Isolation of a Potent (Na-K)ATPase Inhibitor from Striated Muscle†

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ABSTRACT: A potent inhibitor of Na^+- and K^+-stimulated adenosine triphosphatase [(Na-K)ATPase] has been purified from both equine and rabbit muscle. At concentrations between 10^{-8} and 10^{-7} M, this muscle derived inhibitory factor (MIF) produces 50% inhibition of the enzyme in the presence of 100 mM NaCl, 25 mM KCl, 3 mM ATP, and 25 mM $MgCl_2$. Mg^{2+} and K^+ facilitate the inhibition, while ATP protects the enzyme from inhibition. Inhibition can be reversed by the addition of norepinephrine. MIF is similar to ATP with respect to mobility on anion-exchange columns, cation-exchange columns, and thin-layer chromatographic systems. The similarity between ATP and MIF explains the observation that commercial sources of muscle derived ATP contain about 3 parts of MIF per 10 000 parts of ATP. An ultraviolet absorption spectrum of the purified material is presented and its stability in the presence of acid, base, and alkaline phosphatase is investigated. The inhibitory effect of MIF and the reversal by norepinephrine were found to be similar for (Na-K)-ATPases from dog kidney, dog brain, and eel electroplax. However, neither MIF nor norepinephrine had any effect on sarcoplasmic reticulum Ca^{2+}-activated ATPase, mitochondrial ATPase (F_1), or actomyosin ATPase at 20 to 50 times the concentrations which affect the (Na-K)ATPase.

Na^+- and K^+-stimulated adenosine triphosphatase [(Na-K)ATPase][1] prepared from dog or lamb kidney exists in slowly interconverting active and inactive conformational states (Cantley and Josephson, 1976; Fagan and Racker, 1977). The inactive state is characterized by an absence of all activities associated with the enzyme including the Na^+-stimulated ATPase activity, the K^+-stimulated *p*-nitrophenylphosphatase activity and phosphorylation by ATP or inorganic phosphate (Cantley and Josephson, 1976; Fagan and Racker, 1977). A similar slow interconversion between active and inactive states occurs with eel electroplax (Na-K)ATPase (Fagan and Racker, 1977) and may occur with rat skeletal muscle (Na-K)ATPase (Erdmann et al., 1976). Mg-ATP, free Mg^{2+} and K^+ affect the equilibrium between active and inactive states by binding to the sites which affect the initial rates of ATP hydrolysis (Cantley and Josephson, 1976). The ability of high concentrations of catecholamines to stabilize the active state of the enzyme and antagonize this type of inhibition has been noted (Fagan and Racker, 1977).

In recent experiments we have found that ATP prepared from yeast fails to cause the transition to the inactive state of the enzyme. Muscle derived ATP had been used in all our previous experiments as well as those of Fagan and Racker (Dr. E. Racker, personal communication). Similar variations of (Na-K)ATPase activity dependent on the source of ATP have been reported by Charney et al. (1975) and communicated to us by Dr. J. D. Robinson (personal communication, 1977).

In this paper we report the purification of a potent (Na-K)ATPase inhibitor from equine and rabbit muscle. The inhibitor cochromatographs with ATP on anion- and cation-exchange columns and in two thin-layer chromatographic systems. It is present in commercially available ATP prepared from muscle and is required for the conversion of (Na-K)-ATPase to the inactive state. The purified inhibitor is characterized with respect to its stability in the presence of acid, base, and alkaline phosphatase. It does not inhibit other ATPases but inhibits (Na-K)ATPases from a number of sources.

Experimental Section

Materials. ATP derived from yeast or muscle sources was obtained from Sigma Chemical Co. The disodium salt of ATP prepared from equine muscle (Sigma Grade ATP) served as a source of (Na-K)ATPase inhibitor. The Tris salt of ATP was purchased from Sigma and is prepared by ion exchange of the Sigma Grade ATP (personal communication, Sigma Chemical Co.). Yeast derived ATP was Sigma's Grade I and was purchased as the disodium salt. CTP, UTP, adenosine 5′-tetraphosphate, cyclic 3′,5′-AMP and delipidated muscle extract powder were also from Sigma. Acetyl CoA, palmityl CoA, and reduced CoA were from P-L Biochemicals. Highly purified GTP and ITP were a gift from Dr. William McClure (Harvard University). Diadenosine 5′,5‴-P^1,P^4-tetraphosphate and diadenosine 5′,5‴-P^1,P^3-triphosphate were generously provided by Dr. Eliezer Rapaport (Massachusetts General Hospital). Alkaline phosphatase was obtained from Worthington. Chelex-100, Cellex-D, and AG-50 resins were from Bio-Rad, while Sephadex G-10 was from Pharmacia. All other reagents were the best available commercial grades and deionized-distilled water was used to prepare all solutions. All metal salts were the chloride form.

Enzyme Preparations. (Na-K)ATPase from the dog kidney was prepared by the procedure of Jorgensen (1974) as described elsewhere (Cantley and Josephson, 1976). The (Na-K)ATPase from dog brain was a gift from Kathy Sweadner (Harvard University) and was prepared by employing a sodium dodecyl sulfate extraction of brain microsomes. (Na-K)ATPase from the eel electroplax was prepared by the method of Albers et al. (1963). ATPase activity from these preparations was at least 98% inhibitable by 50 μM ouabain.

†From the Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138. *Received April 25, 1977.* This work was supported by Grant HL 08893 from the National Institutes of Health and Grant BMS 73-06752 from the National Science Foundation.

‡National Institutes of Health Postdoctoral Fellow (AM 00899-02).

§National Institutes of Health Postdoctoral Fellow (GM 05508-01).

