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THE EFFECTS OF VANADATE ON RABBIT VENTRICULAR MUSCLE ADENYLATE CYCLASE AND SODIUM PUMP ACTIVITIES

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Abstract—Vanadate in the +5 oxidation state has been reported to have a positive inotropic action on cardiac ventricular muscle. We have investigated the biochemical actions of vanadate on ventricular muscle adenylate cyclase and sodium pump activities in both intact or disrupted cell systems in an attempt to elucidate the mechanism(s) responsible for the physiological response.

Vanadate at concentrations up to 100 μM (K_a = 2 μM) stimulated adenylate cyclase activity in sarcolemmal membrane preparations or disrupted myocytes isolated from rabbit ventricular muscle by 2–3-fold. Increasing the vanadate concentrations above 100 μM resulted in a progressive inhibition of basal or hormone-stimulated adenylate cyclase activity (K_i = 5 mM) which was similar to that found by the reaction product, pyrophosphate (K_i = 0.5 mM). Both activation and inhibition by vanadate was fully reversible. Maximum activation of adenylate cyclase by vanadate and isoprenaline were not additive whereas maximum fluoride activation was decreased (18%) and the forskolin-stimulated response was slightly potentiated. Vanadate reversibly inhibited ouabain-sensitive *p*-nitrophenylphosphatase activity (K_i = 60 nM) in sarcolemmal membrane preparations and disrupted myocytes. Complete inactivation was found at 1 μM vanadate.

Acute or chronic incubation of intact myocytes with vanadate at concentrations up to 0.5 mM had no measurable affect on ouabain-sensitive ^{86}Rb influx or isobutylmethylxanthine, isoprenaline or forskolin-stimulated accumulation of intracellular cAMP concentration. Inhibition of ^{86}Rb influx and cAMP accumulation was found at higher concentrations of vanadate; however, this accompanied the progressive decrease in cell viability as measured by the decrease in percentage of rod-shaped cells.

It is concluded that vanadate, at concentrations which have been reported to induce a positive inotropic action on mammalian ventricular muscle, does not increase adenylate cyclase activity or inhibit the sodium pump activity in intact myocytes. These results show that caution must be applied when extrapolating the actions found with vanadate in broken cell systems to intact tissues.

Vanadium has been found in tissue extracts from animals and man [1]. Although toxic at high concentrations, vanadium appears to be an essential trace element since removal of the low concentrations found in the diet can lead to deficiency disorders [2]. The physiological role of vanadium remains unknown; as the oxide, vanadate has been reported to have actions on many physiological and biochemical processes (see review by Ramasarma and Crane [3]) including a positive inotropic action on ventricular muscle [4, 5] as well as a species dependent positive or negative inotropic action on atrial muscle [4, 6]. These effects may be related to the ability of vanadate to inhibit various phosphate-transferring enzymes [3, 7], including (Na + K)-ATPase [8–10] and to stimulate adenylate cyclase [11]. Vanadium in the +5 oxidation state is the active species, with the more reduced forms (+4 and +3 oxidation states) being ineffective [12]. Vanadate has also been reported to regulate calcium fluxes as a result of interactions with the extracellular membrane bound calcium stores associated with atrial tissue [6]. It is possible that a direct action of vanadate on similar calcium "pools" may be involved in the inotropic response in ventricular muscle.

In this study, we have investigated the actions of vanadate on the adenylate cyclase and ouabain-sensitive *p*-nitrophenylphosphatase (*p*-NPPase) activities of a rabbit ventricular sarcolemmal fraction in an attempt to identify the mechanism(s) of its positive inotropic action. In addition, the responses measured using the sarcolemmal membrane fractions were correlated with the action of vanadate on sodium pump activity (ouabain-sensitive ^{86}Rb influx) and the adenylate cyclase system of intact cells.

A preliminary account of this work has been presented [13].

