Biochemical Pharmacology, Vol. 21, pp. 2951–2960. Pergamon Press 1972. Printed in Great Britain.

ACTION OF AMIODARONE ON GUINEA PIG HEART SODIUM AND POTASSIUM ACTIVATED ADENOSINE TRIPHOSPHATASE

COMPARISON WITH OUABAIN*

J. BROEKHUYSEN, M. CLINET and C. DELISEE
Department of Biochemistry, Labaz Research Centre, B-1120 Brussels, Belgium

(*Received* 11 *February* 1972; *accepted* 24 *May* 1972)

Abstract—The effect of amiodarone [2-butyl-3(3,5-diiodo-4-β-diethylaminoethoxybenzoyl)-benzofuran] *in vitro*, a potent antianginal and antiarrhythmic drug, on guinea pig heart Na^+, K^+-activated and Mg^{2+} dependent ATPases has been studied. The drug has no effect on the Mg^{2+}-dependent enzyme at concentrations up to 0·1 mM but inhibits the Na^+, K^+-activated enzyme. The inhibition is competitive with respect to ATP, K_i is about 0·065 mM (K_m being about 1·23 mM). No competition with respect to Mg^{2+}, Na^+ or K^+ could be observed. K_a values of 0·82, 52·7 and 0·16 mM have been recorded for activation by Mg^{2+}, Na^+ and K^+ ions respectively. Amiodarone differs strikingly from ouabain which is a non-competitive inhibitor with respect to ATP, and the sites of action of both drugs seem to be distinct.

About 0·1 mM amiodarone is needed to achieve 50 per cent inhibition, and this concentration is of the same magnitude as that found in human hearts after medical treatment.

MANY drugs are presently in use for the management of angina pectoris and arrhythmias. One of them, amiodarone† exerts in dogs multiple effects which have been extensively reviewed.[1] Prominent pharmacological properties of the drug include heart rate decrease (which has been shown to be independent of *para-* and *ortho-*sympathetic mechanisms), unchanged cardiac output, damping (but not blocking) of both the α and β cardiovascular effects of catecholamines, coronary vasodilatation and a powerful antiarrhythmic action. It has been shown recently[2] that amiodarone, in anaesthetized closed chest dogs, enhances the action of catecholamines on stroke volume, in spite of its damping properties on heart rate and blood pressure. In other words, the drug enables the heart to do the same work at a lower beating rate, leading to some decrease of oxygen consumption.

Amiodarone has also been shown on rabbits to increase the duration of ventricular and atrial muscle action potential, essentially during the latest part or repolarization, the other features of the action potential being practically unchanged.[3] The reason for this could be a modification of the transport mechanisms of ions across the cell membrane.

* This work was supported in part by a grant of the Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture (IRSIA), to which we express our thanks.

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

The active transport of sodium and potassium ions across the plasmic membrane is known to be controlled by a Na^+ and K^+ activated adenosine triphosphatase (Na^+, K^+-activated ATPase). It has been shown that several drugs are able to modify the Na^+, K^+-dependent ATPase activity from various tissues; for example, cardiac glycosides decrease[4] and Co^{2+} ions increase[5] this activity in the heart.

Because glycosides have typical positive inotropic properties and Co^{2+} ions cause cardiac insufficiency,[6] some correlation may be thought about between the inotropic effect and the influence on Na^+, K^+-activated ATPase.

In this work, we present data showing that amiodarone is an inhibitor of guinea pig heart Na^+, K^+-activated ATPase *in vitro*.

