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THE PRESENCE OF TWO ($Na^+ + K^+$)-ATPase INHIBITORS IN EQUINE MUSCLE ATP: VANADATE AND A DITHIOERYTHRITOL-DEPENDENT INHIBITOR

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Summary

A potent inhibitor of ($Na^+ + K^+$)-ATPase activity was purified from Sigma equine muscle ATP by cation- and anion-exchange chromatography. The isolated inhibitor was identified by atomic absorption spectroscopy and proton resonance spectroscopy to be an inorganic vanadate. The isolated vanadate and a solution of V_2O_5 inhibit sarcolemma ($Na^+ + K^+$)-ATPase with an I_{50} of 1 μM in the presence of 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 145 mM NaCl, 6mM $MgCl_2$, 15 mM KCl and 2 mM synthetic ATP. The potency of the isolated vanadate in increased by free Mg^{2+}. The inhibition is half maximally reversed by 250 μM epinephrine. Equine muscle ATP was also found to contain a second ($Na^+ + K^+$)-ATPase inhibitor which depends on the sulfhydryl-reducing agent dithioerythritol for inhibition. This unknown inhibitor does not depend on free Mg^{2+} and is half maximally reversed by 2 μM epinephrine. Prolonged storage or freeze-thawing of enzyme preparations decreases the susceptibility of the ($Na^+ + K^+$)-ATPase to this inhibitor. The adrenergic blocking agents, propranolol and phentolamine, do not block the catecholamine reactivation. The inhibitors in equine muscle ATP also inhibit highly purified ($Na^+ + K^+$)-ATPase from shark rectal gland and eel electroplax. The inhibitors in equine muscle ATP have no effect on the other sarcolemmal ATPases, Mg^{2+}-ATPase, Ca^{2+}-ATPase and ($Ca^{2+} + Mg^{2+}$)-ATPase.

Introduction

Recently there have been a number of reports claiming that Sigma ATP prepared by extraction from equine muscle contains a specific ($Na^+ + K^+$)-

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid; ANS, 1-amino-2-naphthol-4-sulfonic acid; Ca^{2+}-ATPase, Ca^{2+}-activated, Mg^{2+}-independent adenosine triphosphatase; ($Ca^{2+} + Mg^{2+}$)-ATPase, Ca^{2+}-activated, Mg^{2+}-dependent adenosine triphosphatase; ($Na^+ + K^+$)-ATPase, ($Na^+ + K^+$)-activated adenosine triphosphatase.

ATPase inhibitor [1—4]. This inhibitor is not present in synthetic ATP, suggesting that the inhibitor is extracted from muscle as a contaminant of ATP. Very recently, Josephson and Cantley extracted a ($Na^+ + K^+$)-ATPase inhibitor from rabbit and equine muscle with properties similar to those of the contaminant in Sigma equine muscle ATP [5] and subsequently this inhibitor has been identified as pentavalent vanadate [6].

Inhibition of ($Na^+ + K^+$)-ATPase activity by vanadate is potentiated by $MgCl_2$ and is antagonized by catecholamines [1,4,6]. In addition, vanadate inhibition is potentiated by KCl and is antagonized by NaCl [7,8].

Over the past year we have been involved in isolating and identifying the ($Na^+ + K^+$)-ATPase inhibitor in muscle ATP. In this study, we have independently isolated an inhibitor from equine muscle ATP and have identified it as vanadate, confirming the observations of Cantley et al. [6]. We also provide evidence for the presence of a second ($Na^+ + K^+$)-ATPase inhibitor in muscle ATP which requires a sulfhydryl-reducing agent for potency. The effect of the ($Na^+ + K^+$)-ATPase inhibitors in equine ATP on highly purified ($Na^+ + K^+$)-ATPase and on other ATPases in cardiac sarcolemma is also compared in this study.