[1]Abbreviations used are: (Na-K)ATPase, Na^+- and K^+-stimulated adenosine triphosphatase; MIF, muscle inhibitory factor; (Ca)ATPase, Ca^{2+}-stimulated adenosine triphosphatase; F_1, mitochondrial coupling factor 1; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; CoA, coenzyme A; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; ATP_S, Sigma Grade ATP; ATP_C, Chelex-100 purified ATP.

(Ca)ATPase was the R_2 preparation from rabbit muscle sarcoplasmic reticulum prepared as described by MacLennan (1970) and donated to us by Siu W. Tong (Harvard University). The F_1 ATPase was prepared from beef heart mitochondria by the procedure of Horstman and Racker (1970) and donated to us by Barbara Baird (Cornell University).

Actin was prepared by the method of Spudich and Watt (1971) and myosin was the 40 to 50% ammonium sulfate cut described by Kielley and Harrington (1960). Actin and myosin were gifts from Jim Anderson (Harvard University).

Kinetic Measurements. The coupled assay monitoring NADH oxidation was employed to measure ATPase activities. The reaction was monitored at 340 nm in a Carey 15 spectrophotometer with a thermostated cell holder. All assays were done in the presence of 100 mM NaCl, 25 mM KCl, 1.4 mM phosphoenolpyruvate, 0.26 mM NADH, 1 mM dithiothreitol, 10 μg/mL pyruvate kinase, 10 μg/mL lactic dehydrogenase, 20 mM Hepes-triethylamine, pH 7.4, 37 °C, with the specified concentrations of $MgCl_2$ and other ligands. Enzyme concentrations were between 1 and 3 μg/mL. In experiments where product formation was not linear with time, final steady-state velocities were measured by waiting until a constant velocity occurred. The fraction of active enzyme is determined by dividing final by initial hydrolytic rates (Cantley and Josephson, 1976).

In some experiments the enzyme was incubated in the presence of various ligands and assayed for activity by dilution into the coupled assay system employing 3 mM $MgCl_2$ and 2.5 mM ATP. Enzyme employed in these experiments was washed as previously described to remove contaminating ligands (Cantley and Josephson, 1976). The fraction of active enzyme was determined by dividing the initial velocity obtained after incubation by the initial velocity obtained without incubation. Irreversible loss of enzyme activity during the incubation period was not observed.

Preparation of Nucleotides from Rabbit Muscle. The first stage of purification of (Na-K)ATPase inhibitor from rabbit muscle is a modification of the purification scheme for ATP from that tissue (Berger, 1957). Approximately 200 g of muscle from the hind legs of a rabbit was obtained within 5 min of decapitation. The muscle was dropped into liquid nitrogen to rapidly lower the temperature and then allowed to thaw. The tissue was passed through a meat grinder and homogenized in a Waring blender for 2 min in 400 mL of 10% trichloroacetic acid. The suspension was filtered through three layers of cheesecloth and the insoluble material was again homogenized in 200 mL of 10% trichloroacetic acid. This suspension was filtered as above and the combined filtrate was repeatedly passed through Whatman No. 1 filter paper until a clear solution was obtained.

The precipitation of nucleotides and other highly charged compounds was achieved by addition of 25 mL of 2 M barium acetate followed by centrifugation at 2500*g* for 20 min. The pellet was suspended in 200 mL of 0.1 mM barium acetate followed by centrifugation as above. After the addition of 10 mL of 0.5 M potassium sulfate and 60 mL of H_2O, the pellet was homogenized in a Potter-Elvehjem device by hand. The pH was adjusted to 7.1 with 1 N NaOH and the mixture stirred for 12 h at 4 °C. The resulting barium sulfate precipitate was removed by centrifugation at 12 000*g* for 20 min. The supernatant was then concentrated to 5 mL by lyophilization and applied to the Chelex-100 column described in Figure 3A.

Thin-Layer Chromatographic Systems. Acidic and basic thin-layer chromatographic systems were employed to analyze the chemical nature of the (Na-K)ATPase inhibitor. The acidic system consisted of isobutyric acid–ammonia–water (100:8:52). The basic system employed 1-propanol:ammonia:water (6:3:1). Both employed a cellulose resin of Eastman No. 13254. The R_f for ATP is 0.4 in the acidic system and 0.06 in the basic system. Mobility of the inhibitor was ascertained by cutting the plate into sections, scraping off the cellulose, and eluting with 0.5 mL of water. Inhibitory activity was assayed in the usual fashion (Figure 2).

Protein concentrations were determined by the method of Lowry et al. (1951). ATP concentration was determined as described by Lowry et al. (1964).

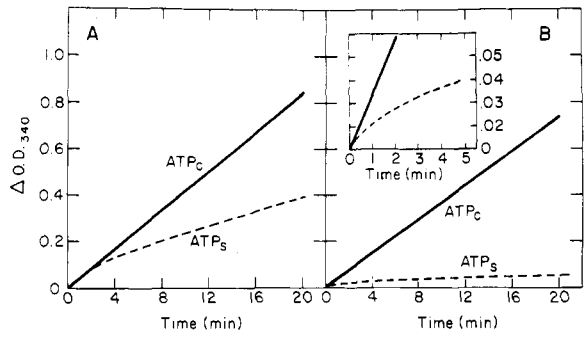

FIGURE 1: The time course of ATP hydrolysis as measured by the coupled assay with either 3 mM Chelex-100 purified ATP (ATP_c, solid lines) or 3 mM Sigma Grade ATP (ATP_s, dashed lines). The $MgCl_2$ concentration was 3.2 mM in A and 28 mM in B. The dog kidney (Na-K)ATPase concentration was 0.7 μg/mL in A and 1.4 μg/mL in B. The inset shows the time course of hydrolysis in the presence of 28 mM $MgCl_2$ at short time periods.