MATERIALS AND METHODS

Preparation of enzyme. Eight to fourteen hearts of guinea pigs (of approx. 400 g) were pooled for each preparation.[7] About 10–15 g of muscle were dried on filter paper, cleaned of adhering tissue, cut into small pieces and homogenized with an Ultra-Turrax (Janke & Kunkel KG, Staufen i-Br.) device (3 times 10 sec duration) in 4 vol. of a solution containing 0·25 M saccharose, 5 mM histidine, 5 mM ethylenediamine tetraacetate (EDTA) and 0·15% sodium deoxycholate and adjusted to pH 6·8 with Tris base. The homogenate was centrifuged for 30 min at 12,000 *g*. The supernatant was then centrifuged for 60 min at 100,000 *g*. The pellet was suspended and recentrifuged at the same speed for the same time, and the final pellet was suspended in 10 ml of a solution containing 0·25 M saccharose, 5 mM histidine, 1 mM Tris–EDTA.

A same volume of 2 M LiBr was added and the mixture stirred for 1 hr. It was then centrifuged twice at 100,000 *g* during 1 hr, resuspending each time with the suspending solution. The final suspension, containing 1 g fresh tissue per ml, was homogenized with a Dounce device and filtered through a gauze layer. All procedures were carried out at 2°.

Activity measurements. For each experiment, 5 sets of 4 tubes were prepared. The first set was used for determination of spontaneous ATP hydrolysis and contained no enzyme. The second set was used for total ATPase activity and contained Na^+ and K^+ ions. The third one was used to determine the effect of drug on total ATPase activity. The fourth set contained no K^+ ions and gave the magnesium-activated ATPase activity. The last one was similar to the fourth except that it contained drug.

The incubation mixture was made up with 0·5 ml 100 mM Tris–HCl buffer (pH 7·5) containing as needed 10 mM $MgCl_2$, 30 mM KCl and 200 mM NaCl, 0·1 ml enzyme preparation, 0·2 ml water and 0·1 ml amiodarone solution or solvent (5% bovine serum albumin in 0·9% NaCl). The tubes were maintained at 37° in a shaking water bath. The reaction was started by adding 0·1 ml 50 mM $ATPNa_2$ (adjusted to pH 7·0 with Tris). After 4, 8, 12 and 16 min the reaction was stopped by adding 2 ml 2% ascorbic acid in 10% trichloroacetic acid and cooling in an ice bath. The tubes were centrifuged for 15 min at 3000 rev/min and 2 × 0·5 ml supernatant were used for orthophosphate determination[8] in duplicate.

Blanks for spontaneous hydrolysis of ATP (set 1) were substracted from the readings at each incubation time, and a regression line was calculated in order to obtain the velocity of the reaction in each experiment. Activities are expressed as micromoles of orthophosphate formed per mg protein and per hr. Protein content of enzyme

preparations was determined by the Folin–Ciocalteu method,[9] with bovine serum albumin as the standard.

Amiodarone being scarcely soluble in water, about 200 mg finely ground powder were mixed on a magnetic stirrer with 50 ml 5% bovine serum albumin in 0·9% NaCl at room temperature during 4 hr. Insoluble material was spun down and amiodarone concentration was measured by its optical absorption at 242 nm against the appropriate blank. Final concentration was usually about 1 mM. This stock solution was diluted as required, producing often a faint turbidity in the incubation mixture. In experiments with ouabain, 0·1 ml of an aqueous solution was substituted for 0·1 ml water in the incubation mixture.

Total ATPase activity refers to the activity measured in the presence of Mg^{2+}, Na^+ and K^+ ions. Mg^{2+}-dependent ATPase activity is that obtained in the absence of K^+ ions (25 mM final Na^+ from $ATPNa_2$ and drug solvent has no activating properties in the absence of K^+ ions).[10,11] Na^+, K^+-dependent ATPase activity is calculated by subtracting Mg^{2+}-dependent ATPase from total activity.

RESULTS

Effect of amiodarone on Mg^{2+}-activated ATPase. The enzyme was assayed in the absence of K^+ ions. It represented 27 ± 13 per cent of the total activity and Fig. 1 shows that the drug has no effect on the reaction at concentrations up to 0·1 mM.

