SANGUINARINE, INHIBITOR OF NA-K DEPENDENT ATP'ASE

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SUMMARY

Sanguinarine, a benzophenanthridine alkaloid, has been found to be an inhibitor of the Na^+,K^+-ATPase isolated from guinea pig brain. It has a pI_{50} of 5.25 (5.62 μM) and exhibits uncompetitive kinetics with respect to $[Na^+]$ and $[K^+]$ and is non-competitive with respect to [ATP]. Preincubation with sanguinarine causes increased inhibition. ATP partially protects the enzyme against this preincubation effect, while Na^+ protects to a lesser extent and K^+ has no effect.

INTRODUCTION

The study of the mechanisms of action of the membrane-bound Na^+,K^+-ATPase has been significantly aided by use of cardiac glycoside (1-4) inhibitors. More recently, Harmaline, a carboline derivative, has been shown to be a competitive inhibitor of the enzyme with respect to Na^+-dependent phosphorylation by ATP (5). Oligomycin, an inhibitor of mitochondria ATP'ase and oxidative phosphorylation, also inhibits the K^+-dependent dephosphorylation of the enzyme, thus suggesting the possibility of some common or similar reaction mechanisms between oxidative phosphorylation and Na^+K^+-ATPase (4,6,7). This is a preliminary report on the inhibition of the Na^+,K^+-ATPase by sanguinarine, a benzophenanthridine alkaloid. This class of compounds has been reported to inhibit oxidative phosphorylation in mitochondria (8) and to uncouple photosynthetic phosphorylation (9).

METHODS

Na^+,K^+-ATPase was prepared from guinea pig brain by the procedure of Akera and Brody (10). The enzyme was diluted to a final volume of 400 ml and kept frozen in 3-5 ml aliquots in capped tubes. The average specific activity of the preparations after being thawed once was 300 µmoles Pi liberated/mg protein/hour.

Protein concentration was determined by the insoluble protein procedure of Lowry (11). Protein concentrations ranged from 20 to 35 ug/ml.

Enzyme incubations were carried out in a total volume of 1 ml. All additions were made at 0°C. Incubations were carried out at 37°C for 30 minutes. The enzyme was incubated with 50 mM imidazole, pH 7.4 at 24°C, and various concentrations of NaCl, KCl, and tris or disodium ATP. The $MgCl_2$ concentration was maintained at 3 mM per incubation except in one set of ATP response curves in which the ATP/Mg^{++} concentration ratio was kept at 1. The reaction was initiated with the addition of ATP except in those experiments which required a preincubation. The order of addition was kept the same where possible. The reaction was terminated by addition of 0.1 ml of 40% cold trichloroacetic acid.

In the series of preincubation experiments the procedure was essentially the same. The only variation was that after adding the desired reactants, the total volume of each tube was brought to 0.5 ml and the tubes were preincubated for the desired length of time at 37°C. At the end of this preincubation, the tubes were returned to the ice bath and the remaining additions were made. The tubes were then returned to the 37°C bath and the incubation was continued as previously described.

Phosphate determination was by the method of Gomori (12) with minor modifications.

MATERIALS

Disodium ATP, tris ATP, free acid EDTA, histidine HCl, and imidazole were purchased from Sigma Chemical Co., St. Louis, Missouri; Sodium

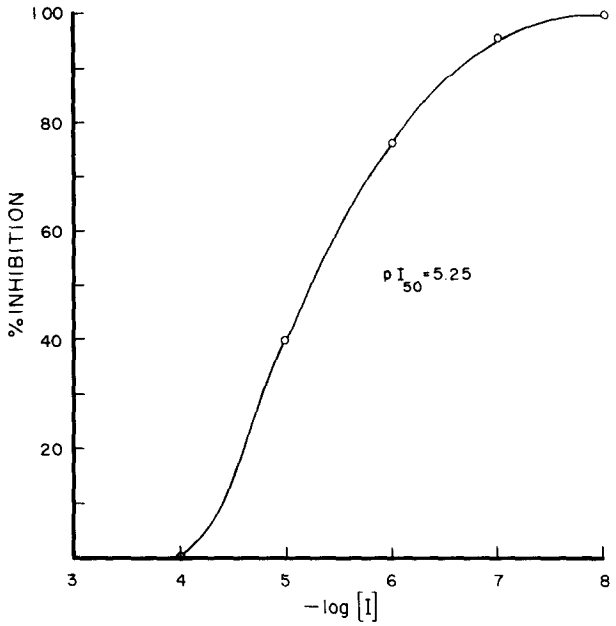

FIGURE 1.

Inhibition of the Na^+,K^+-ATPase as a function of Sanguinarine concentration. Final protein concentrations were 3.2 μg/ml.