Materials and Methods

Preparation of sarcolemma from rabbit heart. Sarcolemma from rabbit heart was prepared by modifications of the method of Hui et al. [9]. Hearts removed from New Zealand white rabbits were perfused with isotonic saline and cooled in ice. All subsequent procedures were carried out at 4°C. Atria, fat and large vessels were removed and the ventricles were minced with scissors. Tissue was suspended in 5 vols. of 10 mM Tris/maleate (pH 7.5) and 0.5 mM dithioerythritol and homogenized for 15 s at a setting of 4 with a polytron PT 20 homogenizer. The homogenate, diluted with an equal volume of 10 mM Tris/maleate and 0.5 mM dithioerythritol, was filtered through cheese-cloth with light suction and centrifuged for 10 min at 2500 rev./min in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet was suspended in 10 vols. of the same buffer and centrifuged at 1500 rev./min for 10 min. This step was repeated three times. The washed pellet was suspended in 5 vols. of 10 mM Tris/maleate and 0.5 mM dithioerythritol and homogenized for 15 s at a setting of 6. To the homogenate an equal volume of 2.5 M KCl, 10 mM Tris/maleate (pH 7.5), 0.1 M sucrose and 0.5 mM dithioerythritol was added dropwise with stirring. The suspension was stirred gently for 10 min and centrifuged at 15 000 rev./min for 10 min. The pellets were washed twice in 10 vols. of 10 mM Tris/maleate and 0.5 mM dithioerythritol and centrifuged at 5000 rev./min for 10 min and once with 10 vols. of 10 mM Tris/maleate (pH 7.5), 0.1 M sucrose, 2 mM $MgCl_2$ and 0.5 mM dithioerythritol and homogenized for 5 s at a setting of 3. The suspension was filtered twice through 250 μm nylon mesh. The sarcolemma membranes were assayed immediately for ATPase activity or could be stored for at least 1 month at −100°C, without significant loss of ($Na^+ + K^+$)-ATPase activity.

Purified ($Na^+ + K^+$)-ATPase preparations Highly purified ($Na^+ + K^+$)-ATPase was prepared from the shark rectal gland of *Squalus acanthias* [10] and the

electric organ of *Electrophorous electricus* [11] as previously described.

ATPase assay. ATPase activity was determined in a final volume of 1 ml containing 30 mM Tris/maleate (pH 7.2), 6 mM $MgCl_2$, 1 mM EGTA, 145 mM NaCl, 5 mM NaN_3, 2 mM Na_2ATP and 100 μg of sarcolemmal protein or 2 μg of the highly purified $(Na^+ + K^+)$-ATPase. Equine muscle ATP from the Sigma Chemical Co. served as the source of $(Na^+ + K^+)$-ATPase inhibitor and synthetic ATP (obtained from Pabst Laboratories, Milwaukee, Wisc.) served as the substrate. For measurement of $(Na^+ + K^+)$-ATPase activity, 15 mM KCl was included in the medium and $(Na^+ + K^+)$-ATPase activity was calculated as the difference between activity in the presence and absence of 0.1 mM ouabain. Ca^{2+}-ATPase activity was determined by excluding $MgCl_2$ and EGTA and adding 2 mM $CaCl_2$ to the medium. $(Ca^{2+} + Mg^{2+})$-ATPase activity was determined by adding $CaCl_2$ to the regular medium. The concentration of Ca^{2+} was calculated using a calcium EGTA stability constant of $10^{10.65}$ as described by Katz et al. [12].

After 10 min preincubation at 37° C the reaction was started by the addition of enzyme. The tubes were incubated for 20 min at 37° C and the reaction was stopped by the addition of 0.8 ml of 5% sodium dodecyl sulfate at room temperature. Inorganic phosphate was determined immediately by the method of Peterson [13]. Briefly, 1 ml of 1.25% ammonium molybdate in 2 M HCl was added to each tube followed by 0.2 ml of 0.04% ANS reagent with vortexing. Color was read after 15 min at 700 nm and K_2HPO_4 was used as a standard.

Protein assay. Sarcolemma membranes were predigested with 1 mM NaOH for at least 1 h to solubilize particulate protein. Protein concentration was estimated by the method of Lowry et al. [14].

Isolation of a $(Na^+ + K^+)$-ATPase inhibitor from muscle ATP. 2 g of Sigma equine ATP were dissolved in 30 ml of H_2O and applied to a 1.5 × 25 cm cation-exchange column (Bio-Rad AG50W-X8, 200—400 mesh) in the hydrogen form. ATP was completely eluted by consecutively applying 30 ml H_2O, 30 ml 0.5 M HCl, and 20 ml 1 M HCl. A $(Na^+ + K^+)$-ATPase inhibitor remaining on the column could be eluted by consecutively applying 50 ml of 2 M HCl and 50 ml 3 M HCl. The final 75 ml of eluant was dried on a roto evaporator (Buchi) at 35° C. This fraction contained NaCl from the Na_2ATP and a number of ultraviolet absorbing substances. This fraction was also found to inhibit $(Na^+ + K^+)$-ATPase activity in the presence of synthetic ATP.

