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ACTION OF AMIODARONE ON RABBIT MUSCLE CALCIUM-DEPENDENT ADENOSINE TRIPHOSPHATASE*

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Abstract—The effects of amiodarone [2-butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)-benzofuran hydrochloride], a potent antianginal and antiarrhythmic drug, have been studied *in vitro* on the ATP splitting activities of several rabbit muscle preparations. The "actomyosin" and myofibrillar heart and crural muscle preparations contained a calcium-dependent ATPase, which was considered as representative of the contractile system, together with other ATPases which could be kept inactive by omitting magnesium ions and by adding sodium azide (mitochondrial ATPase). Amiodarone inhibits the magnesium-dependent enzyme of all preparations in the same way as it acts on guinea pig heart sodium and potassium activated magnesium-dependent ATPase;[6] inhibition was shown, in the case of heart "actomyosin", to be competitive with respect to ATP, not to magnesium. On the "contractile" calcium-dependent ATPase of all preparations, however, amiodarone failed to exert any effect at concentrations up to 0·6 mM. It is concluded that amiodarone is not likely to impair muscular and cardiac contractility.

AMIODARONE† is a potent antianginal and antiarrhythmic drug in use in several countries. Its pharmacological properties have been extensively studied[1–5] and it was recently reported by this laboratory[6] that the drug inhibits guinea pig heart Na^+ and K^+-activated Mg^{2+}-dependent adenosine triphosphatase (Na^+, K^+-activated ATPase), the enzyme which is thought to actuate the active transport of Na^+ and K^+ ions across the cellular membranes.[7] In spite of the lack of any structural relationship between amiodarone and adenosine triphosphate (ATP), it was shown that the drug competes with ATP but not with the activating ions Mg^{2+}, Na^+ and K^+. In this respect, the drug differs from cardiac glycosides like ouabain that do not compete with ATP, but possibly with K^+ ions.[8] The unexpected competition between ATP and amiodarone prompted us to investigate the effect of the drug on another important ATPase system, i.e. the enzyme responsible for the contractile properties of muscle fibers.

MATERIALS AND METHODS

Enzyme preparations

Rabbit heart actomysin. Hearts were obtained from unanaesthetized rabbits and immediately cleaned from adhering tissues, cut into pieces and homogenized during 1 min with a Sorvall Omni mixer at 2°.[9] The homogenate was slowly stirred for 30 min at 2° with 6 vol. of a solution containing 0·5 M KCl, 30 mM $NaHCO_3$ and 2·5

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† 2-Butyl-3-(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)-benzofuran hydrochloride. Cordarone®, Labaz.

mM cysteine, and afterwards left at room temperature for 15 hr. Three to four vol. of the same solution were added and the mixture centrifuged at 25,000 *g* during 10 min. Six to seven vol. of distilled water were then added to the supernatant in order to precipitate the actomyosin, which was isolated by 10 min centrifugation at 25,000 *g*. The preparation was thoroughly washed with distilled water, with 50 mM KCl and, finally dissolved by stirring for 12 hr at 2° in 1 M KCl (same vol. as that of the initial extracting solution). The actomyosin solution was diluted with about one fifth of Tris buffer (pH 7·1) and dialysed in the cold against the same buffer during 40 hr. Tris buffer was made by dissolving 1·55 g Tris base and 9·75 g NaCl in 500 ml water and pH brought to 7·1 with 230 ml 0·05 M HCl ($I = 0{\cdot}35$).

Rabbit heart myofibrillar ATPase. The hearts were cleaned, cut into pieces and squeezed through a Delepine tissue press with 0·7 mm holes.[10] Thirty vol. of cold 0·32 M saccharose were added and the mixture stirred very slowly at 2° for 30 min. It was then filtered through a 80-mesh stainless steel sieve and centrifuged at 50 *g* for 2 min. The supernatant was centrifuged 5 times at 500 *g* for 20 min, resuspending the pellet each time with 0·32 M saccharose. Another centrifugation of 2 min at 50 *g* was performed, and the supernatant subjected to a last run at 500 *g* during 20 min. The pellet was suspended in 177 mM Tris–HCl buffer of pH 7·4 (vol. equal to one third of the initial extraction volume) and kept at 0° for 18 hr. All measurements were made within 12 hr after that time. The same procedure was used to prepare myofibrillar ATPase from rabbit crural muscle.