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deoxycholate and enzyme grade sucrose from Schwartz Mann, Orangeburg, New York; NaCl, KCl and $MgCl_2$ from Fisher Scientific Co., St. Louis, Missouri; and Sanguinarine sulfate from Pfaltz and Bauer, Inc., Flushing, New York.

RESULTS

The Na^+,K^+-ATPase is strongly inhibited by the presence of sanguinarine. As can be seen in Figure 1, the concentration required for half-maximal inhibition is approximately 5×10^{-6} M. The ouabain-insensitive fraction (about 8-10% of the total activity) has a half maximal inhibition of sanguinarine of about 4×10^{-5} M (not shown) The steady state kinetics of this inhibition were further studied by varying Na^+, K^+, and ATP concentrations over the range which excluded low level inhibition by Na^+ or K^+ and high level inhibitions by K^+. Activity of

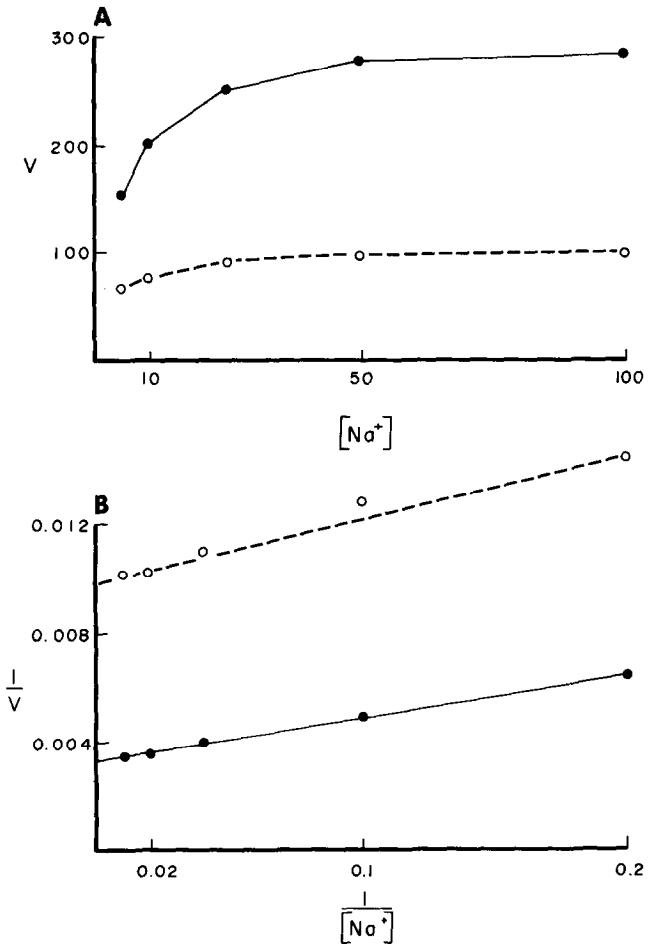

FIGURE 2.

Effects of NaCl concentration on the Na^+,K^+-ATPase activity. The incubations contained 3 mM $MgCl_2$, 50 mM imidazole, 20mM KCl, 3 mM Tris ATP, varying concentrations of NaCl and were run either with (0---0) or without (●---●) 10^{-5}M sanguinarine. In both 1-A and 1-B V, is in μmoles Pi released per mg protein per hour and $[Na^+]$ is in mM. The data shown are representative of the six experiments run. Each point represents the average of three determinations.

the enzyme is plotted against sodium concentrations and in addition presented as a Lineweaver-Burke plot in Figure 2. Over this region of $[Na^+]$, Michaelis-Menton first-order reaction kinetics are observed and sanguinarine is approximately uncompetitive with sodium over this range.

