Cadmium Effect on the Na,K-ATPase System in Cultured Vascular Smooth Muscle Cells

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SUMMARY The present study focuses on the interaction between cadmium (Cd) and the Na, K-ATPase system in in vitro grown vascular smooth muscle cells (VSMCs) derived from the rat carotid artery. In disrupted VSMCs rendered permeable by osmotic shock, Cd inhibited Na, K-ATPase; $I_{50}$ was reached at $10^{-5}$ M Cd. Mg-ATPase was also inhibited by Cd; $I_{50}$ was attained at concentrations of $10^{-4}$ M Cd. Cd inhibition of Na,K-ATPase in the VSMCs was noncompetitive with respect to Na, K, and ATP. Rubidium transport experiments performed with intact VSMCs demonstrated that within an incubation period of 150 minutes, a concentration of $10^{-4}$ M Cd in the extracellular fluid exerted no acute effect on the Na-K pump. Within this time interval, intracellular Cd attained a concentration eightfold higher than the extracellular Cd concentration. Thus, it appears that under acute conditions Cd exerts its inhibitory effect on Na,K-ATPase only in disrupted VSMCs. The data further suggest that, in the VSMC, conditions under which Cd inhibits Na, K-ATPase are consistent with inhibition from the cytoplasmic side of the cell membrane. (Hypertension 6: 20-26, 1984)

KEY WORDS • heavy metals • Mg-ATPase • rubidium uptake • Na-K pump

N A, K-ATPase (the enzymatic correlate of the electrogenic Na-K pump) is an essential factor for the regulation of vascular tone. Cadmium (Cd) has been reported to inhibit Na, K-ATPase in homogenates and subcellular fractions in a variety of organs and tissues originating from different animal species. Several studies have shown that hypertension can be induced by chronic ingestion of Cd or by acute systemic administration of this element. However, a number of other investigators have not been able to induce hypertension in laboratory animals exposed to Cd. It is possible that, if it occurs, hypertension induced by Cd results from inhibition of the vascular smooth muscle cell (VSMC) Na,K-ATPase. Thus, knowledge pertaining to the interaction between Cd and the VSMC Na, K-ATPase could be of substantial importance.

In the present study we have investigated the impact of Cd on the Na, K-ATPase system in in vitro grown VSMCs by examining its uptake by VSMCs and by observing its effect on the following cellular modalities: 1) the specific activity and kinetics of Na, K-ATPase as assayed by the ouabain sensitive hydrolysis of ATP; and 2) the operation of the Na-K pump as measured by the ouabain-sensitive rubidium (60Rb) uptake by VSMCs. The data reported herein demonstrate that Cd exerts a substantial effect on the VSMC Na, K-ATPase only in disrupted VSMCs but has no demonstrable acute effect on the Na-K pump in intact VSMCs.

Methods

The VSMCs in these experiments originated from carotid arteries of male adult Sprague-Dawley rats. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco 320-1885) plus 292 µg/ml of L-glutamine, antibiotics (50 µg penicillin, 150 µg streptomycin, and 150 µg neomycin per ml) and 17% heat-inactivated fetal calf serum (FCS). The techniques for growing the cells and their enrichment have already been described.
The acute effect of the Cd on Na,K-ATPase in the VSMCs was examined as follows. Aliquots consisting of 0.8 to 1.0 × 10^6 cells were inoculated into each well of Costar 24-well plates. DMEM plus 17% FCS without antibiotics was used to grow the cells in each well. The effect of Cd on Na, K-ATPase activity was measured 48 hours after inoculation of the cells when the number in each well was approximately 2.5 × 10^6. The techniques for in situ assays of VSMC Na, K-ATPase have been described. Briefly, the medium was aspirated from each well and the cell layers washed twice with 150 mM Tris-HCl (pH 7.4). The VSMC’s permeability to the reactants (used in the substrate solutions for the enzymatic assays) was then increased by adding distilled water to the wells and placing the plates in dry ice for 30 minutes. Thereafter, the cells were thawed at 37°C.

