Studies on Mechanism of Atabrine Inhibition of ATPase Activity of the Myofibrills.* (26204)

GEORGE KALDOR (Introduced by Bruno W. Volk)
Department of Physical Chemistry, Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn, N. Y.

It has been known since the early work of Wagner-Jauregg(1) that in the physiological pH range acridines precipitate ATP to form salts. Karremann, Mueller and Szent-Gyorgyi(2) found that acridine orange acting as a competitive inhibitor suppressed contraction of glycerol-extracted muscle fibre bundles. Sandow and Isaacson(3) explained the suppressive effect of acridine orange on muscle contraction on the basis of a photodynamic mechanism, and pointed out that some of the non-photodynamic effects of acridine orange on muscle are due to its Zn^{++} content. It therefore seemed to us that a study of the acridine inhibition of ATPase, as it exists in myofibrillar suspensions, would lead to information of greater relevance to the biochemical mechanisms involved. With a myofibrillar suspension, the problem of diffusion of both ATP and the acridine drug is removed. The preparation does not contain free Mg^{++} or Ca^{++} and the separate and combined participation of these cations can be conveniently studied. Atabrine was chosen for this study because it is obtainable as the hydrochloric acid salt which is free of Zn^{++} and because of its pharmacological importance.

Material and methods. Myofibrillar suspension (rabbit skeletal muscle) was prepared as previously described(4) except that 0.05 M borate buffer, pH 7.5, was substituted for the succinate buffer. ATPase determinations were carried out at room temperature (23-25°) in .05 M borate buffer, final volume 1.0 ml, pH 7.5, by measuring the amount of inorganic phosphate liberated in 5 mintues. The reaction was stopped by addition of 5% TCA solution. The deproteinized supernatant of the incubation mixture was treated with 0.1 ml charcoal slurry (1:2, v/v, activated charcoal and water) to absorb atabrine. ATPase activity is given in terms of μM inorganic phosphate liberated per mg of protein per minute (μM/mg/min). The composition of the individual incubation mixtures, in millimoles per liter, is specified below. Atabrine was kindly provided by the Dept. of Medical Research, Winthrop Laboratories, New York. Fluorometric measurements were made with a Coleman Electronic Photofluorometer, Model 12C using 14-212 and 12-221 filters.

Results. Curves A, B, and C of Fig. 1 show that in absence of Mg^{++} and Ca^{++} as activating cations, inhibition of ATPase activity by atabrine was demonstrated at low concentrations of ATP, but reversed at higher levels of ATP. On the other hand, in presence of 5 mM Ca^{++} (curve D, Fig. 1) atabrine at the concentrations indicated, now caused a 30% activation of ATPase (curve E, Fig. 1).

If 5.0 mM Mg^{++} was used instead of Ca^{++} as the activator of myofibrillar ATPase, atabrine behaved as a strong inhibitor. By increasing ATP concentration the inhibition caused by 1.0 and 1.5 mM of atabrine is reversed (curves B and C, Fig. 2). That caused by 2.0 mM or more of the drug is also reversed but to a slight extent (curves D, E and F, Fig. 2). Thus, in presence of Mg^{++}, atabrine behaved as a much stronger inhibitor than in its absence.

A comparison of curve A and B, Fig. 3, demonstrates stimulation of ATPase activity by Mg^{++} in presence of Ca^{++}, and under the conditions shown by curve A pronounced inhibition of ATPase activity could be demonstrated with atabrine as is illustrated in curves C, D, and E of Fig. 3.

In another experiment 300 mM KCl was added to each incubation mixture to insure an ionic strength above 0.40. ATP and Ca^{++} concentrations were fixed at 5.0 mM. Under these conditions 1.0 mM to 5.0 mM ata-