Activity measurements

Colorimetric method for actomyosin. The method has been described earlier,[6] but was modified as follows. Each tube contained 2 ml enzyme preparation (of which the pH was adjusted to 7·4 with Tris), 0·1 ml 0·18 M $CaCl_2$ or $MgCl_2$ and 0·2 ml amiodarone solution or solvent, and kept at 25° in a shaking water bath. The reaction was started by adding 1 ml 6 mM ATP (pH adjusted to 7·0 with Tris). 0·2 ml aliquots were taken at 3 min intervals and immediately mixed with 2 ml ice-cold 2% ascorbic acid in 10% trichloroacetic acid for duplicate phosphate determinations.[6]

Enzymatic method for actomyosin. Was modified from the method of Schwarz *et al.*[11] The reaction was performed directly in 1 cm optical spectrophotometer cuvettes maintained at 25°. Each cuvette contained 1·3 ml 50 mM triethanolamine buffer (pH 7·6) containing 5 mM ethylenediamine tetraacetate (EDTA) and 10 mM $MgCl_2$, 0·5 ml 215 mM phosphoenolpyruvate, 0·03 ml 0·2% pyruvate kinase, 0·1 ml 1% NADH, 0·03 ml 0·5% lactate dehydrogenase, 0·1 ml 110 mM ATP, eventually 0·2 ml 0·18 M $CaCl_2$, 0·2 ml amiodarone solution or solvent and a suitable volume of enzyme extract. NADH disappearance was followed continuously at 340 nm.

Colorimetric method for myofibrillar ATPase. Each tube contained 1 ml enzyme preparation, 0·05 ml 0·15 MCa Cl_2 or $MgCl_2$, 0·05 ml amiodarone solution or solvent and 0·05 ml 0·15 M NaN_3 when needed. The tubes were maintained at 37° in a shaking water bath. The reaction was started by adding 0·3 ml 12 mM ATP (pH adjusted to 7·0 with Tris) and stopped as described earlier[6] by adding 2 ml ice-cold 2% ascorbic acid in 10% trichloroacetic acid.

Protein concentration was assayed by the Folin–Ciocalteu procedure.[12] Activity was calculated as μmole orthophosphate liberated per milligram protein per hr. Reaction velocities were always calculated from the regression lines. Amiodarone

was dissolved either in water or in methanol. Methanol had a slight inhibiting action which was accounted for.

RESULTS

Actomyosin ATPase activation by Ca^{2+} and Mg^{2+} ions. Rabbit heart "actomyosin" preparations displayed a small ATPase activity in the absence of added Ca^{2+} and Mg^{2+} ions. When magnesium chloride was added, activity increased at most by about 50 per cent; the peak of activation was observed with 7·6 mM Mg^{2+}. When calcium ions were added, a maximal 5-fold activation was measured at 5–6 mM Ca^{2+}. It may be concluded therefore that the preparations were contaminated with Mg^{2+}-dependent ATPases.

Action of amiodarone on Mg^{2+}-dependent ATPase activity contaminating the actomyosin preparations. When the "actomyosin" preparations were incubated with 5·5 mM Mg^{2+} ions in the absence of Ca^{2+} ions, amiodarone inhibited the ATPase activity as can be seen from Fig. 1.

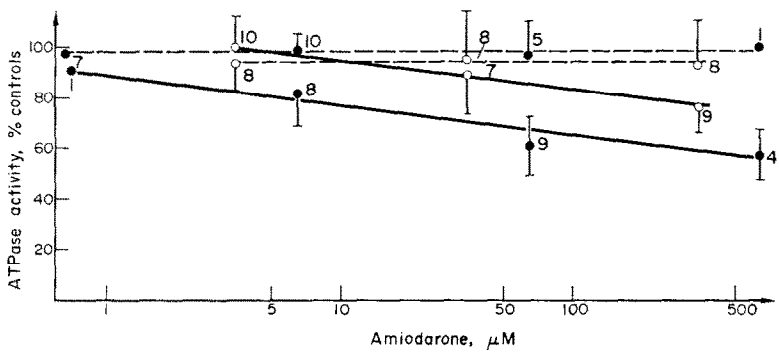

FIG. 1. Action of amiodarone on rabbit muscle ATPases. ●——● Heart actomyosin preparation, at 25°, with 5·5 mM Mg^{2+}, no Ca^{2+}. ● - - - ● Heart actomyosin preparation, at 25°, with 5·5 mM Ca^{2+}, no Mg^{2+}. ○ - - - ○ Heart myofibrillar preparation, at 37°, with 5·15 mM Ca^{2+}, no Mg^{2+} (NaN_3 present). ○——○ Crural muscle myofibrillar preparation, at 37°, with 5·15 mM Mg^{2+}, no Ca^{2+} (NaN_3 present).

A Lineweaver–Burk plot was derived from experiments in which the enzyme preparations were incubated with 5·5 mM Mg^{2+} (no calcium added), 0·08 mM amiodarone and varying concentrations of ATP. Fig. 2 shows that amiodarone competes with ATP, its K_i being about 10 μM. Variations of Mg^{2+} concentration (between 25 and 0·58 mM) resulted always in the same inhibition, indicating that the drug did not compete with the metal.

Action of amiodarone on Ca^{2+}-activated actomyosin ATPase. When the "actomyosin" preparations were incubated with 5·5 mM Ca^{2+} ions, in the absence of Mg^{2+} ions, amiodarone had no influence at concentrations ranging from 0·65 μM to 0·6 mM, as can be seen from Fig. 1.