Results

An Inhibitor of (Na-K)ATPase in Muscle ATP. During the course of our experiments, we observed that the time course of (Na-K)ATPase activity depended drastically on the source of ATP employed in the assay. ATP derived from yeast gave nearly constant hydrolytic rates while ATP prepared from muscle produced a pronounced loss in enzymatic activity during the course of the assay. This difference in enzymatic activity suggested that contaminants in the various commercial sources of ATP were responsible for these effects and prompted us to develop methods for separating muscle derived ATP from the contaminants affecting the (Na-K)ATPase. Figure 1 compares the time course of (Na-K)ATPase activity obtained using commercial muscle ATP with that obtained with muscle ATP purified by passage through a Chelex-100 column (see Figure 3). The commercial preparation is the Sigma Grade product and is denoted ATP_s, while the Chelex-100 purified ATP is denoted ATP_c. Although initial ATPase velocities are identical for the two types of ATP, differences become apparent at long incubation times and at high Mg^{2+} concentrations. These results suggest that treatment with Chelex-100 removes an inhibitor of (Na-K)ATPase from ATP_s.

An Assay for (Na-K)ATPase Inhibitor. In order to isolate and characterize the inhibitor, it was necessary to develop a simple quantitative assay for its biological activity. Figure 2 (inset) illustrates the result of adding 1 mM ATP_s to 3 mM ATP_c. Rather than stimulating enzyme activity, the addition of ATP_s results in a progressive loss of activity until a steady-state rate of ATP hydrolysis is reached. Initial and final hydrolytic rates give the fraction of active enzyme molecules as described above. We have repeated this measurement as a function of ATP_s in the presence of 28 mM $MGCl_2$ as shown in Figure 2. Since the K_m for ATP is 1.1 mM under these conditions (Cantley and Josephson, 1976), increasing concentrations of ATP do not markedly alter the rate of ATP

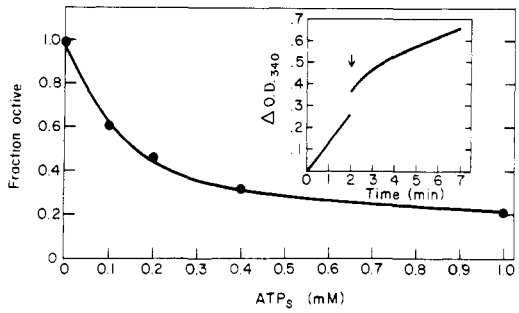

FIGURE 2: The fraction of enzyme active at equilibrium as a function of Sigma Grade ATP (ATP_s) in the assay. The coupled assay was used with 28 mM $MgCl_2$, 3 mM ATP_c, 3 μg/mL dog kidney enzyme, and various concentrations of ATP_s. The active fraction was determined by dividing final linear hydrolysis rates (after approximately 10 min) by initial rates as described in the Experimental Section. The inset shows the time dependence of the inhibition resulting from addition of 1 mM ATP_s at the arrow.

hydrolysis. The high concentration of free Mg^{2+} (about 25 mM) serves to maximize the effects of the inhibitor. One unit of inhibitor is the amount required for 50% inhibition in a 1 mL assay solution containing 3 mM ATP_c and 28 mM $MgCl_2$ with other conditions as given in the Experimental Section. Figure 2 was employed as a standard curve to obtain the amount of inhibitor in samples. Under these assay conditions, the concentration of (Na-K)ATPase, as determined by the number of ouabain binding sites, is well below the dissociation constant for the binding of the inhibitor (see below).

Our assay for inhibitor is based not only on the inhibitory activity itself, but also on the characteristic reversal produced by norepinephrine. Following inactivation, the addition of 2.5 mM norepinephrine restores the enzyme to its initial activity. A complete reactivation occurs with a characteristic relaxation rate when norepinephrine is added to the inhibited enzyme (Cantley et al., manuscript in preparation). All inhibition is reversed by 2.5 mM norepinephrine unless otherwise stated.

The fact that only ATP derived from muscle contained significant amounts of (Na-K)ATPase inhibitor suggested muscle as a source for this compound. A mixture of organic phosphates was prepared from rabbit muscle by barium precipitation as described in the Experimental Section. This muscle extract was assayed as described above and found to contain a norepinephrine reversible inhibitor of (Na-K)-ATPase. The extract was applied to a Chelex-100 column as shown in Figure 3A. The column was eluted with distilled water and the fractions assayed for inhibitory activity. The norepinephrine reversibility of this inhibitory activity is discussed below (see Figure 6D). The inhibitory activity elutes at approximately 1.3 void volumes closely trailing the nucleotides. To further ensure that (Na-K)ATPase inhibitor is present in muscle, all solutions employed for the preparation of inhibitor were tested for a norepinephrine reversible inhibitory activity; the absence of this type of activity clearly demonstrates that the source of the inhibitor is muscle tissue.

A delipidated equine muscle extract used in the preparation of Sigma Grade ATP (personal communication from Sigma Chemical Co.) was obtained from Sigma Chemical Co. and analyzed for inhibitor content. The ratio of inhibitor to ATP was 13.6 units/μmol of ATP in the equine muscle extract, 5.0 units/μmol of ATP in our rabbit muscle extract, and 6.2 units/μmol of ATP in Sigma Grade ATP.

