadium transport by erythrocytes (Cantley et al., 1978b), these results suggest that vanadium gained access to the cellular interior and was probably not bound to the plasma side of the cell membrane.

**Discussion**

Vanadate increases vascular contraction in in vitro preparations of the rat aorta (Rapp, 1981). Acute intravenous infusion of vanadate to the dog induces vasoconstriction and elevation of the systemic blood pressure (Inciarte et al., 1980). Chronic oral administration of vanadate to the rat produces elevated blood pressure (Steffen et al., 1981). These effects could be mediated via inhibition of the VSMC Na,K-ATPase. However, recent observations suggest that the vasoconstrictor effect of vanadate on the vascular smooth muscle may be independent of its inhibitory effect on the Na,K-ATPase. Ouabain, another potent inhibitor of the Na,K-ATPase, produces inhibition of Rb uptake in blood vessels. In red blood cells, vanadate inhibition of the Na,K-ATPase is also associated with a reduction of Rb uptake (Cantley et al., 1978b). On the other hand, vanadate increases tension in the isolated dog saphenous vein at a medium concentration of $10^{-6}$ M, yet it fails to reduce Rb uptake (Huot et al., 1979). Rapp (1981) recently observed that, in vascular rings of rat aortas, vanadate concentration in the medium required to induce vascular contraction was much higher than vanadate levels known to inhibit the Na,K-ATPase in homogenates and subcellular fractions obtained from other tissues. Furthermore, this investigator demonstrated that potassium-induced relaxation of the rat aortic vascular smooth muscle was not affected by high concentrations of vanadate. The potassium-induced relaxation of the vascular smooth muscle has been considered to be mediated via the enzyme Na,K-ATPase. Since vanadate exerts its inhibitory action on Na,K-ATPase from the cytoplasmic side (Cantley et al., 1978a, 1978b), the difference in response of the vascular smooth muscle in comparison to the erythrocyte may relate to different degrees of accessibility of vanadate to its site of action in the two cell types, differences in intracellular binding of vanadion, or differences in the capacity of the two cell types to convert vanadate (+5) to vanadyl (+4) ion. Vanadion in the former oxidation state exerts a potent inhibitory effect on Na,K-ATPase, whereas the vanadyl ion has only a minimal influence on the enzyme (Cantley and Aisen, 1979; Grantham and Glynn, 1979). Another possibility is that, in comparison with other tissues, Na,K-ATPase in the VSMC is less sensitive to vanadate. It has been shown, for instance, that the canine kidney Na,K-ATPase is more sensitive to inhibition by vanadate than the human erythrocyte Na,K-ATPase (Bond and Hudgins, 1981). It is possible, therefore, that any or a combination of these possibilities may modify the inhibitory effect of vanadate on vascular tissue Na,K-ATPase.

Our preparations of in vitro grown VSMC's rendered permeable by osmotic shock made it possible to examine the direct effect of vanadate on the VSMC Na,K-ATPase. The results clearly show that the Na,K-ATPase in the VSMC grown in vitro is quite sensitive to vanadate and that the $I_50$ for vanadate inhibition of the enzyme in the VSMC is similar to that reported in homogenates and subcellular fractions obtained from other tissues (Grantham and Glynn, 1979; Nechay and Saunders, 1979). In contrast, vanadate at a concentration of $10^{-5}$ M in the extracellular fluid of the intact VSMC's had no effect on the Na-K pump as demonstrated by the Rb uptake experiments.

The potassium and sodium kinetics of the Na,K-ATPase inhibition by vanadate have been described in different tissues (Grantham and Glynn, 1979; Bond and Hudgins, 1979, 1981, 1982). It has been generally known that potassium potentiates vanadate inhibition of the Na,K-ATPase. In our studies, we also noted potentiation of vanadate inhibition of the VSMC Na,K-ATPase as the potassium concentration in the substrate solutions was increased from 1.75-20.0 mEq/liter. Thus, the interaction between potassium and vanadate plays an important role in vanadate inhibition of Na,K-ATPase in vitro. However, in whole animal studies, we could not demonstrate any effect of potassium on vanadium action on Na,K-ATPase in vivo (Higashino et al., 1983).