MATERIALS AND METHODS

Biochemicals and reagents. Biochemicals were purchased from Sigma (Poole, Dorset). $^{86}Rb^+$ (specific activity 86–688 Ci/mmole) and ^{3}H-cAMP (specific activity 40 mCi/mmole) were obtained from Amersham International. Forskolin was purchased from Calbiochem (Bishops Stortford, Herts.). Sodium or ammonium *ortho*- or *meta*-vanadate were obtained from BDH (Poole, Dorset) or Aldrich Chemical Co. Ltd. (Gillingham, Dorset). All other chemicals were of analytical grade and supplied by BDH.

Animals. Male New Zealand White rabbits (1–1.5 kg) were obtained from the University of St. Andrews stocks. Animals were kept under a 24 hr day/night cycle and allowed free access to food and water.

Myocyte preparation. Ventricular myocytes were prepared from adult rabbits by the method reported for the rat [14]. The cells, of which at least 60% were rod shaped, were maintained under aerobic conditions at 37° in a Krebs Henseleit bicarbonate buffer containing 118.5 mM NaCl, 14.5 mM $NaHCO_3$, 2.6 mM KCl, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 11.1 mM glucose, 0.5 mM $CaCl_2$ and 2% (w/v) bovine serum albumin. Buffer pH was maintained at 7.4 by gassing with 95% O_2/5% CO_2. Myocyte homogenates were prepared by resuspending myocyte pellets (2×10^6 cells) in 5 ml ice-cold 5 mM Tris, 1 mM dithiothreitol, pH 7.4 and disrupting the cells using a Polytron PT10 homogenizer (3×10 sec bursts at setting 3).

Sarcolemmal membranes. Sarcolemmal membranes were prepared as described previously for the rat [15]. Membranes were stored at −70° in 5 mM Tris, 0.25 M sucrose, 0.5 mM dithiothreitol, pH 7.4 until required. The specific activity of three sarcolemmal marker enzymes, adenylate cyclase (0.28 ± 0.07 nmole/mg protein/min), K^+-stimulated *p*-nitrophenylphosphatase (*p*-NPPase) (4.67 ± 0.49 μmole/mg protein/hr) and (Na^- + K^+)-ATPase (34.8 ± 5.4 μmole/mg protein/hr) were purified at least 10-fold over the initial homogenate.

^{86}Rb *influx.* The method used to measure ^{86}Rb influx was essentially that reported by Powell *et al.* [14]. ^{86}Rb influx was measured in a cell suspension containing $0.3–0.4 \times 10^6$ myocytes/ml, 2 μCi/ml ^{86}Rb and 0.1% bovine serum albumin in Krebs Henseleit Bicarbonate buffer as above. Tracer uptake into myocytes was measured during a 3 min incubation of 200 μl of the cell suspension. Uptake was terminated by the high speed centrifugation (Beckman Microfuge) of the cells through the silicone oil, versilube F50 (Alpha Chemicals Ltd.). The centrifuge tubes were immediately frozen in liquid N_2 and cut directly above the myocyte pellet. The extent of contamination by the external medium was taken as the uptake at zero time and was always less than 30% of the total accumulated activity at 3 min. ^{86}Rb was determined by the Cerenkov method in a Packard liquid scintillation spectrophotometer.

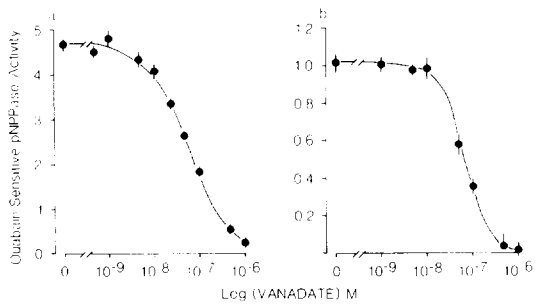

Fig. 1. Effect of vanadate on ouabain-sensitive *p*-nitrophenylphosphatase activity in (a) a sarcolemmal membrane preparation or (b) a myocyte homogenate. Activity was measured at 37° over a 20 min incubation period. Ordinate: μmoles *p*-nitrophenol/mg protein/hr or μmoles *p*-nitrophenol/10^6 cells/hr. Abscissa: vanadate concentration [M]. Results are the means ± S.D. for four measurements using the same membrane preparation or means ± S.D. for three measurements using the same myocyte preparation.