Separation of Inhibitor from Sigma Grade ATP. Initial attempts to separate inhibitor from ATP employing anion-exchange columns (Cellex-D), cation-exchange columns (AG-50), barium precipitation, and organic extractions

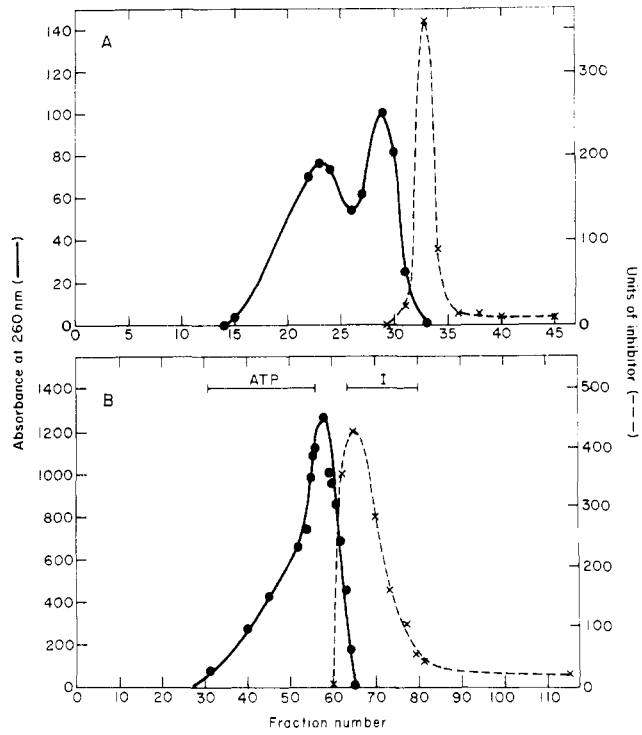

FIGURE 3: (A) The separation of inhibitor from rabbit muscle extract. Three milliliters of the extract described in the Experimental Section was added to a 2 × 55 cm Chelex-100 column (sodium form equilibrated in deionized distilled water). The column was eluted with deionized-distilled water and 3.6-mL fractions were collected and checked for absorbance at 260 nm (●) and inhibitor content (X) as described in the text. (B) The separation of inhibitor from Sigma Grade ATP. Ten milliliters of 0.5 M Sigma Grade ATP (sodium salt, pH 7.0) was added to a 1.5 × 150 cm Chelex-100 column. The column was eluted with deionized-distilled water and 3.6-mL fractions were collected and checked for absorbance at 260 nm (●) and inhibitor content (X).

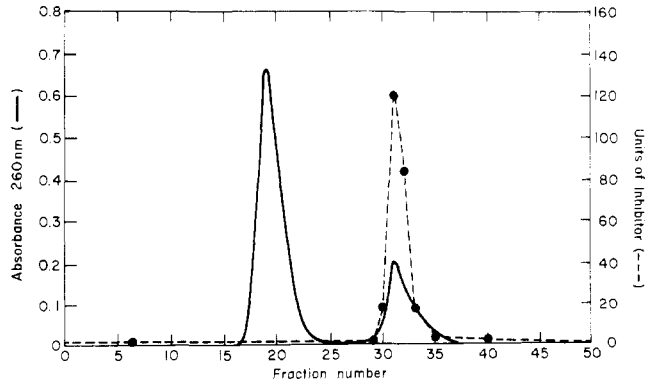

FIGURE 4: Approximately 0.5 mL of inhibitor from the Chelex-100 column in Figure 3B (concentrated to 600 units/mL by lyophilization) was added to a 1.2 × 55 cm Sephadex G-10 column (equilibrated in deionized-distilled water). The column was eluted with deionized-distilled water and 1.5-mL fractions were collected after passing the eluate through a flow cell monitoring at 260 nm. The inhibitor was assayed as described in the text.

(ether/water and chloroform:methanol/water) failed to produce a significant separation of ATP and inhibitor. A Chelex-100 column gave a reasonable separation of ATP and inhibitor (Figure 3B). The fractions richest in inhibitor were lyophilized and added to a Sephadex G-10 column after adjusting the pH to 7.0 with 0.1 N NaOH. Sephadex G-10 produces an excellent separation of the remaining ATP from the inhibitor (Figure 4) but the resin's inability to tolerate high

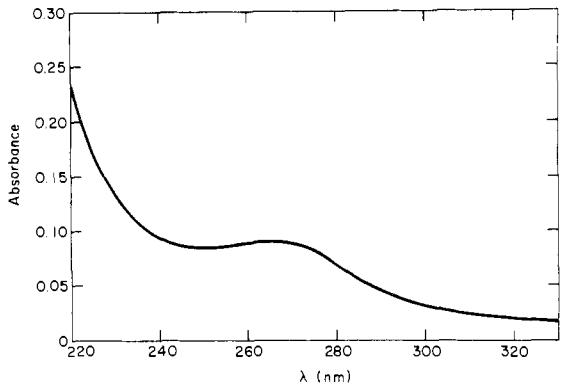

FIGURE 5: An absorption spectrum of the inhibitor after purification as described in the text. The inhibitor concentration was 600 units/mL in 10 mM sodium sulfate at pH 6.0, 25 °C. The reference solution contained 10 mM sodium sulfate.