The inhibitor fraction from the cation-exchange column was suspended in 10 ml H_2O and applied to a 1.5 × 45 cm anion-exchange column (Bio-Rad AG1-X2, 200—400 mesh) in the chloride form. 1.5-ml fractions were collected at a flow rate of 1 ml/min at room temperature. The column was eluted with 50 ml H_2O and 75 ml 0.05 M HCl. The absorbance of the fractions was measured at 260 nm and 0.05-ml aliquots were assayed for the presence of $(Na^+ + K^+)$-ATPase inhibitor. The bulk of the inhibitor was found associated with a broad ultraviolet absorbing peak eluting with the second void volume of H_2O. There was also some inhibitor associated with a peak eluting with 0.05 M HCl.

Fractions comprising the first peak were combined and evaporated to dryness. The fraction was dissolved in 10 ml H_2O and applied to the same AG1-X2 column. The column was eluted with 100 ml H_2O and 75 ml 0.05 M

HCl. Fractions (1.5 ml) were collected, their absorbance was measured at 260 nm, and 0.05-ml aliquots were assayed for $(Na^+ + K^+)$-ATPase inhibitor. The inhibitor was associated with peak II which elutes with 0.05 M HCl (Fig. 4). Presumably the removal of NaCl by the first pass over the anion-exchange column allowed separation of the inhibitor from other ultraviolet absorbing components. The lyophilized fraction was yellow in color.

Other methods. Paper chromatography: isolated unknowns were chromatographed on Whatman No. 1 paper using H_2O adjusted to pH 10 with NH_4OH as the solvent [15].

Proton magnetic resonance spectroscopy: Approx. 100—200 μg of isolated unknowns were suspended in 0.5 ml of deuterium oxide. NMR spectra were measured on a Bruker 70 MHz nuclear magnetic spectrophotometer.

Atomic absorption spectroscopy: samples for vanadium determination were sent to the WARF Institute, Madison, Wisc. Analytical method used, Perkin-Elmer.

Materials. Equine disodium ATP, 1-epinephrine bitartrate, (—)-norepinephrine · HCl, (—)-isoproterenol · HCl and (±)-propranolol · HCl, 5′-AMP, ADP, adenosine, adenine, dithioerythritol, and EGTA were purchased from the Sigma Chemical Co., St. Louis, Mo. Yeast disodium ATP and pyrocatechol were from PL laboratories, Milwaukee, Wisc.; deuterium oxide, Aldrich; phentolamine, CIBA-Geigy. All other chemicals used in this study were reagent grade.

Results

Properties of $(Na^+ + K^+)$-ATPase inhibitors in muscle ATP

In preliminary experiments, it was found that sarcolemma $(Na^+ + K^+)$-ATPase activity was linear for up to 30 min at 37°C in the presence of both muscle and synthetic ATP (Fig. 1). However, $(Na^+ + K^+)$-ATPase activity was 20—25% less in the presence of muscle ATP at a $MgCl_2$ concentration of 6mM. Addition of 0.1—0.5 mM dithioerythritol to the assay medium decreased $(Na^+ + K^+)$-ATPase activity a further 25—30% in the presence of muscle ATP but not synthetic ATP. Ascorbic acid (0.1—0.5 mM) had no effect on $(Na^+ + K^+)$-ATPase activity in the presence of either muscle or synthetic ATP. Sarcolemma membranes which were freeze-thawed twice were not sensitive to the extra $(Na^+ + K^+)$-ATPase inhibition observed by including dithioerythritol with muscle ATP. Freeze-thawing did not significantly affect the specific activity of $(Na^+ + K^+)$-ATPase in the membranes or the inhibition found with muscle ATP in the absence of dithioerythritol.