Enzyme assays. Adenylate cyclase activity was determined by the method of Luzio *et al.* [16] in an assay medium comprising 32 mM Tris/β-glycerophosphate, 0.5 mM ATP, 2.5 mM $MgSO_4$, 0.5 mM $MnSO_4$, 0.5 mM DTT, 6 mM theophylline, 2.5 mM phosphoenolpyruvate, 10 units/ml pyruvate kinase pH 7.4. Ouabain-sensitive *p*-NPPase was determined in an assay medium comprising 100 mM Tris/HCl, 200 mM KCl, 5 mM $MgCl_2$, 10 mM *p*-nitrophenylphosphate pH 7.4, in the absence or presence of 4 mM ouabain as outlined by Bers [17]. Cyclic AMP was determined by the protein binding assay of Brown *et al.* [18]. Protein was measured by the method of Lowry *et al.* [19] using bovine serum albumin as the standard.

RESULTS

Although results were qualitatively the same irrespective of the source of the vanadate salt, quantitative differences were found in the K_m calculated for vanadate action between the *ortho-* and *meta-*species from the same supplier as well as the same salts obtained from different suppliers. Although values for half-maximal inhibition or activation varied by nearly an order of magnitude, there was no significant change in the V_{max}. The reason for the variations found are unknown, however, as the manufacturers stated that the salts were at least 98% pure, the results may possibly reflect the extent of dimerization of the VO_4^{3-} or VO_3^- species to V_2O_5 during manufacture. As a consequence all results reported refer to experiments carried out using sodium *ortho*-vanadate supplied by BDH (batch no. 5633210A).

Addition of vanadate (oxidation state, +5) to sarcolemmal membranes or homogenates prepared from intact myocytes results in a marked inhibition of ouabain-sensitive *p*-NPPase activity (Fig. 1) with half maximal inhibition at 6×10^{-8} M. Ouabain-sensitive activity was measured in the presence of 5 mM Mg^{2+}, a concentration which has previously been shown to modulate the affinity of vanadate for the sodium pump [20]. Both sodium and ATP which antagonize vanadate action [21], were absent. The inhibitory action of vanadate was reversible with full enzyme activity being recovered on dilution followed by re-centrifugation to pellet the membranes.

Vanadate also has a biphasic action on the adenylate cyclase activity of both sarcolemmal membrane preparations and disrupted myocytes (Fig. 2). At low concentrations (0.5–50 μM), vanadate stimulates adenylate cyclase activity by 2–3-fold with half maximal activation occurring at 2 μM. Increasing the vanadate concentrations to above 100 μM, however, results in an inhibition of adenylate cyclase activity with half maximal inhibition at 5 mM. Millimolar concentrations of vanadate also inhibit isoprenaline, fluoride and forskolin stimulated activity (results not shown). Similar inhibition of basal or stimulated activity is also found with pyrophosphate (Fig. 3).