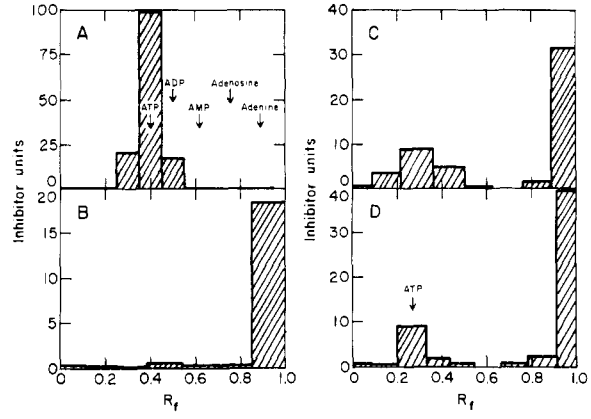

FIGURE 6: Thin-layer chromatography of the inhibitor in the acidic chromatographic system described in the Experimental Section. (A) Purified inhibitor (the arrows show mobilities of successive hydrolysis products of ATP). (B) Inhibitor after 3 h in 1 N NaOH (Table I). (C) Inhibitor after alkaline phosphatase treatment (Table I). (D) Inhibitor from rabbit muscle after a Chelex-100 column (Figure 3A). ATP (arrow) moved slightly slower in this chromatogram than in Figure 3A because of a higher salt content. R_f is the fractional distance to the solvent front.

concentrations and large quantities of ATP precludes its use for the entire purification scheme. Fractions containing the inhibitor were again concentrated by lyophilization and water was added to give an activity of approximately 6×10^3 units/mL. An equal volume of 1 M barium acetate was added to precipitate the inhibitor and the supernatant was discarded. The precipitate was washed with 1 M barium acetate. Resolubilization of the inhibitor was accomplished by adding a volume of 0.1 M sodium sulfate equal to the original volume. The yield of purified inhibitor is approximately 20% of that in ATP_S. Purified inhibitor is stored at −20 °C.

A spectrum of the purified inhibitor is shown in Figure 5. The absorption peak at 265 nm clearly differs from the 259-nm absorption peak of ATP. In addition, the ratio of the absorption at shorter wavelengths, i.e., 240 nm, to that at 260 nm exceeds that seen with ATP (Bock et al., 1956).

Stability of the Inhibitor. The inhibitory activity is not labile at neutral pHs or moderate temperatures (37 °C). The inhibitory activity is destroyed by a 24-h incubation with 1.0 N NaOH but is remarkably stable to acid and treatment with alkaline phosphatase (see Table I).

Mobility of the Inhibitor on Thin-Layer Chromatographic Systems. Figure 6A compares the mobility of purified inhibitor with the mobility of a variety of compounds resolved by the acidic thin-layer system. The inhibitory activity and a UV-absorbing spot co-chromatograph with ATP in the acidic system or in the basic thin-layer system (data not shown).

The mobility of the (Na-K)ATPase inhibitor activity obtained from the purification described may be altered by a 3-h incubation with 1 N NaOH (Figure 6B). A 24-h incubation with 1 N HCl produces a similar result (data not shown) as does incubation with alkaline phosphatase (Figure 6C). The results suggest that the removal of one or more phosphate groups increases the mobility of the inhibitor without destroying its activity.

Figure 6D shows the chromatogram of inhibitor from rabbit muscle after partial purification on Chelex-100 (Figure 3A). The inhibitory activity migrates at two positions much like the alkaline phosphatase treated inhibitor. It appears that a large portion of the rabbit muscle inhibitor may be hydrolyzed either before or during the purification but that rabbit muscle does contain an inhibitory activity that cochromatographs with ATP.

The kinetics of inactivation and norepinephrine produced reactivation obtained with the NaOH-treated form of the inhibitor differed from those observed with the untreated form. Inhibitory activity migrating near the solvent front produced

TABLE I: Stability of Inhibitor.

Treatment[a]	Remaining inhibitory act.	Fraction with R_f 0.4[b]	Fraction with R_f >0.9[b]
	1.0	1.0	0
1 N NaOH, 3 h, 120 °C	1.0	0.02	0.98
1 N NaOH, 24 h, 120 °C	0.02		
1 N HCl, 24 h, 120 °C	1.0	0.3	0.7
Alkaline phosphatase[c]	0.9	0.35	0.65

[a] Inhibitor purified from Sigma Grade ATP was used. [b] From Figure 6. [c] 0.1 mg/mL alkaline phosphatase, 65 °C, pH 8.0 for 90 min. In a parallel experiment, >95% of ATP was converted to adenosine by this treatment.

a more rapid inhibition and reactivation than that obtained with comparable inhibitory activities of untreated inhibitor. In addition, the reactivation by norepinephrine was far from complete with the NaOH-treated form of the inhibitor (20 to 40% of the inhibition was reversed by 2.5 mM norepinephrine). All inhibitory activity migrating at the solvent front gave similar kinetics regardless of the treatment employed. The inhibitory activity of the untreated form of the inhibitor was completely norepinephrine reversible and we shall refer to this compound as muscle inhibitory factor (MIF).

A variety of ATP like compounds were tested for their ability to cause a norepinephrine reversible inhibition of the (Na-K)ATPase at concentrations up to 100 μM, employing the assay in Figure 2. Of the compounds tested (GTP, ITP, CTP, 3′,5′-cAMP, adenosine 5′-tetraphosphate, diadenosine-5′,5‴-P^1,P^4-tetraphosphate, diadenosine 5′,5‴-P^1,P^3-triphosphate, reduced CoA, acetyl CoA, and palmityl CoA), only diadenosine-5′,5‴-P^1,P^4-tetraphosphate and palmityl CoA produced more than 10% inhibition and this inhibition was not reversed by norepinephrine.

A variety of metal salts was also tested for MIF-like inhibition including $CaCl_2$, $Ba(CH_3COO)_2$, $FeSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Hg(CH_3COO)_2$, $CuCl_2$, K_2CrO_4, and $K_2Cr_2O_7$. No norepinephrine reversible inhibition was observed, although up to 50% inhibition of initial velocities was observed at 100 μM concentrations (1 mM dithiothreitol was present in all assays).