The substrate solution used to measure Na, K-ATPase consisted of the following, in mM: NaCl 100, KCl 10, MgCl_2 5, imidazole-HCl 100, ATP 3 (pH 7.4). Ouabain (10^{-5} M) was added and KCl omitted to inhibit Na, K-ATPase activity. Variable concentrations of ATP, Na, and K were used for the enzyme kinetic analyses. Blank wells containing cells treated prior to incubation with 30% trichloroacetic acid (TCA) were used for measurements of the nonenzymatic hydrolysis of ATP. The reaction in all other wells was terminated after the incubation period at 37°C for 30 minutes by placing the plates on ice and by the addition of 250 μl ice cold 30% TCA.

The inorganic phosphate (P_i) generated was measured using the Fiske and Subbarow method, a protein determination by the Lowry et al. method, and the cell number was counted by a Coulter Counter (Model ZBI). The specific activities of Na, K-ATPase and the ouabain insensitive Mg-ATPase were expressed as μmol P_i generated per mg cell protein or per 10^6 cells per hour. In preliminary experiments, we demonstrated no effect of different concentrations of Cd (in the form of CdCl_2) on the activity of Na, K-ATPase when it was mixed in the substrate solutions used to assay the enzyme. The most likely reason for this finding was the binding of Cd to imidazole, which was used as the buffer system in the substrate solutions. The inhibition by Cd of Na, K-ATPase in the VSMCs could, however, be easily demonstrated when Cd was added during the process of disrupting the cells prior to the introduction of the substrate solutions. Thus, prior to the enzymatic assays, the cells were preincubated in the presence or absence of Cd for 20 minutes at 37°C.

The effect of Cd on Rb uptake by intact VSMCs was measured as follows. The medium containing FCS was aspirated and fresh DMEM (without FCS) containing approximately 1 μCi/ml of ^82Rb and 0.1 mM RbCl was added to the wells. When appropriate, CdCl_2, at a concentration of 10^{-4} M was also added. The K and Na concentrations in the DMEM were 7.0 and 140 mEq/liter, respectively. The cells were incubated at 37°C and 5% CO_2-air for various time intervals. The uptake experiments were stopped by aspiration of the medium and rapidly washing the cells four times with ice cold 0.01 M MgCl_2. The cells were then extracted for 1 hour with 5% TCA: aliquots were added to Aquasol-2 (New England Nuclear, Billerica, Massachusetts) and measured in a liquid scintillation counter.

The uptake of Cd by the VSMCs was measured using the following method. DMEM (without FCS) containing 10^{-4} M CdCl_2, with approximately 0.4 μCi ^105Cd/ml was added to the wells. The cells were incubated for various time intervals, at 37°C and 5% CO_2-air, and then rapidly washed four times with a solution of 150 mM NaCl with/without 10^{-4} EDTA. The VSMCs were then subjected to either one of the following treatments: extraction with 5% TCA, or trypsinization (0.01% trypsin in phosphate buffer). Aliquots of these preparations were counted in a gamma counter. In this set of experiments we also examined the intracellular water space using 3-0-methyl-D-glucose, as described by Kletzien et al. for liver parenchymal cells and Brock and Smith for cultured VSMCs. Briefly, the cells were incubated for various time intervals (2 to 20 minutes) in phosphate buffer containing 1-2 μCi/ml of 3-O[D-14C]D-glucose and either 2 or 10 mM of unlabeled 3-O methyl-D-glucose. The equilibrium distribution of 3-O-methyl-D-glucose was reached after 10 minutes of incubation. Following this period, the cells were washed four times with an ice cold phosphate buffer containing 1 mM phloretin. The cells were then either extracted with 5% TCA or trypsinized. Aliquots of the preparation were counted in a liquid scintillation counter using Aquasol-2 as the scintillation fluid. There were no differences between the activities of the trypsinized samples and samples extracted with TCA. The availability of the measurements of the intracellular water space made it possible to express the amount of Cd gaining access to the interior of the VSMCs as concentration per unit of cellular water volume. Data are presented as means ± SEM. Statistical analyses have utilized the Student’s t test.

