# VANADATE EFFECTS ON $Na^+$+$K^+$-ATPASE AND $Na^+$ TRANSPORT IN MEMBRANE VESICLES FROM CRAB (CARCINUS MAENAS) GILL

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Vanadium is widespread as a trace element in the natural environment, and is believed to be an essential element for normal physiological function. It is also used as an alloy and catalyst in industrial applications and is bioaccumulated in the environment (Phillips, Nechay and Heidelbaugh, Fed. Proc. 42:2969-2973, 1983). In the 5+ oxidation state, vanadium (as vanadate) is an inhibitor of the sodium pump, acting from the cytoplasmic side of the plasma membrane (Cantley, Resh and Guidotti, Nature 272:552-554, 1978a). In preparations of purified $Na^+$+$K^+$-ATPase, vanadate appears to compete at ATP binding sites and enhances ouabain binding to the enzyme (Cantley, Cantley and Josephson, J. Biol. Chem. 253:7361-7368, 1978b; Wallick, Lane and Schwartz, J. Biol. Chem. 254:8107-8109, 1979). In intact red blood cells or ghosts, vanadate inhibits ouabain-sensitive $^{86}Rb^+$ uptake after a period of incubation to allow vanadate to cross the plasma membrane (Cantley et al., 1978a). In cultured myocardial cells, however, vanadate stimulates ouabain-sensitive $^{86}Rb^+$ uptake (Werdan, Bauriedel, Bozsik, Krawietz and Erdmann, Biochim. Biophys. Acta 597:364-383, 1980). The contrast between the universally-observed inhibition of purified $Na^+$+$K^+$-ATPase by vanadate and the discrepancies observed with intact cells prompted us to examine the effect of vanadate at an intermediate level, namely partially purified membrane vesicles. The effects of vanadate have been studied with purified $Na^+$+$K^+$-ATPase reconstituted into liposomes (Karlish and Pick, J. Physiol. 312:505-529, 1981), but few studies with native membrane vesicles have been reported.

Green shore crabs (Carcinus maenas) were collected from intertidal and subtidal areas of Salsbury Cove, Maine, and were maintained for at least one week in recirculating sea water of 10 o/oo salinity at 13-15°C. Basolateral $Na^+$+$K^+$-ATPase activity in gill, the organ responsible for $Na^+$ uptake from dilute seawater, is maximally stimulated under these conditions (Siebers, Leweck, Markus and Winkler, Mar. Biol. 69:37-43, 1982; Towle and Kays, J. Exp. Zool. 239:311-318, 1986). Plasma membrane vesicles were prepared from the three most posterior gills by sucrose density gradient centrifugation (Towle and Hølleland, Am. J. Physiol. 252:R479-R489, 1987). Catalytic activity of $Na^+$+$K^+$-ATPase was measured by the colorimetric determination of inorganic phosphorus (Towle, Palmer and Harris, J. Exp. Zool. 196:315-322, 1976), and protein was determined by coomassie blue dye binding (Bradford, Anal. Biochem. 72:248-254, 1976). Membrane vesicles were loaded by dilution and centrifugation with 100 mM KCl, 50 mM sucrose and 10 mM Tris (pH 7.4 with HEPES) (Towle and Hølleland, 1987). Initial rates of $^{22}Na^+$ uptake were determined by placing a 5-ul drop of vesicle suspension (5-10 mg protein/ml) adjacent to a 100-ul drop of uptake medium [230 mM sucrose, 2 mM $MgCl_2$, 4 mM $^{22}NaCl$ (0.25 Ci/mol) and 10 mM Tris (pH 7.4 with HEPES), with and without 4 mM ATP (Tris salt)] in the bottom of a polystyrene

test tube. Uptake was initiated by mixing with a vortexer and was stopped at 2, 4, and 6 seconds by adding 1 ml of cold stop solution [125 mM NaCl and 10 mM Tris (pH 7.4 with HEPES)], using an electronic metronome set at 60 beats/sec as a timer. The mixture was immediately filtered on a Schleicher & Schuell BA83 (0.2 um) filter and the test tube and filter were washed with two 2-ml aliquots of stop solution. Filters were counted in 10 ml Ecolume liquid scintillation medium (ICN). Zero-time values (less than 10% of the counts at 6 sec under maximally-stimulated conditions) were subtracted and initial rates of uptake were determined by linear regression and expressed as nmol $Na^+$/sec per mg protein.