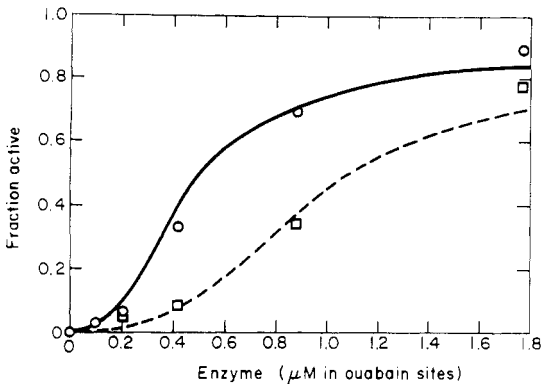

FIGURE 7: The fraction of dog kidney enzyme active following a 30-min incubation in 20 mM Tris-Cl (pH 7.4), 20 mM KCl, 20 mM $MgCl_2$, 25 °C, and either 1 mM (○) or 2 mM (□) of the Tris salt of Sigma Grade ATP (ATP_s) as a function of (Na–K)ATPase concentration. The enzyme concentration is expressed in ouabain binding sites (see text). The fraction active was calculated as described in the Experimental Section. The lines were drawn using eq 2 and the parameters K_I = 6 nm and (I_t) = 0.27 μM (solid line) or (I_t) = 0.54 μM (dashed line).

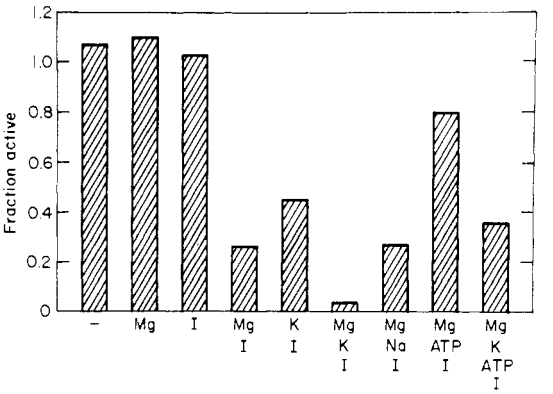

FIGURE 8: The fraction of dog kidney enzyme active following a 30-min incubation in 20 mM Tris-Cl (pH 7.4) at 37 °C. The fraction active was calculated as described in the Experimental Section. Where indicated to be present, the ligand concentrations were 12 units/mL inhibitor (I), 25 mM $MgCl_2$, 25 mM KCl, 100 mM NaCl, and 3 mM Chelex-100 purified ATP.

The Affinity of MIF for the (Na–K)ATPase. The K_I of MIF and the concentrations added with known concentrations of ATP_s can be estimated if one assumes that the number of inhibitor binding sites is equal to the number of ouabain binding sites (the concentration of ouabain binding sites on the dog kidney enzyme is 2.5 ± 0.5 nmol/mg; Cantley, unpublished results). The fraction of active enzyme at equilibrium may be expressed by eq 1 where

$$\text{fraction active} = (E)/(E_t) = [K_I(E \cdot I)]/[(E_t)(I_f)] \quad (1)$$

(E) is the concentration of active enzyme, (E_t) is the total concentration of enzyme, (I_f) is the concentration of free MIF, (E·I) is the concentration of inhibited enzyme, and K_I is the dissociation constant for the E·I complex. The relationship between (I_t), the total concentration of inhibitor, (E_t), and the fraction of active enzyme molecules is given by eq 2.

$$\text{fraction active} = [-A + \sqrt{A^2 + 4K_I(E_t)}]/[2(E_t)] \quad (2)$$

$$A = (I_t) + K_I - (E_t)$$

Figure 7 shows the variation in the fraction of active enzyme molecules resulting from varying enzyme concentration in the presence of 1 or 2 mM ATP. The data were fit to eq 2 using a nonlinear least-squares computer program assuming that (I_t) is proportional to ATP_s. From this procedure, (I_t) = 0.27 μM per 1 mM ATP_s and K_I = 6 nM. The value of (I_t) may then be employed to estimate the affinity of the inhibitor under standard assay conditions (Figure 2). Under these conditions, 50% inhibition of enzyme activity occurs at 40 nM MIF.

The estimate of K_I obtained in this fashion is in agreement with the results of a total phosphate analysis using the procedure of Fiske and SubbaRow (1957). One unit of purified MIF per mL is 43 nM in phosphate. If the absorbance at 265 nm is due to a nucleotide base with a typical extinction coefficient of 1.5×10^4 M^{-1} cm^{-1}, then the concentration of MIF causing 50% inhibition is 10 nM. These results are consistent with the presence of phosphate in MIF.

The molecular weight of MIF was estimated by eluting it through a Sephadex G-25 column (1.2 × 55 cm) with 20% acetic acid to eliminate hydrophobic or ionic interactions (data not presented). The MIF activity eluted between the markers tyrosine and NADH with an apparent molecular weight of 330.

Ionic Requirements for Inhibition of (Na–K)ATPase by MIF. The ionic requirements for inhibition were examined by incubating the enzyme for 30 min in the presence of various combinations of Mg^{2+}, K^+, Na^+, and ATP as shown in Figure 8. The ligands were added at concentrations sufficient to achieve saturation of their respective sites (Cantley and Josephson, 1976) and 10 units of purified MIF per mL was added as indicated. In the absence of ligands, MIF had no effect on enzyme activity and only partial inhibition occurred in the presence of K^+ alone. Significant inhibition occurred in the presence of Mg^{2+} alone and inhibition was most pronounced in the presence of Mg^{2+} and K^+. K^+ also potentiated the inhibition in the presence of ATP, while Na^+ had no effect under these conditions. ATP exerted a protective effect in the presence of Mg^{2+}.

