(Mg^{2+} + K^{+})-DEPENDENT INHIBITION OF NaK-ATPase DUE TO A CONTAMINANT IN EQUINE MUSCLE ATP

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SUMMARY: A contaminant present in commercial ATP isolated from equine muscle induces a (Mg^{2+} + K^{+})-dependent inhibition of human red cell NaK-ATPase (EC 3.6.1.3). This inhibition is completely reversed by isoproterenol (1 mM). The contaminant (inhibitor) is essentially lacking in ATP prepared synthetically by phosphorylation of adenosine. A procedure is described for separation of the inhibitor from equine muscle ATP with strongly acidic cation exchange resin. In the presence of isolated inhibitor, a (Mg^{2+} + K^{+})-dependent inhibition of NaK-ATPase can be demonstrated with synthetic ATP as the substrate.

INTRODUCTION: We previously described an unusual inhibition of red cell NaK-ATPase by Mg^{2+} and K^{+} in combination (1,2). Inhibition becomes more pronounced with increasing concentrations of Mg^{2+} and K^{+}, and is antagonized by Na^{+}. The kinetics of inhibition are not consistent with competition on the part of either Mg^{2+} or K^{+} at Na^{+} activation sites. Others have recently reported similar results with NaK-ATPase preparations from other tissues (3-5).

The ATP used in our studies was obtained from equine muscle (Sigma Chemical Co., St. Louis, MO). In this paper we will show that the inhibition described above is caused by a contaminant in the ATP. The contaminant is essentially lacking in ATP prepared synthetically by phosphorylation of adenosine (Sigma I ATP). This investigation was prompted by two observations. First, in a footnote to the paper by Fagan and Racker (4), it was mentioned that a nucleotide other than ATP in commercial ATP preparations, and difficult to separate from ATP, was responsible for inhibition by Mg^{2+} and K^{+}. Second, Charney et al. (6) reported the presence of an inhibitor of NaK-ATPase in equine muscle ATP (Sigma Chemical Co.), which was not found in synthetic ATP (Sigma II ATP). The latter report did not, however, note an

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ionic dependence for inhibition by the contaminant in equine muscle ATP.

The purpose of this report is to describe: 1) specific assay conditions under which inhibition can be demonstrated when equine muscle ATP is used as the substrate; 2) reversal of inhibition by isoproterenol; and 3) a simple procedure for removal of the inhibitor from equine muscle ATP.

METHODS: Red cell membranes were prepared from freshly outdated human blood as previously described (7). The protein content of each preparation was measured by the method of Lowry et al. (8). ATPase activity was assayed by measuring the production of inorganic phosphate (Pi) according to modification of the method of Fiske and SubbaRow (9).

The following conditions were common to all experiments: disodium or Tris·ATP 2 mM; Tris·HCl (pH 7.4) 63 mM; and Tris·EGTA [ethylene glycol-bis (β-aminoethylether) N,N'-tetraacetic acid] 0.25 mM. For measurement of Mg-ATPase activity, K^+ was omitted and 0.25 mM ouabain was added. The volume was 2 ml and the incubation temperature was 38°C. Other conditions are described in the text. NaK-ATPase activity was taken as the difference between the total ATPase activity in the presence of Na^+ and K^+, and the Mg-ATPase activity measured concurrently. All figures represent the average of two or more experiments run in duplicate.

Removal of the inhibitor from equine muscle ATP was accomplished by batch or column chromatography with Dowex 50x8 cation exchange resin in the H^+-form. In the batch procedure, equine muscle ATP was exposed to the resin for 45 min. The supernatant fluid was removed and the ATP was neutralized to pH 7.8 with Tris base. This ATP was essentially free of the inhibitor. The resin was repeatedly washed to remove residual ATP as verified by monitoring the absorbance of the washes at 259 nm. The inhibitor was then eluted from the resin by addition of Tris base until the pH of the slurry was 7.8. Supernatant fluid (Dowex eluate) contained the inhibitor.

RESULTS AND DISCUSSION: Figure 1 shows NaK-ATPase activity at three Mg^{2+} concentrations and with combinations of equine muscle and synthetic ATP selected to maintain a constant total ATP concentration of 2 mM. As the concentration of equine muscle ATP increased, a Mg^{2+}-dependent inhibition was evident. With 4 mM Mg^{2+}, a nearly saturating concentration of the inhibitor was contributed by 2 mM equine muscle ATP. At this point inhibition was not complete, and was limited by the availability of Mg^{2+} (1,2). With 2 mM synthetic ATP there was only a small effect of Mg^{2+} variation, and optimal activity was obtained with equimolar Mg^{2+} and ATP, in agreement with past observations (10-12).