In the present experiments, increasing the sodium concentration in the substrate solution partially counteracted vanadate inhibition of VSMC Na,K-ATPase. This phenomenon has been demonstrated by others using Na,K-ATPase in kidney and erythrocyte preparations (Bond and Hudgins, 1982).

Our Rb uptake experiments demonstrate that vanadate at high concentrations in the extracellular fluid has no effect on the VSMC Na-K pump. Studying aortic segments derived from the rat, Rapp (1981) has recently speculated that although it is a potent inhibitor of the enzyme in isolated membrane preparations, vanadate does not inhibit Na,K-ATPase in intact vascular smooth muscle cells. Our findings in vitro grown VSMC's support this contention and observations made by others in regard to Rb uptake in intact blood vessels (Hout et al., 1979). Thus, it is logical to conclude that vascular contraction induced by acute administration of vanadate is likely to be exerted via a mechanism other than inhibition of the Na,K-ATPase.

It should be noted that, in our experiments, vanadium concentrations in the intact VSMC's after 120 minutes of incubation reached levels higher than concentrations in the medium containing $10^{-6}$ M vanadate. Despite such high intracellular concentrations, no demonstrable inhibition of Rb uptake by the VSMC's was noticed. These results are compatible with conclusions arrived at by us (Higashino et al., 1983) and others, that once gaining access into the intracellular compartment, vanadium may be either bound to cellular proteins and/or converted from the vanadate form to the vanadyl form. Both of these possibilities may also favor the uptake of vanadate from the extracellular fluid into the cellular interior against a concentration gradient for vanadium, a phenomenon demonstrated in our experiments.

Finally, despite belonging to the same chemical
family as vanadate, tantalate and niobate did not exert any inhibitory effect on the Na,K-ATPase in the disrupted VSMC's. This finding underscores the uniqueness of vanadate as an inhibitor of Na,K-ATPase. It is possible that steric factors are involved in preventing the larger niobate and tantalate anions from binding to the Na,K-ATPase at the critical binding site(s).

In summary, our experiments have indicated that vanadate exerts a potent inhibition of Na,K-ATPase in disrupted VSMC's and that the $I_0$ for vanadate inhibition of the enzyme in the VSMC is similar to that of Na,K-ATPase in homogenates or subcellular fractions of other tissues. The potassium dependency and sodium antagonism of this inhibition have also been demonstrated. However, despite high concentrations of vanadium in the intracellular compartment, Rb uptake was not altered in intact VSMC's exposed to high concentrations of vanadate in the extracellular fluid. It is concluded that the reported discrepancy between the results of Rb fluxes and the contractile response to vanadate in vascular tissue is neither related to a lesser sensitivity of the VSMC Na,K-ATPase to vanadate inhibition nor is it a function of poor access of vanadate to its cellular sites of action. The most likely explanation for the aforementioned discrepancy is either binding of vanadium to intracellular proteins and/or the conversion of vanadate to the vanadyl ion in intact VSMC's. This conclusion is based on the tacit assumption that the Na,K-ATPase in the VSMC grown in vitro retains the same biological characteristics as its in vivo counterpart.

We thank Patricia Asconi, Laura Sarokin, and Diana Aviv for their technical help, and Nancy Deegan for her expert secretarial skills. This study was supported in part by Grant IR 23-HL-29221-01 from the National Heart, Lung, and Blood Institute. Address for reprints: Abraham Aviv, M.D., Division of Pediatric Nephrology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School 100 Bergen Street Newark, New Jersey 07103.

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Received August 19, 1982; accepted for publication June 15, 1983.

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INDEX TERMS: Rubidium uptake • Sodium • Potassium • Vanadum uptake


*Circ Res.* 1983;53:186-191
doi: 10.1161/01.RES.53.2.186

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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