The specific activities of Ca^{2+}-ATPase (12 μmol P_i/mg per h), $(Ca^{2+} + Mg^{2+})$-ATPase (4 μmol P_i/mg per h) and Mg^{2+}-ATPase (2 μmol P_i/mg per h) in cardiac sarcolemma were the same in the presence of muscle or synthetic ATP indicating that the contaminants in muscle ATP are specific for $(Na^+ + K^+)$-ATPase activity. The contaminants in muscle ATP also inhibited highly purified $(Na^+ + K^+)$-ATPase from shark rectal gland and eel electroplax, although higher concentrations of $MgCl_2$ were required to observe inhibition in the absence of dithioerythritol (see below). Prolonged storage of the highly purified $(Na^+ +$

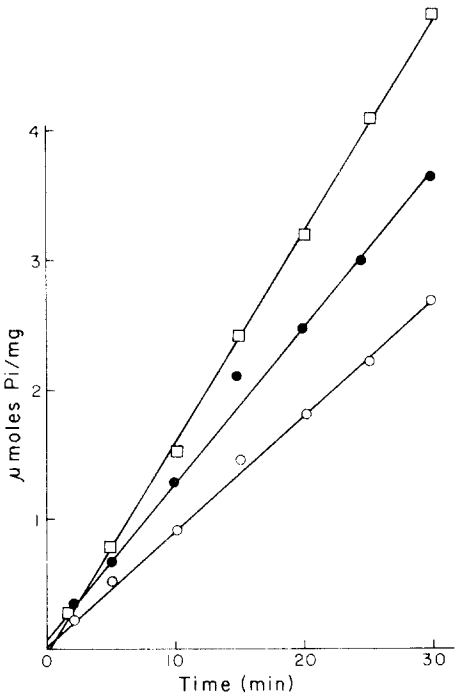


Fig. 1. Time course of $(Na^+ + K^+)$-ATPase activity in cardiac sarcolemma in the presence of 2 mM synthetic ATP (□), 2 mM equine muscle ATP (●) and 2 mM equine muscle ATP + 0.5 mM dithioerythritol (○).

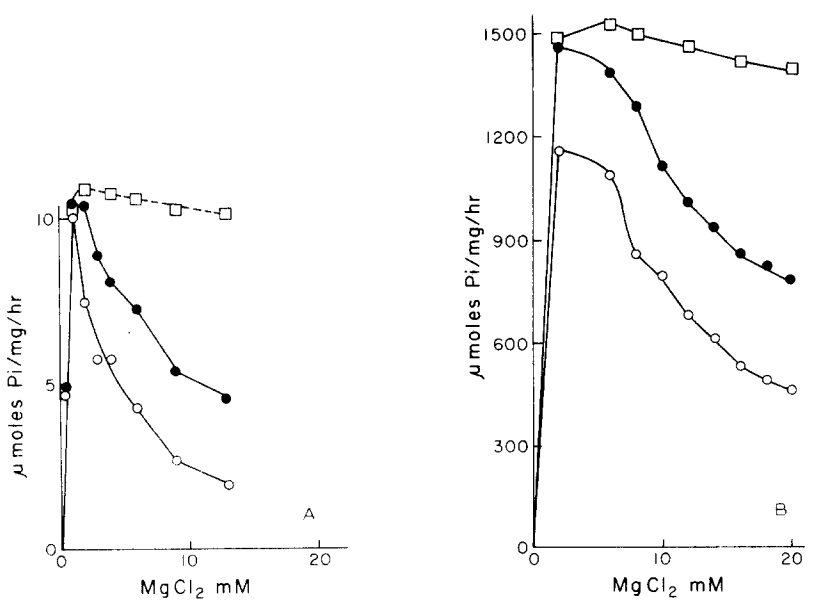


Fig. 2.(A) $MgCl_2$ dependence of sarcolemma $(Na^+ + K^+)$-ATPase in the presence of 2 mM synthetic ATP ±0.5 mM dithioerythritol (□), 2 mM equine muscle ATP (●) and 2 mM equine muscle ATP + 0.5 mM dithioerythritol (○). (B) $MgCl_2$ dependence of shark rectal gland $(Na^+ + K^+)$-ATPase in the presence of 2 mM synthetic ATP ±0.5 mM dithioerythritol (□), 2 mM equine muscle ATP (●) and 2 mM equine muscle ATP + 0.5 mM dithioerythritol (○).