The Effects of MIF and Norepinephrine on a Variety of ATPases. We have attempted to define the possible physiological role of the effects of MIF and norepinephrine on the (Na–K)ATPase by examining their effects on other ATPases as shown in Table II. All assays were done in the presence of 25 mM $MgCl_2$ and 3 mM ATP_c, conditions which maximize the effects of MIF on the (Na–K)ATPase. (Ca)ATPase and actomyosin ATPase gave linear rates of hydrolysis under all conditions. The progressive loss of F_1 ATPase activity has been previously described (Cantley and Hammes, 1973; Moyle and Mitchell, 1975; Fagan and Racker, 1977) and was insensitive to the presence of norepinephrine or MIF. (Na–K)ATPase from the electroplax resembles dog kidney (Na–K)ATPase by exhibiting linear rates in the absence of MIF but a progressive inactivation in its presence. Norepinephrine blocks the time-dependent effects of MIF but has no effect on the initial velocity. Dog brain (Na–K)ATPase differs from the eel or dog kidney enzymes by inactivating even in the absence of MIF; this inactivation was blocked by norepinephrine. Addition of MIF accelerated this inactivation and resulted in a substantial inhibition of enzyme activity. Inhibition by MIF and the characteristic protection by norepinephrine occur with (Na–K)ATPases from a number of sources but not with other ATPases.

Discussion

Rabbit muscle contains two compounds detectable by thin-layer chromatography capable of inhibiting dog kidney (Na–K)ATPase. One of these compounds (MIF) cochromatographs with ATP on anion-exchange columns, cation-exchange columns, and in two thin-layer chromatographic sys-

TABLE II: The Effects of MIF and Norepinephrine on Various ATPases.

ATPase	MIF (units/mL)	Norepinephrine (mM)	Initial[a] velocity	Steady-state[b] velocity
F_1			1.00	0.25
	22		0.87	0.22
		2.5	0.80	0.18
Actomyosin[c]			1.00	1.00
	22		1.00	1.18
		2.5	1.00	0.98
(Ca)ATPase[d]			1.00	1.13
	55		0.94	0.97
		2.5	1.12	1.28
(Na-K)ATPase (electroplax)			1.00	1.11
	2.0		0.96	0.58
	6.0		0.96	0.27
	6.0	2.5	0.89	0.97
(Na-K)ATPase (brain)			1.00	0.75
	2.0		1.07	0.29
		2.5	1.07	1.07
	2.0	2.5	1.07	0.88
(Na-K)ATPase (kidney)			1.00	1.00
	6.0		0.88	0.18
	6.0	2.5	0.88	1.03
		2.5	1.00	1.03

[a] Assays were performed under the standard assay conditions given in the Experimental Section, except when 100 mM NaCl was omitted from actomyosin and (Ca)ATPase assays. All velocities for a particular enzyme are relative to the initial velocity in the absence of effectors. [b] The relative velocity after the assay became linear. For F_1 and brain (Na-K)ATPase, the velocity after 5 min was taken since both assays decayed to less than 10% activity without becoming linear. [c] At least 90% of the ATPase was stimulated by actin (2 mg/mL). [d] At least 90% of the ATPase activity was dependent on the presence of 200 μM $CaCl_2$.

tems. It occurs in commercial sources of muscle derived ATP and is necessary for the (Na-K)ATPase inactivation reported earlier (Cantley and Josephson, 1976; Fagan and Racker, 1977). We have developed a method of purifying MIF by the use of Chelex-100 and Sephadex G-10 and have examined the ultraviolet spectrum of MIF. The material is characterized by an absorption maximum at 265 nm. The inhibitory activity is stable to acid or base treatment, although prolonged exposure to acid, base, or alkaline phosphatase alters its mobility on a thin-layer chromatographic system and reduces the characteristic norepinephrine reversibility of its inhibition. A variety of compounds thought to be capable of copurifying with ATP were tested for MIF-like inhibition of (Na-K)ATPase. None significantly mimicked the effects of MIF.

The chemical nature of MIF is as yet unresolved. The failure of EDTA to prevent inhibition except at concentrations stoichiometric with Mg^{2+} (Cantley and Josephson, 1976; Fagan and Racker, 1977) and the failure of dithiothreitol to effect inhibition at millimolar concentrations make a metal unlikely. The ability of MIF to precipitate with Ba^{2+} and its migration with ATP on anion-exchange columns clearly indicate a highly negatively charged compound. The absorbance spectrum is unlike any common nucleotide and the resistance of MIF to acid, base, and alkaline phosphatase treatments (conditions which would hydrolyze ATP) make it unlikely that MIF's *essential* structure is a nucleoside phosphate. An unusually stable, nonexchangeable metal-nucleotide complex cannot be ruled out at this point. MIF migrates on a Sephadex G-25 column with an apparent molecular weight of 330 and phosphate copurifies with MIF activity in stoichiometric amounts. A more detailed chemical analysis of MIF is now in progress.

The assay we have developed for MIF activity maximizes the inhibitory effects by employing high concentrations of free Mg^{2+} and long incubation periods (i.e., 10 to 20 min). Provided that assays are done in this fashion, MIF inhibition and the characteristic norepinephrine antagonism of this inhibition occur with a variety of (Na-K)ATPases. Other ATPases, including the (Ca)ATPase from sarcoplasmic reticulum, fail to respond to these effectors.