**Results**

The specific activity of Na, K-ATPase in the VSMCs was 2.37 ± 0.05 μmol P_i/mg protein/hr or 0.82 ± 0.02 μmol P_i/10^6 cells/hr. The specific activity of Mg-ATPase was 2.44 ± 0.06 μmol P_i/mg protein/hr or 0.82 ± 0.02 μmol/10^6 cells/hr. These results are quite similar to those observed in our previous studies. Cd exerted a potent inhibitory effect on the VSMC Na, K-ATPase. Figure 1 depicts the dose response curve for Cd inhibition of the VSMC Na, K-ATPase. Figure 1B shows the dose response curve for Cd inhibition of the VSMC Na, K-ATPase. The I_{50} (50% inhibition) by Cd was reached at concentrations of approximately 10^{-3} M and almost complete inhibition of the enzyme was evident at 10^{-3} M. Mg-ATPase was approximately 10-fold more resistant to inhibition by Cd than the Na, K-ATPase (Figure 1).

Enzyme kinetic experiments (Figure 2) demonstrated that inhibition of the VSMC Na, K-ATPase by Cd at concentrations of 10^{-3} M resulted from an acute reduction in the V_{max} (maximal rate of the enzymatic reac-
tion) rather than as a consequence of alteration in the apparent $K_0$ (criterion for affinity) of the enzyme to $K$, $Na$, or ATP. When measuring the effect of Cd on the ATP kinetics, the specific activity of Na, K-ATPase in the VSMCs was somewhat lower than in the other kinetic experiments. However, the inhibitory effect of Cd on the enzyme was quite apparent. At ATP concentrations of 7 mM, there was a marked decline in the specific activity of the enzyme in the presence or absence of Cd. This was observed previously by us and others.\textsuperscript{18, 23} This decline in the activity of the enzyme at higher ATP concentrations is likely to be due to substrate inhibition.

The capacity of the chelating agent EDTA to prevent or reverse Cd inhibition of the VSMC Na, K-ATPase and Mg-ATPase is shown in Figure 3. EDTA was partially effective in preventing the inhibitory effect of $10^{-5}$ and $10^{-4}$ M Cd when $10^{-4}$ M of the chelator was introduced into the medium concurrently with Cd. However, the addition of $10^{-4}$ M EDTA after 20 minutes of preincubation with $10^{-5}$ M Cd (third, cross-hatched, bar in Figure 3 top) was less effective in reversing the inhibitory effect of the element than when introduced concurrently with Cd. The addition of EDTA at $10^{-4}$ M concentrations after 20 minutes incubation with $10^{-4}$ Cd (sixth, cross-hatched, bar in Figure 3) was ineffective in reversing Cd inhibition of the enzyme. Similar observations were noted in regard to the effect of Cd on Mg-ATPase. EDTA at a concentration of $10^{-4}$ M had no effect on the basal activity of Na, K-ATPase.

![Figure 1](http://hyper.ahajournals.org/)

**Figure 1.** Dose response curve for the effect of cadmium (Cd) on the VSMC Na, K-ATPase (●) and Mg-ATPase (△). The y axis represents the percentage of activity of the enzyme with respect to control. Each point represents seven to eight pairs of wells. Horizontal bars represent SEM.

![Figure 2](http://hyper.ahajournals.org/)

**Figure 2.** Kinetics of Na, K-ATPase under control conditions (●), or in the presence of $10^{-3}$ M Cd (○). A. Potassium kinetics. B. Sodium kinetics. C. ATP kinetics. Each point represents eight to 12 pairs of wells.
Rb uptake experiments (Figure 4) demonstrated no effect of $10^{-4}$ Cd on the rate of ouabain-sensitive Rb uptake within the minimum incubation time of 150 minutes. There was also no effect of Cd on the ouabain-insensitive uptake of Rb (not shown in figure 4). Despite the presence of Cd in the medium, the pump demonstrated a full capacity to increase its activity in response to an increase in the intracellular Na concentration, which was induced by introducing 5.0 µg/ml of monensin. As shown, Rb uptake by the VSMCs was increased to the same extent in the presence or absence of Cd in the medium when the ionophore was added into the medium. After 150 minutes of incubation, the cumulative uptake of Rb by the VSMC was 9.6 ± 0.5 nmol/mg protein. Addition of monensin to the medium resulted in almost doubling the rate of ouabain-sensitive Rb uptake to 18.5 ± 1.4 nmol/mg protein in 150 minutes. Cd did not alter this response.