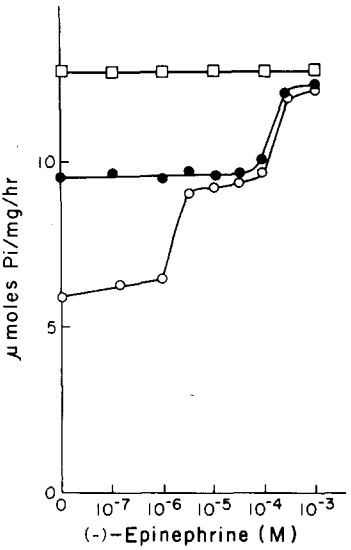


Fig. 3. Effect of (—)-epinephrine on cardiac sarcolemma ($Na^+ + K^+$)-ATPase activity in the presence of 2 mM synthetic ATP ±0.5 mM dithioerythritol (□), 2 mM equine muscle ATP (●) and 2 mM equine muscle ATP + 0.5 mM dithioerythritol (○).

K^+)-ATPase preparations resulted in a loss of the dithioerythritol-dependent inhibition in the presence of muscle ATP.

The inhibition of sarcolemma and shark rectal gland ($Na^+ + K^+$)-ATPase by a contaminant in muscle ATP was potentiated by high concentrations of $MgCl_2$ (Figs. 2A and 2B). In the presence of 2 mM muscle ATP, sarcolemma ($Na^+ + K^+$)-ATPase activity was inhibited 50% in the presence of 9 mM $MgCl_2$ (Fig. 2A). In contrast, shark rectal gland ($Na^+ + K^+$)-ATPase activity required 20 mM $MgCl_2$ to achieve 50% inhibition under the same conditions (Fig. 2B). The decrease in ($Na^+ + K^+$)-ATPase activity found by including dithioerythritol with muscle ATP was not potentiated by $MgCl_2$ concentrations greater than 2 mM. It is apparent that expression of dithioerythritol-dependent inhibition is not dependent on high concentrations of free Mg^{2+} like vanadate as most of the Mg^{2+} would be chelated by ATP and EGTA. However, at least 2 mM was required for maximal inhibition by the dithioerythritol inhibitor in the presence of 2 mM ATP.

It has been reported that ($Na^+ + K^+$)-ATPase activity in various preparations is activated by catecholamines [6,17—19]. We found that cardiac sarcolemma was also activated by catecholamines but only in the presence of muscle ATP (Fig. 3). In the absence of dithioerythritol, the (—)-epinephrine activation curve of ($Na^+ + K^+$)-ATPase was monophasic, and half maximal reactivation of ($Na^+ + K^+$)-ATPase activity occurred at a concentration of 200—300 μM. Pyrocatechol was found to reactivate ($Na^+ + K^+$)-ATPase activity with similar potency. In the presence of dithioerythritol and muscle ATP, the epinephrine reactivation curve was biphasic. The dithioerythritol-dependent inhibition was half maximally reactivated by 2—3 μM epinephrine. Other catecholamines, (+)-epinephrine, (—)-isoproterenol and (—)-norepinephrine reactivated the dithioerythritol-

dependent inhibition with similar potency. However, at least a 100-fold higher concentration of pyrocatechol was required to reactivate the dithioerythritol-dependent inhibition. The maximal ($Na^+ + K^+$)-ATPase activity obtained in the presence of catecholamines and muscle ATP was equal to the activity obtained in the presence of yeast ATP indicating that catecholamines activate ($Na^+ + K^+$)-ATPase activity by antagonizing the inhibitors in muscle ATP. Phentolamine and propranolol at 100 μM did not block reactivation of ($Na^+ + K^+$)-ATPase activity by catecholamines in the presence of muscle ATP and dithioerythritol.

Isolation of a ($Na^+ + K^+$)-ATPase inhibitor from muscle ATP

A potent inhibitor of ($Na^+ + K^+$)-ATPase activity was purified from equine muscle ATP by a second passage through a AG1-X2 anion-exchange column (see Methods). The ($Na^+ + K^+$)-ATPase inhibitor was found associated with peak II which elutes off the column with 0.05 M HCl (Fig. 4). Peaks I and III were identified by NMR spectroscopy and paper chromotography as adenine and AMP, respectively. The ultraviolet spectrum of the unknown in peak II was unlike any purine or pyrimidine [15]. The unknown had an absorption maximum at approx. 263 nm and the absorbancy ratios, A_{250nm}/A_{260nm} and A_{280nm}/A_{260nm} were 0.97 and 0.77, respectively. Furthermore, the isolated inhibitor had no NMR spectrum, suggesting that it is inorganic and not an organic-inorganic complex. Boiling the inhibitor for 2 h in 60% $HClO_4$ also did not destroy its inhibitor potency, further identifying it as an inorganic inhibitor. A number of polyvalent metals which were inhibitors of ($Na^+ + K^+$)-ATPase activity [2] were tested as possible candidates for the isolated unknown (see below). Analysis of the unknown by atomic absorption confirmed that the isolated inhibitor was vanadium. Because vanadium is readily oxidized, it is