In view of the high affinity of this inhibitor for the (Na-K)ATPase and its inability to inhibit other ATPases (Table II), it is of interest to compare its properties with the cardiac glycoside, ouabain. Like ouabain, muscle derived inhibitor eliminates all observed activities of the (Na-K)ATPase including (Na)ATPase activity, (Na-K)ATPase activity, K^+-stimulated *p*-nitrophenylphosphatase activity, and Na^+-dependent phosphorylation (Cantley and Josephson, 1976; Fagan and Racker, 1977). Both inhibitors depend for their action on the presence of ligands required for ATP hydrolysis. Under certain conditions inhibition can occur slowly or not at all. The combination of ligands favoring the inhibitory action of each compound differs greatly, however. Ouabain binds most strongly in the presence of Mg^{2+}, Na^+, and Mg-ATP (Lane et al., 1973), while MIF fails to inhibit under these conditions (linear Na^+-stimulated ATPase activity is obtained even in the presence of high concentrations of free Mg^{2+} and Sigma Grade ATP; Cantley and Josephson, 1976). MIF inhibits more strongly in the presence of K^+, a ligand which antagonizes ouabain binding under all conditions (Schwartz et al., 1975).

The presence of MIF in muscle tissue and its high specificity as a (Na-K)ATPase inhibitor suggest that it may play a role in regulating active transport in vivo.

Note Added in Proof

While this paper was in press we were able to determine that MIF is vanadium in the 5+ oxidation state (vanadate). Sodium *o*-vanadate has an absorption spectrum similar to that presented in Figure 5 and migrates with ATP on the acidic thin-layer chromatography system. It exhibits a K_I of 40 nM for the dog kidney (Na-K)ATPase under standard assay conditions and its inhibition is reversed by norepinephrine. A manuscript containing the data supporting this conclusion and a discussion of the physiological significance has been accepted for publication [Cantley, L. C., Jr., et al. (1977), *J. Biol. Chem.*, in press]. Two other papers have been published acknowledging the presence of a (Na-K)ATPase inhibitor in Sigma Grade ATP while this paper was in press [Beaugé, L. A., and Glynn, I. M. (1977), *Nature (London) 268*, 355; Hudgins, P. M., and Bond, G. H. (1977), *Biochem. Biophys. Res. Commun. 77*, 1024].

Acknowledgment

We would like to thank Professor Guido Guidotti for his continued support and helpful suggestions throughout this work.

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Selective Phosphorylation of Erythrocyte Membrane Proteins by the Solubilized Membrane Protein Kinases†

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ABSTRACT: This report describes the substrate and phosphoryl donor specificities of solubilized erythrocyte membrane cyclic adenosine 3′,5′-monophosphate (cAMP)-independent protein kinases toward human and rabbit erythrocyte membrane proteins. Three types of substrate preparations have been utilized: heat-inactivated ghosts, isolated spectrin, and 2,3-dimethylmaleic anhydride (DMMA)-extracted membranes. A 30 000-dalton protein kinase, extracted from either human or rabbit erythrocyte membranes, catalyzes the phosphorylation of heat-inactivated membranes in the presence of ATP. The resulting phosphorylation profile is analogous to that of the autophosphorylation of membranes with ATP (in the absence of cAMP). These kinases also phosphorylate band 2 of isolated spectrin and band 3, but not glycophorin, in the DMMA-extracted ghosts. The ability of the 30 000-dalton kinases to use GTP as a phosphoryl donor appears to be related to the substrate or some other membrane factor. A second kinase, which is 100 000 daltons and derived from rabbit erythrocyte membranes, uses ATP or GTP to phosphorylate membrane proteins 2, 2.1, 2.9–3 in heat-inactivated ghosts, band 2 in isolated spectrin, glycophorin, and to a lesser extent, band 3 in the DMMA-extracted ghosts.

Erythrocyte membrane proteins can be phosphorylated by membrane-bound (Avruch and Fairbanks, 1974; Fairbanks and Avruch, 1974; Hosey and Tao, 1976) and soluble (Hosey and Tao, 1977a) cAMP[1]-dependent and -independent protein kinases. Multiple protein kinase activities are found in rabbit and in human red cell membranes. The autophosphorylation of human erythrocyte membranes is catalyzed by cAMP-dependent and -independent protein kinases. In contrast, the autophosphorylation of rabbit erythrocyte membranes appears to be catalyzed by two protein kinases both of which are independent of cAMP. The cAMP-independent protein kinases from rabbit and human erythrocyte membranes have been solubilized and partially purified (Hosey and Tao, 1977b).

This report deals with the phosphorylation of red cell membrane proteins by these solubilized enzymes. The data suggest that a 30 000-dalton kinase, found in human and in rabbit erythrocyte membranes, can elicit a pattern of phosphorylation in heat-inactivated membranes similar to that obtained in membrane autophosphorylation in the presence of either ATP or GTP. A second kinase of approximately 100 000 daltons, which is extracted from rabbit erythrocyte membranes, uses either ATP or GTP to phosphorylate glycophorin in 2,3-dimethylmaleic anhydride extracted ghosts and to a lesser extent, bands 2–2.1 and 2.9–3 in heated membranes.

Experimental Procedures

Materials. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from either New England Nuclear or Amersham/Searle. ATP and GTP were purchased from P-L Biochemicals. 2,3-Dimethylmaleic anhydride (DMMA) was purchased from Sigma Chemical Co. Electrophoresis supplies were obtained from Bio-Rad Laboratories. Frozen rabbit red blood cells were supplied by Pel-Freez.

Methods. Protein kinases were prepared from membranes of frozen rabbit or outdated human red blood cells as previously described (Hosey and Tao, 1977b). Briefly, the enzymes were extracted from erythrocyte ghosts with 0.5 M NaCl, concen-

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; DMMA, 2,3-dimethylmaleic anhydride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid–Schiff base; Tris, tris(hydroxymethyl)aminomethane.