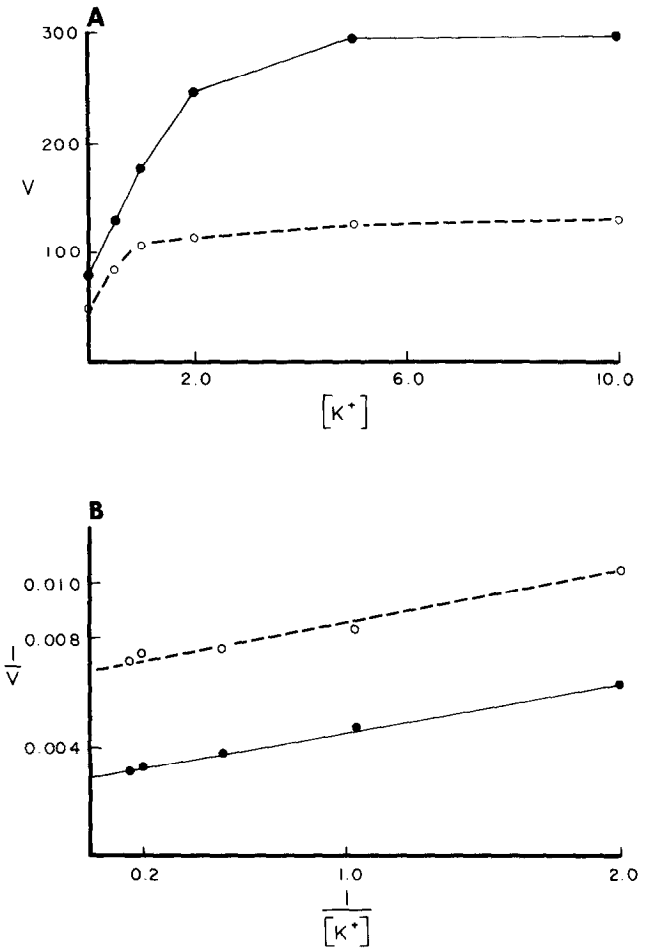

FIGURE 3.

Effects of KCl concentration on the Na^+,K^+-ATPase activity. The incubations contained 3 mM $MgCl_2$, 50 mM imidazole, 100 mM NaCl, 3mM Tris ATP, plus the concentrations of KCl shown and were run either with (0---0) or without (●---●) 10^{-5}M sanguinarine. In each plot V is in μmoles Pi/mg/hour aud KCl concentration is in mM. The data shown are representative of five experiments. Each point is the average of 3 separate incubations.

The same graphical presentation of activity as a function of K^+ concentrations is seen in Figure 3; sanguinarine also seems to be uncompetitive for $[K^+]$.

However, velocity as a function of ATP concentration shows definite

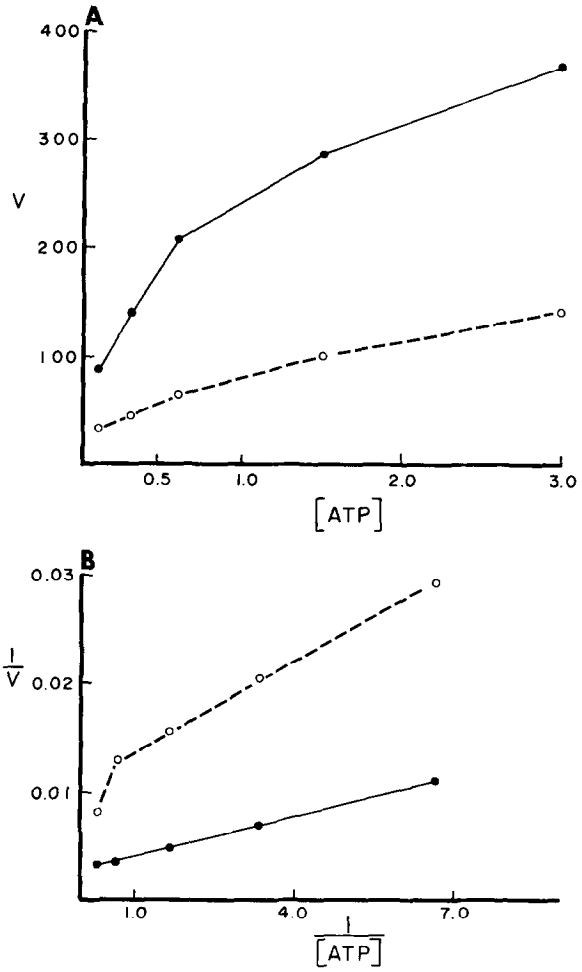

FIGURE 4.

Effects of ATP concentration on the Na^+,K^+-ATPase activity. The incubations contained 3 mM $MgCl_2$, 50 mM imidazole, 100 mM NaCl, 20 mM KCl, plus the concentrations of tris ATP shown and were run either with (0---0) or without (●---●) 10^{-5}M sanguinarine. In both plots v is in μmoles Pi released per mg protein per hour and ATP is in mM. The data presented is representative of 6 separate experiments. Each point represents the average of 3 separate incubations.

non-competitive inhibition by sanguinarine as shown in Figure 4. In these experiments Mg^{++} was always in excess of ATP but the same kinetics are observed when Mg^{++} and ATP are used in equivalent amounts as shown in Figure 5. Unfortunately the Lineweaver-Burke plots are curved over