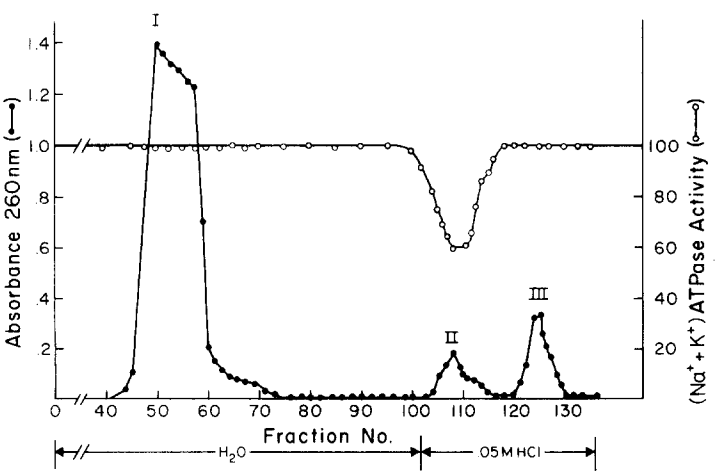


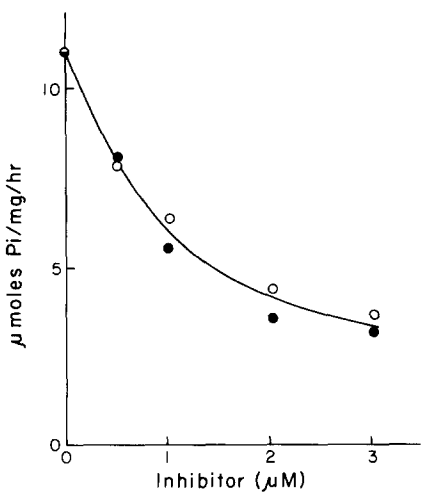
Fig. 4. Separation of an inhibitor from equine muscle ATP by anion-exchange chromotography (AG1-X2). The absorbance was monitored at 260 nm (●) and 0.05-ml aliquots of various fractions were assayed under standard ($Na^+ + K^+$)-ATPase assay conditions for presence of inhibitor (○———○) using synthetic ATP as substrate. Peak I elutes with approximately the second void volume with H_2O. Peak II and III elute with 0.05 M HCl. The ATPase assay was corrected for slight lowering of pH due to addition of 0.05 ml 0.05 M HCl to 1.0 ml assay mixture.

very likely a vanadate in solution. Muscle ATP was found to contain 38 ppm vanadium, and synthetic ATP less than 12 ppm vanadium by atomic absorption spectroscopy. Therefore, the concentration of vanadium in muscle ATP would yield a vanadate concentration of approx. 0.6 μM in the presence of 2 mM muscle ATP. As further proof, the ultraviolet spectral constants for the isolated inhibitor are almost identical to those obtained for a solution of V_2O_5 at pH 5.0. The fact that the inhibitor is retained on the anion-exchange column suggests that it is a negatively charged vanadate. The fact that the inhibitor is also retained by the strongly acidic-exchange column (see also ref. 4) suggests that a different form of the ion (i.e., VO_2^{2+}) may be present under those conditions.

Effects of the isolated inhibitor on ($Na^+ + K^+$)-ATPase activity

The isolated vanadate and V_2O_5 inhibit sarcolemma ($Na^+ + K^+$)-ATPase activity with an I_{50} of approx. 1 μM (Fig. 5) in the presence of synthetic ATP. The concentration of isolated vanadate was estimated from the extinction coefficient obtained for V_2O_5 at pH 5.0. Dithioerythritol had no effect on the K_i of the isolated vanadate. This finding indicates that the vanadate does not account for the dithioerythritol-dependent inhibition of ($Na^+ + K^+$)-ATPase activity in the presence of muscle ATP. The potency of the isolated vanadate was increased by higher concentrations of $MgCl_2$ in an identical manner to that observed in the presence of muscle ATP (Fig. 2), and is therefore not shown.