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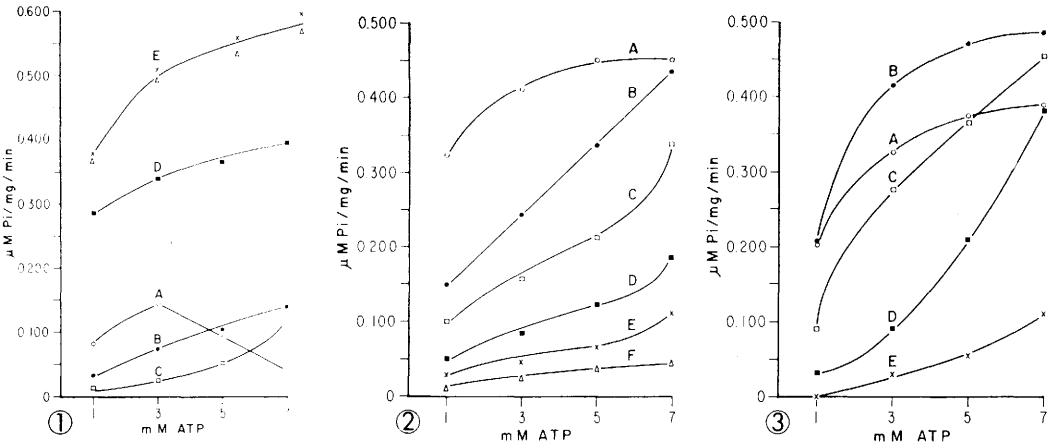

FIG. 1. Curves A, B, C show effect of substrate concentration on atabrine inhibition of myofibrillar ATPase in absence of any added Mg^{++} or Ca^{++}. Each tube contained about 0.3 mg myofibrillar protein to which was added: Curve A (○) none, curve B (●) 2.5 mM atabrine, curve C (□) 5.0 mM atabrine. Curves C and D show effect of atabrine on 5 mM Ca^{++} activated myofibrillar ATPase at various substrate concentrations. Each tube contained about 0.3 mg myofibrillar protein and 5.0 mM Ca^{++} to which was added: Curve D (■) none, curve E (×) 2.5 mM atabrine, and (△) 5.0 mM atabrine.

FIG. 2. Inhibition of 5.0 mM Mg^{++} activated myofibrillar ATPase by atabrine at various ATP concentrations. Each tube contained about 0.3 mg myofibrillar protein and 5 mM Mg^{++} to which was added: Curve A (○) none, curve B (●) 1.0 mM atabrine, curve C (□) 1.5 mM atabrine, curve D (■) 2.0 mM atabrine, curve E (×) 2.5 mM atabrine, curve F (△) 5.0 mM atabrine.

FIG. 3. Effect of 0.5 mM Mg^{++} on 5 mM Ca^{++} activated myofibrillar ATPase at various ATP and or atabrine concentrations. Each tube contained about 0.3 mg myofibrillar protein and 5.0 mM Ca^{++} to which was added: Curve A (○) none, curve B (●) 0.5 mM Mg^{++}, curve C (□) 0.5 mM Mg^{++} and 1.0 mM atabrine, curve D (■) 0.5 mM Mg^{++} and 2.0 mM atabrine, curve E (×) 5.0 mM atabrine.

brine failed to produce any inhibition. If 0.01 mM to 0.5 mM Mg^{++} was added to incubation mixtures, 15-40% inhibition occurred, which remained unchanged when 2.5 mM atabrine was added together with the Mg^{++}. At high ionic strength atabrine, with or without Mg^{++}, was not inhibitory towards the Ca^{++} activated myofibrillar ATPase (myosin ATPase).

Karremann, Mueller and Szent-Gyorgyi (2) found that acridine orange can form a red fluorescent complex with ATP. They were able to show a similar red fluorescent complex in well-washed glycerol-extracted muscle fibres. Because of these results, they assumed that acridine orange and ATP might be bound by the same groups of the contractile proteins.

It was therefore thought desirable to investigate the influence of ATP, Ca^{++}, Mg^{++} and K^+ upon the fluorescence of the atabrine. Experiments were carried out in 1 x 10^{-5}M borate buffer, pH 7.5. The fluorescence of the atabrine is considerably increased by ATP and this increased fluorescence is quenched by Mg^{++}, Ca^{++} or K^+ (Table I). On the other hand, the same cations can quench the original fluorescence of the atabrine. These findings indicate formation of an atabrine-ATP complex which is destroyed by Mg^{++}, Ca^{++} or K^+ due to possible formation of a metal-ATP chelate complex and/

TABLE I. Influence of Presence of ATP, Ca^{++}, Mg^{++} and K^+, Either Alone or in Combination, on Fluorescence of Atabrine.