Cd uptake by the VSMCs is presented in Figure 5. Approximately 50% of the Cd taken up by the cells was chelated by EDTA when the latter was added to the washing solutions. After 150 minutes of incubation, cells washed with 150 mM NaCl and extracted with 5% TCA showed a cumulative uptake of 3.50 ± 0.20 nmol Cd/mg protein or 1.30 ± 0.01 nmol Cd/10^6 cells. Cells washed with 150 mM NaCl + $10^{-4}$ M EDTA and then extracted with TCA demonstrated Cd uptake of 1.70 ± 0.07 nmol/mg protein or 0.66 ± 0.02 nmol Cd/10^6 cells in 150 minutes of incubation. The most likely explanation for this discrepancy is that a substantial percentage of Cd was bound to the plasma side of the cellular membrane and that this bound Cd was chelated by the EDTA. There were no statistically significant differences in cellular Cd values obtained from measurements of Cd by its extraction from the cells with TCA or by measurements of total Cd in the trypsinized cells (Figure 5). These observations suggest that within the experimental time period Cd gaining access into the cell interior was reversibly or loosely bound to cellular elements.
It is well recognized that some of the heavy metals inhibit Na, K-ATPase in homogenates and subcellular fractions derived from a variety of tissues. Brain and kidney exhibit high activity of Na, K-ATPase. Thus, investigators have often used tissues obtained from these organs as model systems to study the interaction between heavy metals and Na, K-ATPase. Homogenates and subcellular fractions of blood vessels are not due only to this enzyme in VSMCs but are rather a function of a myriad of cellular and extracellular components of blood vessels. Tissue culture preparations can provide a useful model to study the effect of various compounds on the VSMC Na, K-ATPase. In vitro preparations of VSMCs are devoid of influences of non-VSMCs and extracellular factors. Therefore, such preparations are ideally suited to study the effects of agents on the VSMC Na, K-ATPase. We recently developed methods to assay Na, K-ATPase in preparations of in vitro grown VSMCs. These techniques made it possible to examine, among other factors, the effect of metal ions on the VSMC Na, K-ATPase system. Our initial investigations have focused primarily on Cd because divalent cadmium has been reported to produce hypertension in laboratory animals. This effect could be mediated via inhibition of VSMC Na, K-ATPase.

The present study demonstrates that VSMC Na, K-ATPase is quite sensitive to Cd inhibition. The \( I_0 \) for Cd inhibition of the enzyme in our experiments was reached at approximately \( 10^{-5} \) M. Other investigators reported \( I_0 \) values of \( 10^{-6} \) M for Cd inhibition of Na, K-ATPase in microsomal preparations of the rat kidney and rat brain synaptosomes. \( I_0 \) values being attained at \( 10^{-4} \) M. Our observation that Mg-ATPase is more resistant to Cd inhibition than Na, K-ATPase concurs with previous findings demonstrating that Mg-ATPase derived from the brain and kidney was 10- to 1000-fold more resistant to Cd inhibition than Na, K-ATPase.

It is very unlikely that the inhibitory effect of Cd on the VSMC Na, K-ATPase is specific to this enzyme system. Heavy metals exert their toxicity at the cellular level by several mechanisms that may result in altering the activities of a variety of enzymes. Perhaps the most likely mode of inhibition of VSMC Na, K-ATPase relates to the high affinity of Cd and other heavy metals for sulfhydryl groups. Na, K-ATPase is rich in sulfhydryl groups, and sulfhydryl reagents inhibit the enzyme. We have shown that other heavy metals such as lead and mercury also inhibit the Na, K-ATPase. Similar observations were reported by others who examined the effect of heavy metals on Na, K-ATPase in a variety of tissues. The inhibitory effect of Cd on the VSMC Na, K-ATPase was probably mediated via toxic damage to the enzyme units, as evidenced by the following observations: 1) the inhibition of the enzyme by Cd was exerted by alterations of the \( V_{\text{max}} \); 2) at \( 10^{-5} \) M Cd, \( 10^{-4} \) M EDTA was only partially capable in reversing the inhibitory effect of the element; and 3) at \( 10^{-4} \) M Cd, \( 10^{-4} \) M EDTA was ineffective in reversing Cd inhibition. In the disrupted VSMCs, reversal of Cd inhibition by EDTA would have been expected had Cd not exerted irreversible toxic effect on the Na, K-ATPase system.