Epinephrine half maximally reactivated ($Na^+ + K^+$)-ATPase in the presence of yeast ATP and 1 μM vanadate at a concentration of 200—300 μM (Fig. 6).



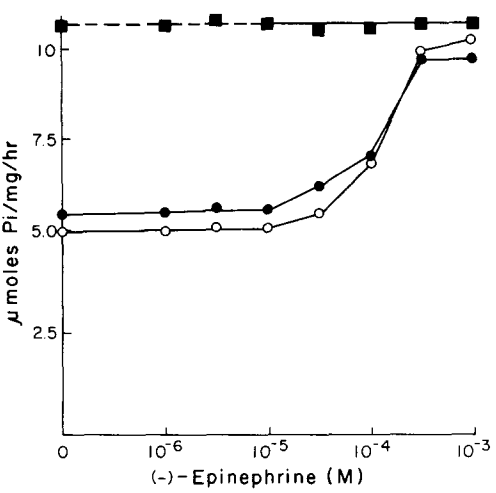


Fig. 5. Effect of vanadate isolated from equine muscle ATP ± 0.05 mM dithioerythritol (○) and 1 μM V_2O_5 ±0.5 mM dithioerythritol (●) on sarcolemma ($Na^+ + K^+$)-ATPase activity assayed in the presence of synthetic ATP.

Fig. 6. Effect of (—)-epinephrine on sarcolemma ($Na^+ + K^+$)-ATPase activity in the presence of 2 mM synthetic ATP (■), 2 mM synthetic ATP + 1 μM isolated vanadate (●) and 2 mM synthetic ATP + 1 μM V_2O_5 (○).

Pyrocatechol reactivated with a similar potency and dithioerythritol was found to have no effect on the K_A of catecholamine reactivation.

Effects of polyvalent metals on ($Na^+ + K^+$)-ATPase activity

A number of metals were tested to determine their effects on ($Na^+ + K^+$)-ATPase activity using synthetic ATP as the substrate. At 0.1 mM, $CdCl_2$, $BaCl_2$, $AlCl_3$, $CuSO_4$, $CoCl_2$, zinc acetate, $HgCl_2$ and $CaCl_2$ were potent inhibitors of ($Na^+ + K^+$)-ATPase activity in the absence of EGTA, in agreement with Hexum [2]. However, EGTA (1.0 mM) normally used in the ($Na^+ + K^+$)-ATPase medium in this study completely blocked the inhibition by the above metal cations. The exceptions were 100 μM $FeCl_3$ and $FeSO_4$ which inhibited ($Na^+ + K^+$)-ATPase activity in the presence of 1 mM EGTA. Inhibition by Fe^{3+}, like vanadate, was completely reversed by catecholamines. However, the concentration of Fe^{3+} in muscle ATP (0.003%) is too low to account for the inhibition of ($Na^+ + K^+$)-ATPase activity.

Discussion

In this study, the $MgCl_2$-dependent ($Na^+ + K^+$)-ATPase inhibitor reported to be in commercial equine muscle ATP [1,3,4,6] was isolated by column chromatography. The inhibitor was identified as an inorganic vanadate which is not complexed to an organic molecule. The isolated vanadate inhibits ($Na^+ + K^+$)-ATPase activity with an I_{50} of 1 μM at a $MgCl_2$ concentration of 6 mM. This I_{50} is about 20 times higher than that reported by Cantley et al. [6]. However, these workers determined their I_{50} at a much higher concentration of $MgCl_2$ (25 mM), and the vanadate inhibition is potentiated at higher $MgCl_2$ concentrations. The I_{50} for vanadates is also dependent on the source of ($Na^+ + K^+$)-ATPase. In the presence of an equal concentration of vanadate, twice as much $MgCl_2$ is required to inhibit shark rectal gland ($Na^+ + K^+$)-ATPase activity compared to sarcolemma ($Na^+ + K^+$)-ATPase (Fig. 2A and B). In agreement with a previous report [1,4], catcholamines reverse the inhibition of the $MgCl_2$-dependent inhibitor in muscle ATP. In this study, in the presence of the isolated vanadate and yeast ATP or muscle ATP, ($Na^+ + K^+$)-ATPase activity was half maximally reactivated at 250 μM (—)-epinephrine or pyrocatechol (Figs. 3 and 6). These catechols presumably reactivate ($Na^+ + K^+$)-ATPase activity by chelating vanadates, as catechols are known to be strong chelators of a number of transition metals [22].