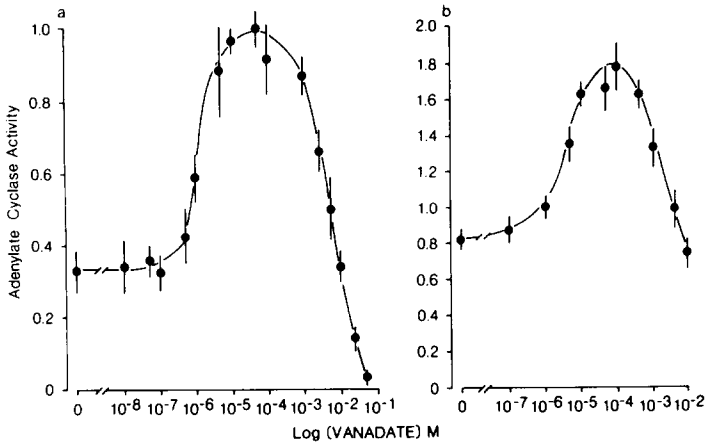

Fig. 2. Effect of vanadate on adenylate cyclase activity in (a) a sarcolemmal membrane preparation or (b) a myocyte homogenate. Activity was measured at 30° over a 10 min incubation period. Ordinate: nmoles cAMP/mg protein/min or nmoles cAMP/10^6 cells/min. Abscissa: vanadate concentration [M]. Results are the means ± S.D. for six measurements from two sarcolemmal membrane preparations or means ± S.D. for three measurements from the same myocyte homogenate.

presumably as a result of product inhibition. Inhibition by vanadate or pyrophosphate cannot be overcome by increasing the isoprenaline, fluoride or forskolin concentrations. In the presence of 100 μM GTP, the β-receptor agonist isoprenaline stimulates adenylate cyclase activity to the same extent as vanadate whereas maximum fluoride activation increases activity by over 4-fold and the plant diterpene forskolin stimulates activity up to 8-fold over basal levels. Maximum activation of adenylate cyclase activity by vanadate and isoprenaline were not additive (Fig. 4). Vanadate did, however, cause a small (18%) but significant reduction in maximum fluoride stimulated activity while at the same time, causing a slight potentiation of the forskolin response (Fig. 4). Simultaneous additions of isoprenaline, fluoride and forskolin were not additive. Both activation and inhibition of adenylate cyclase activity by low (100 μM) and high (5 mM) concentrations of vanadate were reversible following dilution/recentrifugation of the membranes. In fact, incubations in the presence of millimolar concentrations of vanadate or pyrophosphate appeared to preserve the highly labile enzyme activity.

Acute or chronic (pre-incubations up to 60 min) exposure to vanadate at concentrations of up to 500 μM had no measurable effect on ouabain-sensitive ^{86}Rb influx (Fig. 5) or isobutylmethylxanthine (IBMX), isoprenaline or forskolin stimulated accumulation of intracellular cAMP (Figs. 6 and 7) in intact myocytes. Higher concentrations of vanadate (1 mM) did, however, cause a slight decrease in the intracellular cAMP concentration in forskolin treated cells (Fig. 7) and also reduced ^{86}Rb influx (Fig. 5). Prolonged incubation of myocytes with high concentrations of vanadate (>500 μM) caused a progressive decrease in cell viability as determined by the reduction in the percentage of rod shaped cells in the suspension. Myocyte viability was reduced from 70% to less than 30% after 1 hr incubation in the presence of 1 mM vanadate. Reductions in cell viability with high concentrations of vanadate have also been found in cultured cardiac cells [22].

DISCUSSION

Vanadate has been reported to be present in animal tissues in the concentration range 10^{-7} to 10^{-6} M [23]. In our experiments with disrupted myocytes and purified sarcolemmal preparations, similar concentrations of vanadate caused maximal inhibition

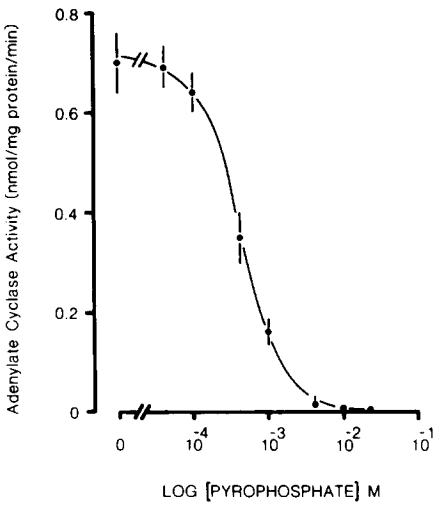

Fig. 3. Pyrophosphate inhibition of fluoride-stimulated adenylate cyclase activity. Sarcolemmal membranes were incubated in the presence of 10 mM fluoride and the pyrophosphate concentrations indicated for 10 min at 30°. Basal activity was 0.21 nmoles cAMP/mg protein/min. Ordinate: nmoles cAMP/mg protein/min. Abscissa: pyrophosphate concentration [M]. Results are means ± S.D. for three measurements from the same sarcolemmal membrane preparation.