Atabrine	ATP	Ca^{++}	Mg^{++}	K^+	Relative fluorescence units
		M/l			
1×10^{-5}	0	0	0	0	50
"	1×10^{-4}	0	0	0	90
"	"	5×10^{-5}	0	0	55
"	"	0	5×10^{-5}	0	52
"	"	0	0	1×10^{-4}	54
"	0	5×10^{-5}	0	0	22
"	0	0	5×10^{-5}	0	21
"	0	0	0	1×10^{-4}	22

or a metal-atabrine coordination complex.

Discussion. According to the results of ATPase determinations and fluorescence measurements, the inhibition of the myofibrillar ATPase at low ionic strength and in the absence of any added Mg^{++} or Ca^{++} might be explained satisfactorily by assuming complex formation between ATP and atabrine and/or between atabrine and the active center of the myofibrills. Mg^{++} and Ca^{++} are both known to be activators of myofibrillar ATPase at low ionic strength. They were equally effective in quenching the increased fluorescence of the ATP-atabrine complex, and so presumably reduce the stability of that complex. ATPase determinations have shown that atabrine was not inhibitory in the Ca^{++} activated system, but that its inhibitory action was potentiated if Mg^{++} was present. Klotz and Ming(5) found that Zn^{++} increased the binding of azo dyes to several proteins including enzymes. According to their interpretation, this effect can be adequately explained by coordination complex formation without assuming chelation. The experimental results described above led us to the assumption that a mechanism similar to that described by Klotz and Ming(5) is responsible for the increased myofibrillar ATPase inhibition in presence of atabrine plus Mg^{++}. (Fig. 2 and 3). It is interesting to point out the differences which exist between the mechanism of action of atabrine and several other known inhibitors of the myofibrillar ATPase system. According to the explanation offered by Perry and Grey (6), pyrophosphate and excessive amounts of ATP inhibit myofibrillar ATPase by binding Mg^{++} so that it is no longer available to the enzyme. The inhibitory action of ethylenediamine-tetraacetic acid is assumed to be a function of its great capacity to chelate Ca^{++} (7). The inhibition of these compounds was reversible if concentration of Mg^{++} and/or Ca^{++} was increased(6,7).

None of the above theories, except that of Klotz and Ming, are applicable to the phenomena observed in the study of the atabrine inhibition of myofibrillar ATPase. The chemical structure of atabrine makes it improbable that chelate formation with Ca^{++} or Mg^{++} can occur. Addition of a small amount of Mg^{++} (0.5 mM) in the 5.0 mM Ca activated system (Fig. 3), induced a similar degree of inhibition in presence of a given amount of atabrine as occurred in the 5.0 mM Mg^{++} activated system (Fig. 2) and in absence of Ca^{++}. This would indicate that under our experimental conditions 0.5 mM Mg^{++} was as equally effective as was 5.0 mM in producing a strong inhibitory compound with atabrine. This in turn indicates that the increased concentration of Mg^{++} or Ca^{++} did not reverse the inhibition of the atabrine plus Mg^{++}. This effect could be due to formation of a coordination complex between Mg^{++} and atabrine which is firmly bound to the myofibrillar ATPase, thus inhibiting the binding and splitting of the ATP. The negative results at high ionic strength are difficult to explain. By dissociating the actomyosin to actin and myosin, we deal with a different enzyme. The characteristics of the myosin ATPase concerning its ion activators, optimal pH etc., are quite different from that of the actomyosin(8). On the other hand, presence of the large amount of KCl (300 mM) greatly reduces the possibility of formation of an Mg atabrine coordination complex. Because of these considerations the lack of atabrine inhibition at high ionic strength can be explained in either of two ways, *i.e.,* the presence of a different enzyme or lack of formation of the Mg-atabrine coordination complex.

Summary. At low ionic strength, atabrine inhibition of myofibrillar ATPase activity was potentiated by Mg^{++}. Ca^{++} counteracted atabrine inhibition in absence but not in presence of Mg^{++}. The assumption of the occurrence of an Mg-atabrine coordination complex as the strong inhibitor is proposed as the best explanation of the experimental results. At high ionic strength, atabrine failed to inhibit Ca^{++} activated myofibrillar ATPase (myosin ATPase) with or without Mg^{++} being present.