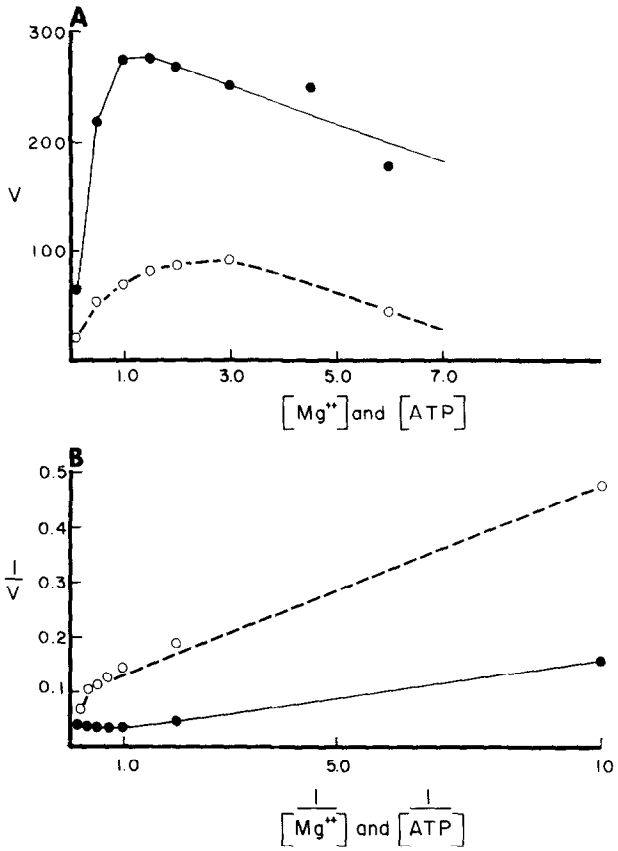

FIGURE 5.

Effect of various equimolar concentrations of Mg^{++} and ATP on the Na^{+},K^{+}-ATPase activity. The incubations contained 50 mM imidazole, 100 mM NaCl, 20 mM KCl, plus the concentrations of ATP and Mg^{++} shown and were run either with (0---0) or without (●---●) 10^{-5}M sanguinarine. In both plots V is in μM Pi/mg/hour. The data shown are representative of 2 separate experiments. Each point represents the average of 3 separate incubations.

the range of ATP used indicating some form of multiple site interaction such as allosteric modifications by ATP. Replots of the data in the form of Hill equations have revealed n values which are always reduced in the presence of sanguinarine; however, since V_{max} is subject to considerable error from experiment to experiment, we do not regard our Hill plots on these data to be accurate and thus are only crude approximations of the actual steady state kinetics of the enzyme.

All the previous experiments were performed with no preincubation. The effects of preincubation are shown in Table I. When the enzyme is preincubated at 37° for 15 min. in the presence of sanguinarine a much greater inhibition can be observed; this effect of preincubation can be prevented by ATP and to a much lesser extent by Na^+, but K^+ is completely ineffective. In addition, ATP will protect in the presence and absence of Mg^{++} in the preincubation mixture.

DISCUSSION

Sanguinarine has been shown to be an inhibitor of the Na-K ATP'ase from guinea pig brain with a pI_{50} of 5.25. Sanguinarine is uncompetitive

TABLE I

EFFECTS OF PREINCUBATION ON SANGUINARINE INHIBITION

Shown as % inhibition of optimal activity

Preincubated 15 min at 37°C	
Enz[(a)]	59%
Enz + Sang + ATP	65%
Enz + Sang + ATP + Mg^{++}	67%
Enz + Sang	92%
Enz + Sang + Na^+	85%
Enz + Sang + K^+	92%

All incubations were run with 100 mM Na^+, 20 mM K^+, 3 mM Mg^{++}, 3 mM Tris ATP, 50 mM imidazole and 10^{-5} sanguinarine when used. All final incubations were for 30 minutes at 37°C.

(a) - No preincubation under these conditions with all reagents added at 4°C gives a 59% inhibition by 10^{-5}M sanguinarine.

with sodium and potassium and non-competitive for ATP while, in contrast, ouabain is non-competitive for [K^+] (13), oligomycin is also non-competitive for [K^+] and uncompetitive for [Na^+] and [ATP] (14), and harmaline is competitive for [Na^+] (5).

In addition, preincubation of the enzyme with ATP in the presence of Na^+ and Mg^{++} increases inhibition by ouabain (3), while preincubation in the presence of ATP alone protects against sanguinarine inhibition. Na^+ protects to a much lesser degree than ATP against the sanguinarine preincubation effect while K^+ alone has no effect. It seems likely, therefore, that sanguinarine inhibits at a site or step of the enzyme sequence different from the cardiac glycosides, oligomycin and the carboline derivatives.

ATP has been reported to protect against N-ethylmaleimide (NEM) inhibition (15) similar to protection by ATP against sanguinarine inhibition. However, preincubation of ATP and Mg^{++} lessens the protection against NEM (15) but there is no difference between ATP and ATP + Mg^{++} in protection against sanguinarine.

In conclusion, sanguinarine, a benzophenanthridine alkaloid, has been shown to be an inhibitor of the Na^+,K^+-ATPase from guinea pig brain. Since steady state kinetic analysis indicate that sanguinarine inhibits at a site different from ouabain, oligomycin or harmaline, this compound may prove a useful tool for further elucidating the mechanism of action of Na^+,K^+-ATPase.

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