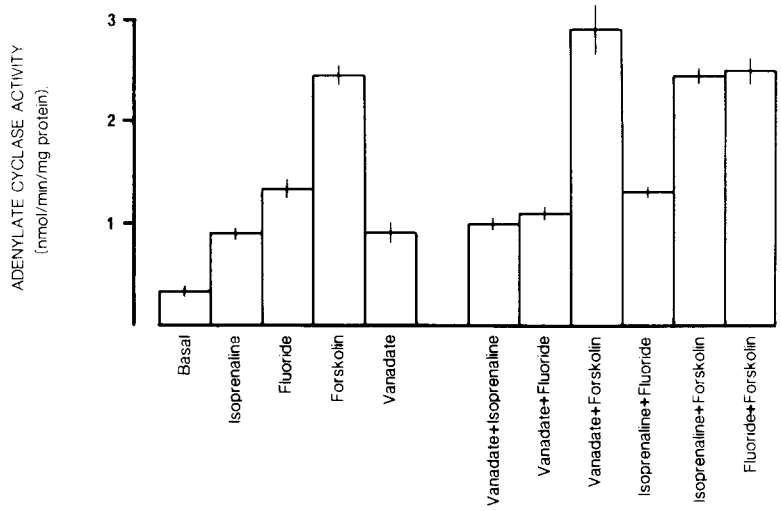

Fig. 4. Maximum stimulation of adenylate cyclase activity by isoprenaline, fluoride, forskolin and vanadate. Activity was determined at 30° in the presence of 100 μM isoprenaline, 25 mM fluoride, 50 μM forskolin and 50 μM vanadate. Ordinate: nmoles cAMP/mg protein/min. Results are means ± S.D. for three measurements using the same sarcolemmal membrane preparation.

of the oubain-sensitive *p*-NPPase activity and a significant stimulation of adenylate cyclase activity. Parallel experiments carried out with intact ventricular myocytes indicated that these vanadate concentrations (10^{-6} M) had no effect on either the activity of the sodium pump (as measured by the oubain-sensitive ^{86}Rb influx) or the activity or the adenylate cyclase system (as measured by the change in the intracellular cAMP concentration). These findings therefore suggest that the actions of vanadate in broken cell systems cannot be directly extrapolated to the intact tissue.

Although vanadate was without effect in intact myocytes its actions on membrane systems are interesting with regard to the mechanistic aspects of enzyme action. The biphasic action of vanadate on adenylate cyclase activity may be a result of the changes in the coordination chemistry of vanadate (+5) as in dilute aqueous solutions of neutral pH [24, 25]. At concentrations below 0.5 mM the major ionic species present in solution are the tetrahedral

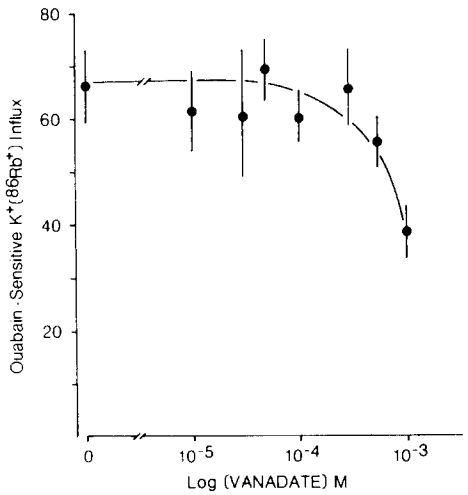

Fig. 5. Effect of vanadate on ouabain-sensitive K^+ (Rb^+) influx in intact myocytes. Myocytes were preincubated for 15 min at 37° in the presence of vanadate at the concentrations indicated. Uptake of radioactive tracer was measured over a subsequent 3 min period. Ordinate: nmoles $Rb^+/10^6$ cells/min. Abscissa: vanadate concentration [M]. Results are means ± S.D. for six meausrements from two myocyte preparations.