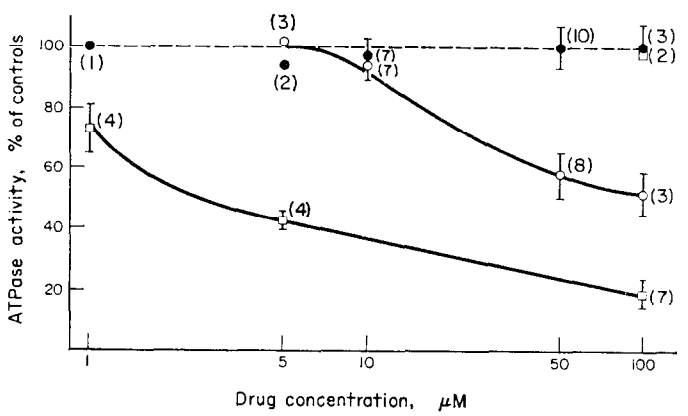

FIG. 1. Action of amiodarone and ouabain on guinea pig heart ATPases. Mg^{2+}-dependent ATPase with ● amiodarone; ■ ouabain. Na^+, K^+-activated ATPase. with ○ amiodarone; □ ouabain; ( ) number of experiments. Vertical bars represent standard deviation.

Effect of amiodarone on Na^+, K^+,-activated ATPase. The enzymatic activity was assayed in the presence of 125 mM Na^+ and 15 mM K^+, with various concentrations of amiodarone. Na^+, K^+-dependent activity was calculated by substracting the Mg^{2+}-dependent activity from the total activity, taking into account that amiodarone has no inhibitory effect on the Mg^{2+}-activated enzyme. Fig. 1 shows that the drug inhibits Na^+, K^+-dependent activity from 0·01 mM up.

Effect of ouabain on Na^+, K^+-activated ATPase. Ouabain is known to inhibit Na^+, K^+-dependent ATPase,[12] and a concentration of 0·1 mM has been claimed[13]

to suppress the activity completely. Fig. 1 shows that in our experimental conditions, 0·1 mM inhibits only 80 per cent of the activity, whereas lower concentrations gave inhibition values well in the range of those observed by others.[7] The drug has no effect on the Mg^{2+}-dependent activity at 0·1 mM.

Interaction between amiodarone and ouabain on Na^+, K^+-activated ATPase. One would expect that, if two inhibitors interact with the enzyme at overlapping sites, sequential treatment of the enzyme with these inhibitors would result in the second inhibitor acting only on those sites left intact by the first one; a cumulative inhibition pattern would be obtained. On the other hand, if two inhibitors interact with the enzyme at distinct sites, sequential treatment of the enzyme would result in the second inhibitor acting on all enzyme molecules. Expected residual activity can be calculated for both cases. For overlapping sites, it would be; $A_1 + A_2 - A_0$, where A_1 is the residual activity with inhibitor 1 only, A_2 the residual activity with inhibitor 2 only, and A_0 the initial activity. For distinct sites, the residual activity would be $A_1 + A_2/A_0$. Table 1 summarizes the results obtained.

TABLE 1. INTERACTION BETWEEN AMIODARONE AND OUABAIN ON GUINEA PIG HEART Na^+, K^+-ACTIVATED ATPase

Initial activity	Residual activity		Residual activity		Expected residual activity with drugs acting on		Measured residual activity with both drugs
	μM	Ouabain	μM	Amiodarone	Distinct sites	Overlapping sites	
6·4	100	1·2	50	2·8	0·5	0·0	0·5
31·2	100	6·2	50	18·1	3·6	0·0	4·5
25·5	100	4·8	10	20·3	3·9	0·0	3·9
12·0	5	4·9	100	5·9	2·4	0·0	2·5
12·0	5	4·9	50	6·5	2·6	0·0	2·4
13·8	5	5·9	50	6·7	2·9	0·0	2·3
13·8	5	5·9	25	9·6	3·1	1·7	3·1
12·0	5	4·9	25	10·5	4·3	3·4	4·1
13·8	5	5·9	10	11·8	5·1	3·9	3·5
11·8	1	8·2	100	5·4	3·7	1·8	3·6
11·8	1	8·2	50	6·7	4·6	3·1	6·7
13·2	1	7·8	50	6·8	4·0	1·4	2·8
13·2	1	7·8	25	9·3	5·5	3·9	5·1
11·8	1	8·2	25	8·6	6·0	5·0	5·8
13·2	1	7·8	10	10·0	5·9	4·6	7·0