Figure 2 compares the kinetics of activation and inhibition of NaK-ATPase by K^+, at two Mg^{2+} concentrations, with equine muscle and synthetic ATP. With equine muscle ATP (Fig. 2 A), the response to increasing

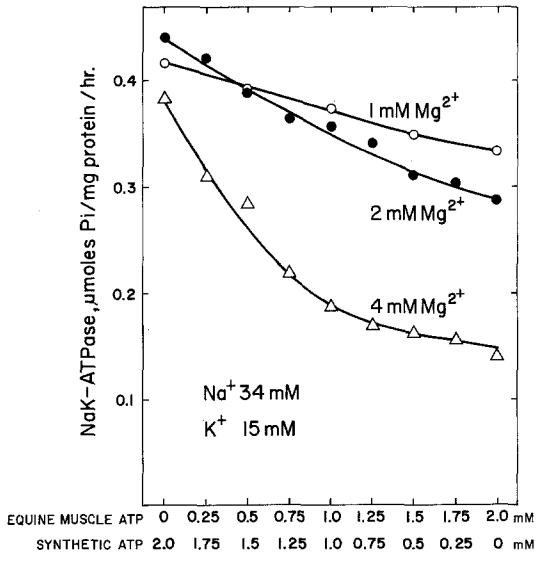

Fig. 1. NaK-ATPase activity with reciprocal variation of equine muscle ATP and synthetic ATP to maintain a total ATP concentration of 2 mM. Other conditions are described in Methods.

K^+ concentration was biphasic at the higher Mg^{2+} concentration. Activity rose to a peak, then declined sharply to a plateau. This is characteristic of inhibition by Mg^{2+} and K^+ (1,2,5). Inhibition was only slightly detectable at the lower Mg^{2+} concentration. It is, therefore, a combined effect of both cations. With synthetic ATP (Fig. 2 B), inhibition by Mg^{2+} and K^+ was not seen; there was no biphasic response to K^+ at the higher Mg^{2+} concentration. Clearly, an inhibitor in equine muscle ATP markedly alters the response of NaK-ATPase to Mg^{2+} and K^+.

It has been reported that catecholamines activate NaK-ATPase derived from brain (13). Fagan and Racker (4) found that catecholamines prevented inhibition of kidney NaK-ATPase by Mg^{2+} and K^+, and suggested that the activation could simply be a reversal of inhibition. The results of Fig. 3 support this conclusion. With 4 mM Mg^{2+} and equine muscle ATP, inhibition by K^+ was prevented by isoproterenol (Fig. 3 A). With synthetic ATP as the substrate, however, isoproterenol had little effect (Fig. 3 B). That some

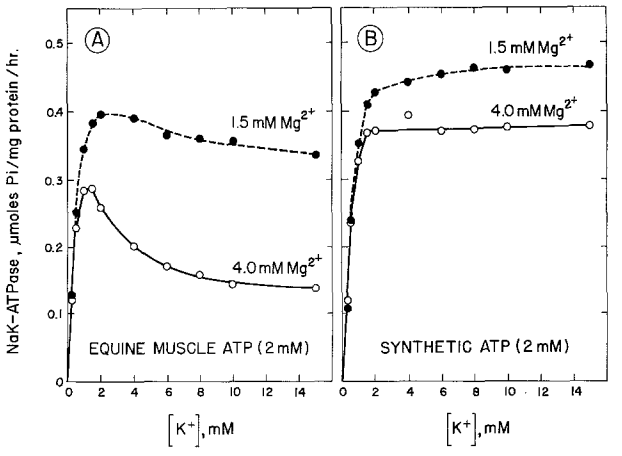

Fig. 2. NaK-ATPase activity as a function of K^+ concentration with equine muscle ATP (panel A) and synthetic ATP (panel B). The Na^+ concentration was 34 mM.