Action of sodium azide on myofibrillar ATPase activity. Heart myofibrillar preparations are known to be contaminated with mitochondrial ATPase which can be inhibited by azide.[13] It was found in four independent experiments that 5·25 mM NaN_3 inhibited 30 $\pm$ 12 per cent of the heart ATPase activity measured in the presence of 5·15 mM calcium. Crural muscle ATPase activities were inhibited by about 20 per cent when assayed under the same conditions.

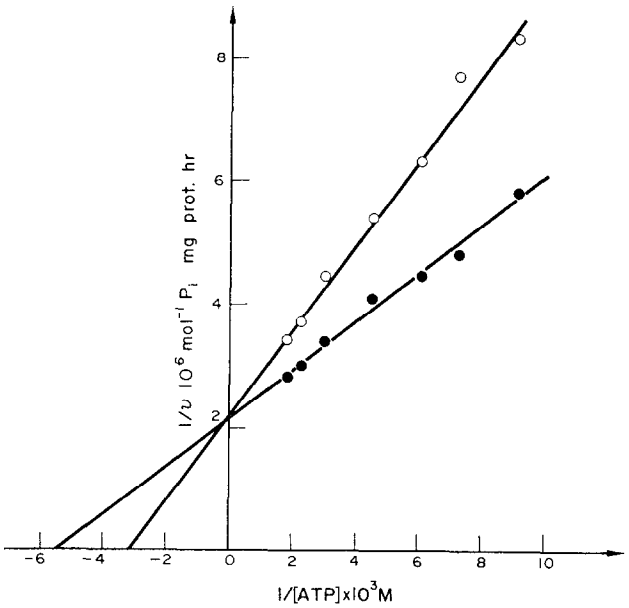

FIG. 2. Lineweaver–Burk plot of the inhibition by 80 μM amiodarone of rabbit heart actomyosin Mg^{2+}-dependent ATPase at 25° as a function of ATP concentration. ● Controls, ○ amiodarone.

Action of amiodarone on myofibrillar ATPase activity. When the enzyme preparations from heart were incubated in the presence of 5·25 mM NaN_3 and 5·15 mM Ca^{2+}, amiodarone had no effect at concentrations ranging from 0·35 mM to 0·35 μM, as can be seen from Fig. 1.

In the presence of Mg^{2+} ions instead of Ca^{2+} ions, the same kind of inhibition occurred as with "actomysoin" preparations. Essentially the same results were obtained with crural muscle preparations assayed in exactly the same conditions (six experiments for each amiodarone concentration). It should be mentioned, moreover, that when magnesium ions (5·15 mM) were used instead of calcium ions, a slight inhibition by amiodarone could be observed (Fig. 1).

DISCUSSION

Amiodarone has been shown[6] to be a potent competitive inhibitor of guinea pig heart Na^+, K^+-activated ATPase with respect to its substrate. This effect might be related somehow to the influence of the drug on the heart muscle action potential of treated rabbits.[5] Amiodarone being used as a therapeutic agent in angina pectoris,[1] it seemed important to know if the drug also inhibits the contractile mechanism of heart muscle.

Muscular contraction is known to depend upon the splitting of ATP by actomyosin fibres. The enzyme system can be purified to a state where it superprecipitates in the presence of ATP,[14] but crude preparations like those used in this work are only active in the presence of calcium ions, which relieve the inhibitory action of the troponin–tropomyosin system.[15] Moreover, these preparations are usually contaminated with other ATP splitting enzymes from cellular membranes and mitochondria.

Mg^{2+}-dependent ATPases can be kept inactive by incubation in the absence of Mg^{2+} ions, whereas the mitochondrial ones are thoroughly inhibited by sodium azide.[13]

Both "actomyosin" and myofibrillar preparations from rabbit heart have been shown in this work to be insensitive to amiodarone in the presence of about 5 mM calcium ions. However, when magnesium replaced calcium, inhibition occurred. Just as in the case of Na^+, K^+-activated ATPase of guinea pig heart, the drug competes with the substrate, not with magnesium. It is therefore very likely that the preparations were contaminated with Na^+, K^+-activated Mg^{2+}-dependent ATPase. In fact, the preparations contained enough Na^+ and K^+ to activate the enzyme.

Guinea pig heart Na^+, K^+-activated ATPase has been shown[6] to have a K_m of 1·23 mM at 37°, whereas the value found here for the rabbit "actomyosin" preparation at 25° was much smaller, 0·018 mM. Similarly, the K_i for amiodarone was 0·065 mM in the case of the guinea pig, and amounted here to 0·01 mM. About 0·02 mM amiodarone is needed to inhibit 25 per cent of the guinea pig heart enzyme at 37°, 0·01 mM for rabbit "actomyosin" at 25°, 0·1 mM for rabbit heart myofibrils at 37° and 0·3 mM at 37° for rabbit crural muscle myofibrillar preparations. There can be no doubt that amiodarone inhibits rabbit heart Na^+, K^+-activated ATPase, and probably also the striated muscle enzyme which seems somewhat less sensitive. Amiodarone has no action on the contractile calcium-dependent ATPase enzyme system of rabbit heart and striated muscle, and is therefore not likely to impair contractility.

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