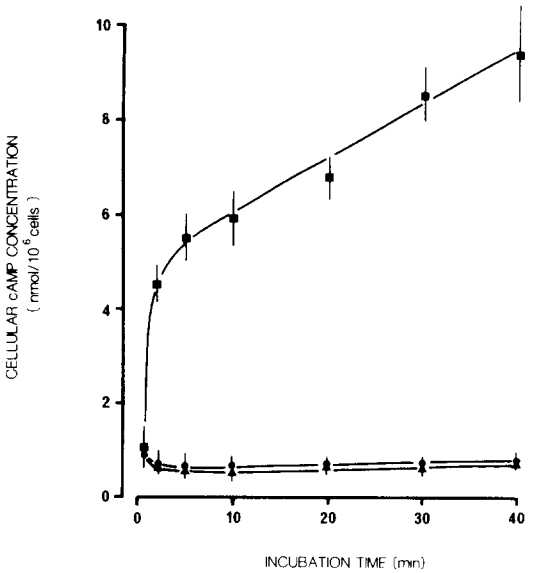

Fig. 6. Effect of vanadate and isoprenaline on intracellular cAMP production in intact myocytes. Myocytes were incubated for the times indicated in the presence of 1 mM isobutylmethylxanthine (IBMX) (●——●) or IBMX or 100 μM vanadate (▲——▲) or IBMX plus 10 μM isoprenaline (■——■). Ordinate: nmoles cAMP/10^6 cells. Abscissa: incubation time (min). Results are means ± S.D. for four measurements from the same myocyte preparation.

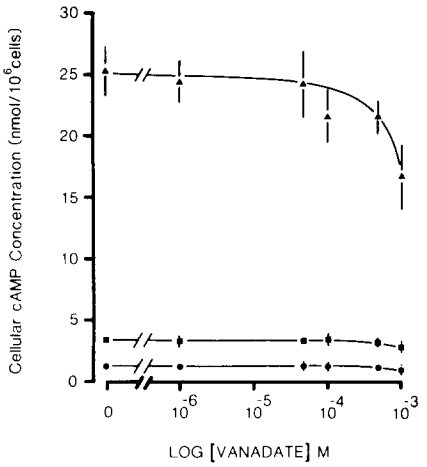

Fig. 7. Effect of vanadate on isoprenaline and forskolin-stimulation of cAMP production in intact myocytes. Myocytes were preincubated for 15 min at 37° in the presence of vanadate at the concentrations shown. cAMP production in the presence of 1 mM IBMX (●——●) or IBMX plus 10 μM isoprenaline (■——■) or IBMX plus 50 μM forskolin (▲——▲) was measured over a subsequent 2 min incubation period. Ordinate: nmoles cAMP/10^6 cells. Abscissa: vanadate concentration [M]. Results are means ± S.D. for three measurements using the same myocyte preparation.

anion $H_2VO_4^-$ (of similar coordination and size to phosphate, $H_2PO_4^-$) and the trigonal bipyramidal structure of $H_3VO_5^{2-}$. It is at these concentrations that vanadate is found to inhibit many enzymes involved in the hydrolysis and transfer of phosphate i.e. ($Na^+ + K^+$)-ATPase [23], acid [26] and alkaline [27] phosphatase, phosphofructokinase [28] and adenylate kinase [29]. The stable 5 coordinate (trigonal bipyramidal) vanadate species has structural similarities with the proposed 5 coordinate transition state formed during hydrolysis of a phosphate ester bond by nucleophilic attack [30]. Vanadate inhibition of enzyme activity is generally considered to be a result of vanadate binding at the active site possibly in the 5 coordinate form thus preventing phosphate hydrolysis. This suggests that vanadate activation of adenylate cyclase activity may result from the inhibition of an endogenous phosphate transferring enzyme (a phosphatase or kinase) associated with the sarcolemmal membrane. Vanadate potentiates forskolin activation of adenylate cyclase activity and, as previously reported in membranes isolated from rat fat cells [11] and turkey erythrocytes [31], inhibits fluoride-stimulated adenylate cyclase activity. This is in contrast with adenylate cyclase activity isolated from guinea-pig ventricular membranes where vanadate and fluoride were found to be additive [32]. The way in which vanadate interacts with the adenylate cyclase system is poorly understood. Stimulation is generally considered to be the result of vanadate binding to the guanine nucleotide regulatory component with the resulting inhibition of the GTPase activity [11, 32]. Similarities in structure indicate that vanadate may act at the same site as molybdate (a potent phosphatase inhibitor) on the guanine nucleotide regulatory component [33]. This site appears to be distinct from both the GTP and fluoride binding sites [31].