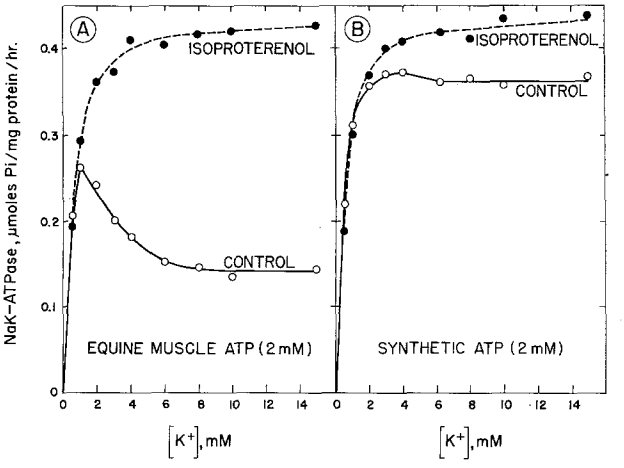

Fig. 3. NaK-ATPase activity as a function of K^+ concentration ± isoproterenol (1.0 mM) with equine muscle ATP (panel A) and synthetic ATP (panel B). The Na^+ concentration was 34 mM.

effect was seen could be taken as an indication that synthetic ATP may not be entirely free of inhibitor.

Table 1 summarizes the results of experiments comparing NaK-ATPase activity under various assay conditions. The first column shows that under

conditions routinely employed for assay of NaK-ATPase, activities with equine muscle ATP and synthetic ATP are the same. This is because inhibition by Mg^{2+} and K^{+} is less pronounced at a higher Na^{+} concentration (1,2). At a lower Na^{+} concentration, there was significant inhibition with equine muscle ATP as compared to synthetic ATP even with 1 mM Mg^{2+} (second column). The inhibition was greater with 4 mM Mg^{2+} (third column). The depression of activity with 4 mM Mg^{2+} as compared to 1 mM Mg^{2+} is a convenient measure of inhibition by Mg^{2+} and K^{+} (see Figs. 1 and 2 A).

In the course of these experiments we found a simple procedure for removal of the inhibitor from equine muscle ATP. This involves exposure to Dowex 50 cation exchange resin in the H^{+}-form, as described in Methods. The inhibitor was not removed by resin in the $Tris^{+}$-form. The success of this treatment is illustrated in Table 1 by comparing activity with Dowex-treated equine muscle ATP at 1 and 4 mM Mg^{2+}. There was some inhibition with 4 mM Mg^{2+} as compared to 1 mM Mg^{2+}, indicating that the inhibitor had not been completely removed, but the inhibition was markedly reduced in comparison to untreated equine muscle ATP. When the inhibitor was eluted from the resin and added to an incubation system containing synthetic ATP, inhibition by Mg^{2+} and K^{+} was identical to that seen with untreated equine muscle ATP.

Charney et al. (6) performed atomic absorption spectrophotometry on equine muscle and synthetic ATP, and reported the iron contents to be 6.5 and 2.3 mmoles/mole ATP, respectively. The content of other heavy metals was the same in each. We found that Fe^{2+} or Fe^{3+}, at concentrations of 10 μM, were not inhibitory in assays conducted with synthetic ATP at 1 and 4 mM Mg^{2+}. It does not appear, therefore, that the inhibitor is a heavy metal. The fact that the inhibitor associated with Dowex 50 in the H^{+}-form, but not in the $Tris^{+}$-form, suggests that it may be an organic base which can accept a proton to become positively charged.

If the inhibitor is a natural constituent of mammalian tissue, it could function as an endogenous regulator of the enzyme. Further characterization

Table 1. Comparison of human red cell NaK-ATPase activity with synthetic and equine muscle ATP as substrates. Assay and chromatography procedures are described in Methods.

	Cation Concentration (in millimoles/liter)		
	Na^+ 120, K^+ 10, Mg^{2+} 2	Na^+ 34, K^+ 15, Mg^{2+} 1	Na^+ 34, K^+ 15, Mg^{2+} 4
Substrate (2 mM)	NaK-ATPase Activity (μmoles Pi/mg protein/hr)		
Synthetic ATP	0.43 ± .01 (3)[a]	0.40 ± .01 (5)	0.38 ± .01 (5)
Equine muscle ATP	0.41 ± .01 (3)	0.33 ± .01 (5)	0.14 ± .01 (5)
Dowex-treated Equine muscle ATP	-	0.37 ± .01 (8)	0.31 ± .02 (8)
Dowex-eluate + Synthetic ATP	-	0.32 ± .02 (5)	0.15 ± .03 (5)

[a] Mean ± S. E. (number of duplicate determinations)

may provide significant information concerning the control of the NaK-pump *in vivo*.

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