Na, K-ATPase is an enzyme that is primarily associated with the plasma cell membrane. Its inhibition can be exerted from the extracellular (plasma) side of the membrane or from the intracellular (cytoplasmic) aspect of the membrane. Ouabain and vanadate, two inhibitors of the enzyme, exert their effect from the extracellular and intracellular sides of the cell membrane, respectively. Using our methods to assay the enzyme in disrupted VSMCs made it impossible to determine whether Cd inhibition of Na, K-ATPase in the VSMC was exerted from the cytoplasmic or plasma sides of the membrane. To measure the hydrolysis of...
ATP by Na, K-ATPase, we subjected the cells to an osmotic shock prior to the enzymatic assays, causing cellular disruption. This procedure was necessary in order to make ATP and other reactants in the substrate solutions freely accessible to the interior of the cell. The increased VSMC permeability was also likely to alter cellular mechanisms and to render the vast extracellular Cd pool readily accessible to the cell interior. Studying Rb uptake by the intact VSMCs concurrently with the enzymatic assays enhanced our understanding of the mode of Cd interaction with the VSMC Na, K-ATPase.

Since Cd in the extracellular fluid of the intact VSMCs appeared to exert no effect on the Na-K pump, it was essential to measure the uptake of Cd by the intact VSMCs within the given experimental time intervals. It has been shown that EDTA does not cross the skeletal muscle cell membrane. The same is likely to be the case with respect to the VSMC. Thus, our observations that washing the VSMCs with solutions containing EDTA resulted in less Cd presence in these cells suggest that the Cd removed by the EDTA was bound primarily to the exterior of these cells.

The Rb uptake experiments clearly showed that, at 10⁻⁴ M concentrations in the medium, Cd exerted no detectable inhibition of the activity of the Na-K pump. (At this concentration of Cd in the medium, more than 50% of Na, K-ATPase was inhibited when the cells were rendered permeable by osmotic shock.) That the Na-K pump was operating normally in the presence of Cd in the extracellular fluid was demonstrated by the response of the VSMCs to the introduction of monensin to the medium. It has been shown previously that monensin rapidly increases the intracellular sodium and that associated with this increase was an augmented activity of the Na-K pump. Our data demonstrate that Cd did not alter the Na, K-ATPase response to monensin. In other experiments we showed that vanadate was a potent inhibitor of Na, K-ATPase in VSMC preparations rendered permeable by osmotic shock. The I₅₀ for vanadate inhibition was reached at 10⁻⁴ M, and almost complete inhibition was achieved at 10⁻⁵ M concentrations of vanadate. However, in a manner similar to the present experiments on Cd, vanadate at 10⁻⁵ M in the extracellular fluid concentration did not alter Rb transport by the intact VSMCs.

The most likely explanation for the inability of Cd to inhibit the Na-K pump in intact VSMCs, despite its high intracellular concentration, is its binding to other intracellular proteins and/or its sequestration in subcellular organelles. These may be the reasons for the intracellular accumulation of Cd to concentrations higher than its extracellular levels. Another less likely possibility is that a specific pump actively transporting Cd against its concentration gradient exists in the VSMC membrane.

The present study does not attempt to support or refute any of the opposing observations relating to the in vivo effect of Cd on blood pressure because it is difficult to extrapolate in vitro findings of the acute effect of Cd on VSMC ATPase to the in vivo effects of this heavy metal. Our data demonstrate, however, that Cd exerts an acute inhibitory effect on Na, K-ATPase in disrupted VSMCs and that the Na-K pump is not inhibited in intact VSMCs by Cd concentrations that generally inhibit Na, K-ATPase in disrupted VSMCs or in homogenates and subcellular fractions derived from a variety of organs and tissues. Finally, the study underscores the necessity to assess the function of the Na, K-ATPase (Na-K pump) by independent techniques.

References


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Hypertension. 1984;6:20-26
doi: 10.1161/01.HYP.6.1.20

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1984 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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