It is quite conceivable that the vanadate in muscle ATP is indeed extracted from equine muscle, as vanadates have been found in a number of animal tissues [20]. Josephson and Cantley [5] also recently isolated vanadate from rabbit and equine skeletal muscle. Although vanadates are potent physiological regulators of a large number of enzymes and cellular functions, their physiological significance is undetermined [20]. We found that the K_i of vanadate is not altered in the presence of 10^{-4} M ascorbate or potassium ferricyanide, suggesting that it does not inhibit by a redox mechanism. Furthermore, vanadate inhibition is readily reversed by catecholamines. Other workers have suggested that because vanadates resemble inorganic phosphates structurally, they may inhibit alkaline phosphatase by binding at P_i sites on the enzyme [21].

The interaction of vanadates with Mg^{2+} and K^+ on $(Na^+ + K^+)$-ATPase suggests that there may be a physiological binding site on $(Na^+ + K^+)$-ATPase for vanadate or perhaps for some other transition metal.

A dithioerythritol-dependent $(Na^+ + K^+)$-ATPase inhibitor in muscle ATP

In this study, we have also found that equine muscle ATP contains an $(Na^+ + K^+)$-ATPase inhibitor in addition to vanadate which is dependent on dithioerythritol for inhibition. This inhibitor is not found in synthetic ATP. The dithioerythritol-dependent inhibitor, unlike vanadate, is not potentiated by high concentrations of $MgCl_2$ (Fig. 2A and B). It was also found that ascorbate does not further decrease $(Na^+ + K^+)$-ATPase activity in the presence of muscle ATP, possibly indicating a specific dependence for sulfhydryl-reducing agents. Aging or freeze-thawing $(Na^+ + K^+)$-ATPase preparations abolished the sensitivity of the enzyme to dithioerythritol-dependent inhibition. This observation suggests that dithioerythritol acts by reducing a functional group on the $(Na^+ + K^+)$-ATPase rather than directly reducing the inhibitor to an active form. The loss of dithioerythritol-dependent inhibition could be due to the formation of disulfide bonds. Kalawek et al. [23] have suggested that during storage of cytochrome *P*-448 intra-or intermolecular disulfide bonds form which are not readily reduced by dithioerythritol.

Dithioerythritol dependent inhibition is also reversed by catecholamines but at a 100-fold lower concentration than with vanadate (Fig. 3). Catecholamines have no effect on $(Na^+ + K^+)$-ATPase in the presence of synthetic ATP, indicating that the catecholamine activation is due to reversal of inhibition by contaminants in muscle ATP. A number of other workers have also reported that catecholamines activate $(Na^+ + K^+)$-ATPase activity at μM concentrations [19,24] and it has been suggested that α- or β-adrenergic receptors are linked to the catecholamine activation [18,25]. We found that the adrenergic blocking agents, propranolol and phentolamine, did not block the reactivation of sarcolemmal $(Na^+ + K^+)$-ATPase activity by catecholamines in the presence of muscle ATP and dithioerythritol. Other catecholamines tested, (—)-isoproterenol, (—)-norepinephrine or (+)-epinephrine also activated $(Na^+ + K^+)$-ATPase activity with similar potency to (—)-epinephrine, indicating a lack of stereo or structural specifity for the activators. Therefore, reactivation of cardiac sarcolemma and shark rectal gland $(Na^+ + K^+)$-ATPase activities in the presence of muscle ATP and dithioerythritol does not appear to be associated with α-or β-adrenergic receptor function. However, dithioerythritol-dependent inhibition requires a 100-fold higher concentration of pyrocatechol for reactivation, suggesting that an additional moiety is required on the pyrocatechol nucleus for high affinity reactivation. Presently we are trying to find suitable conditions in which to isolate this inhibitor from muscle ATP.

Acknowledgements

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