1. Albert, A., *The Acridines,* Edward Arnold and Co., London, England, 1951.

2. Karremann, G., Mueller, H., Szent-Gyorgyi, A., *Proc. Nat. Acad. Sci.,* 1957, v43, 373.

3. Sandow, A., Isaacson, A., *Biochem. Biophys. Res. Comm.,* 1960, v2, 455.

4. Gergely, J., Kaldor, G., Briggs, F. N., *Biochim. Biophys. Acta,* 1959, v34, 218.

5. Klotz, I. M., Loh-Ming, W. C., *J. Am. Chem. Soc.,* 1954, v76, 805.

6. Perry, S. V., Grey, T. C., *Biochem. J.,* 1956, v64, 184.

7. Weber, A., *J. Biol. Chem.,* 1959, v234, 2764.

8. Szent-Gyorgyi, A., *Chemistry of Muscular Contraction,* Academic Press, New York, 1951.

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Ability of Isopregnenolone-21-Carboxylates to Block Renal Effects of Deoxycorticosterone and Aldosterone in Rats. (26205)

C. M. KAGAWA AND E. A. BROWN

Divisions of Biological and Chemical Research, G. D. Searle and Co., Chicago, Ill.

Previous reports described the synthesis of a number of steroidal 17-spirolactones(1-3) with ability to block the acute renal electrolyte effects of aldosterone and aldosterone-like steroids in laboratory studies(4-7). The compounds studied clinically caused diuresis in edematous patients with associated hyperaldosteronism(8,9), demonstrating the therapeutic value of aldosterone-blocking agents (10). Our effort was recently directed toward synthesis of isopregnenolone-21-carboxylic acids, and the present report demonstrates that these compounds are potent deoxycorticosterone- and aldosterone-blocking agents in laboratory studies.

Materials and methods. The procedures for evaluating aldosterone-blocking activity have been described(5,6). Briefly, the method consisted of subcutaneously injecting groups of adrenalectomized rats (150 to 200 g) with isotonic saline and with an oil solution of 12 μg of deoxycorticosterone acetate (DCA). Test compounds were given additionally either as an injection or an oral dose in saline.* The animals were placed in metabolism cages after treatment for collection of a 4-hour sample of urine. No food or water was provided during collection period. In this assay, DCA alone reduces the urinary ratio of Na/K ions by causing simultaneously Na retention and K loss. Blocking activity was determined by reversal of the Na/K response and expressed in terms of proportion of the standard dose of DCA inhibited by the compound. Na and K analyses were done on a Beckman flame photometer.

Isopregnenolone-21-carboxylates were obtained by treatment of steroidal 17-spirolactones with a molar equivalent of methanolic Na or K hydroxide at reflux temperature for 30 minutes, and identified by elemental analysis and infrared spectral data. The following compounds were prepared for tests in animals: 3-oxo-17β-hydroxy-19-nor-17α-pregn-4-ene-21-carboxylic acid as the Na salt (I); 3-oxo-17β-hydroxy-17α-pregn-1,4-diene-21-carboxylic acid as the Na (IIa) and K (IIb) salts; and 3-oxo-9α-fluoro-11β, 17β-dihydroxy - 17α - pregn - 4 - ene - 21 - carboxylic acid as the K salt (III).

Results. Data demonstrating the DCA-blocking properties of the 4 compounds in adrenalectomized rats are shown in Table I, together with electrolyte data of untreated and DCA controls for reference. DCA alone reduced the Na/K ratio by inducing a strong retention of Na and some K loss. It can be noted that Compound I, IIb and III at the given doses blocked Na/K response primarily by changing Na output, with only slight and variable effects occurring on K excretion. At times, however, reversal of Na/K was obtained because of a greater effect on K than on Na excretion, as shown with a dose of 0.1 mg of Compound IIa. Over a wide range of effective doses, the maximal Na, K and Na/K responses achieved with the compounds approximated, but did not represent

* Compounds are soluble in water to extent of about 15% by weight.