Vanadate (100 uM) completely blocked ouabain-sensitive $Na^+$+$K^+$-ATPase activity in membrane vesicle preparations from posterior Carcinus gill (Table 1). Residual $Mg^{2+}$-ATPase was less affected by vanadate. The $K_{1/2}$ for inhibition of $Na^+$+$K^+$-ATPase by vanadate was about 1 x $10^{-6}$ M, corresponding to the low affinity vanadate site described for $Na^+$+$K^+$-ATPase purified from vertebrate kidney (Cantley et al., 1978b).

Vanadate also inhibited ATP-dependent $^{22}Na^+$ uptake by vesicles, but only after preincubation in uptake medium containing $Na^+$ and $Mg^{2+}$ (Table 2). In these assays, ATP-dependent uptake was initiated by vortexing an aliquot of ATP solution with the preincubated mixture of vesicles and uptake medium. Preincubation of vesicles with vanadate in $K^+$-containing resuspension medium was not sufficient to demonstrate vanadate inhibition. Apparently $Mg^{2+}$ is required, in agreement with previous studies on reconstituted liposomes (Karlish and Pick, 1981). Kinetic studies of the effect of varying vanadate concentrations indicated a $K_{1/2}$ value of approximately 1 x $10^{-6}$ M, identical to that for inhibition of $Na^+$+$K^+$-ATPase catalytic activity.

**Table 1.** Effect of vanadate on ATPase activities of plasma membrane vesicles from posterior Carcinus gill. Vanadate was incubated with the membrane preparation for 12 min in the complete assay medium lacking ATP, and the reaction was started with ATP. Means ± S.E. of triplicate determinations; representative of three experiments.

| Vanadate Concentration (umol/l) | ATPase Specific Activities (nmol $P_i$/min per mg protein) $Na^+$+$K^+$-ATPase | $Mg^{2+}$-ATPase |
|---|---|---|
| 0 | 87.5±6.1 | 91.5±2.8 |
| 100 | 0±0 | 70.5±3.4 |

**Table 2.** Effect of vanadate, with and without preincubation, on $^{22}Na^+$ uptake by $K^+$-loaded membrane vesicles from Carcinus posterior gill. Initial rates were calculated from uptake values at 0, 2, 4, and 6 secs by linear regression and are presented as the X coefficient (slope) ± S.E. of the coefficient. Representative of three experiments. N.D.= not determined.

| Preincubation | Vanadate (umol/l) | Initial Rate of $^{22}Na^+$ Uptake (nmol/sec per mg protein) +ATP | -ATP |
|---|---|---|---|
| No | 0 | 3.36±0.50 | 0.13±0.18 |
| No | 100 | 4.24±0.42 | 3.06±0.93 |
| Yes | 0 | 3.13±0.36 | N.D. |
| Yes | 100 | 0.21±0.25 | N.D. |

Interestingly, vanadate consistently enhanced ATP-independent $^{22}Na^+$ uptake by vesicles from Carcinus gill (Table 2). Such enhancement of uptake in the absence of ATP was observed with or without preincubation and often approached rates observed in the presence of ATP without vanadate. These rates were far in excess of equilibrium values (Towle and Hølleland, 1987), indicating that vanadate was not simply permeabilizing the membrane. Without the regulatory presence of ATP, vanadate may be interacting with the ATP-binding site to permit the $K^+$ gradient to drive $Na^+$ countertransport into the vesicle. These results may help to explain vanadate activation of the sodium pump previously observed in certain intact cells.

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