Increasing vanadate concentrations above 0.1 mM results in a progressive inhibition of stimulated adenylate cyclase activity. This has previously been reported in membranes isolated from erythrocytes [31]. As the vanadate concentrations are increased above 0.5 mM, polyionic species predominate in solution [25, 34]. Although the trimer and tetramer are more stable species the dimer $H_5V_2O_9^{3-}$ has structural features in common with pyrophosphate $H_2P_2O_7^{2-}$ which inhibits ($K_i = 0.5$ mM) both basal and hormone-stimulated adenylate cyclase activity possibly as a result of a product inhibition. We feel that the inhibitory action of vanadate on adenylate cyclase activity may be associated with this structural similarity of the polymeric vanadate species to pyrophosphate.

In the presence of 5 mM Mg^{2+} half maximal inhibition of ouabain-sensitive *p*-NPPase activity by vanadate was found at 6×10^{-8} M which agrees with the inhibition constant found for ($Na^+ + K^+$)-ATPase activity [23] indicating that vanadate has possibly the same binding site for inhibition of both the enzyme activities associated with the sodium pump. As with the other enzyme activities, inhibition may be the consequence of competition between vanadate and a phosphate moiety at the active site.

Our results show that in a purified membrane preparation and a disrupted cell system, vanadate has access to both the sodium pump and the adenylate cyclase complex. However, despite reports of vanadate increasing intracellular cAMP in cat papillary muscle [35] and inhibiting ouabain-sensitive ^{86}Rb influx in erythrocytes [20], incubation of myocytes with vanadate, at concentrations two orders of magnitude higher than required for effects on membrane enzymes, had no effect on either intracellular cAMP accumulation or ouabain-sensitive ^{86}Rb influx. Since vanadate had been reported to act at the cytoplasmic surface of the plasma membrane in erythrocytes [20], the fact that, in these experiments vanadate has no effect on intact cells suggests that either ventricular myocytes are impermeable to the anion or, as reported for intact erythrocytes [36, 37], the conditions found in the intracellular environment reduces vanadate to the inactive vanadyl (+4) form [12]. An extracellular action of vanadate on a membrane-bound calcium ion "pool" cannot be excluded as a possible mechanism by which the positive inotropic response is induced [6, 21].

Vanadate has been shown to be taken up into cultured cardiocytes via an energy independent transport system [38] and this uptake was associated with both positive inotropic and chronotropic responses. In these studies a stimulation rather than an inhibition of ouabain-sensitive ^{86}Rb influx was reported, indicating that inhibition of the sodium pump may not always be associated with the physiological action of vanadate. The increased ^{86}Rb influx, mediated by the sodium pump, was said to be a consequence of the increased rate and force of contraction. Similar conclusions have been reported using rat ventricular muscle strips [39]. Likewise, the reports of increased tissue cAMP do not always

parallel the inotropic effect said to be due to vanadate [35, 40]. Since vanadate has no effect on the sodium pump or the adenylate cyclase system of intact myocytes, the results suggest that other mechanisms may be responsible for the inotropic response. Clearly, concentrations of vanadate which are known to induce positive inotropic actions in mammalian ventricular muscle do not cause a general increase in the adenylate cyclase activity and/or inhibition of the sodium pump of intact myocytes.

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