Activities in μmole P_i liberated/milligram protein/hr.

Inhibitory action of amiodarone on Na^+, K^+-activated ATPase in the presence of various concentrations of substrate (ATP). The enzyme was incubated with 0·69 and $1·15 \times 10^{-4}$ M amiodarone and with ATP concentrations ranging from 0·5 to 4 mM. In five independent experiments, amiodarone appeared to act as a competitive inhibitor of Na^+, K^+-dependent ATPase. K_m (for ATP) was $1·23 \pm 0·10$ mM, whereas K_i had a mean value of $0·066 \pm 0·010$ mM (Table 2). Kinetic constants were obtained by the graphical method of Lineweaver and Burk[14] and by calculation. Figure 2 depicts the results of a typical experiment.

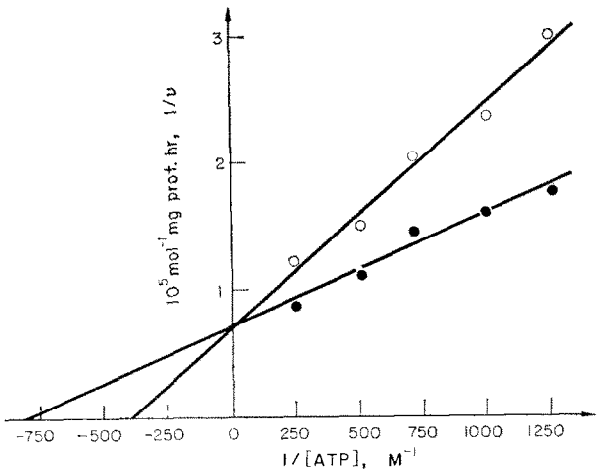

FIG. 2. Lineweaver–Burk plot of the inhibition by 69 μM amiodarone of guinea pig heart Na^+, K^+-activated ATPase as a function of ATP concentration. ● Controls, ○ amiodarone.

TABLE 2. KINETIC DATA OF THE ACTION OF AMIODARONE ON Na^+, K^+-DEPENDENT ATPase, WITH VARYING CONCENTRATIONS OF ATP

Expt. No.	ATP conc. range (mM)	K_m (mM)	V	Drug conc. (μM)	K_i	V_i
1	0·8–4	1·38	16·4	69	60	20·8
2	0·8–4	1·24	13·9	69	68	14·3
3	0·8–2	1·23	16·6	115	83	14·3
4	0·8–2	1·15	19·2	115	60	17·9
5	0·8–2	1·14	16·7	115	61	12·2
Mean ± S.D.		1·23 ±	0·10		66 ±	10

V and V_i as μmole P_i liberated/milligram protein/hr.
Measured at 37° with 5 mM Mg^{2+}, 15 mM K^+ and 125 mM Na^+.

Effect of amiodarone on the activating action of Na^+, K^+ and Mg^{2+} ions

Mg^{2+} ions. In the presence of Na^+ and K^+ ions, the enzyme requires also Mg^{2+} ions to display its activity. Figure 3 shows that an excess of Mg^{2+} ions is unable to compete with amiodarone to relieve its inhibition. The K_a for Mg^{2+} was 0·82 mM and did not change in the presence of amiodarone. K_i has about the same value as that given above (73 instead of 66 μM). Table 3 contains the results of two experiments and Fig. 4 depicts one of them.

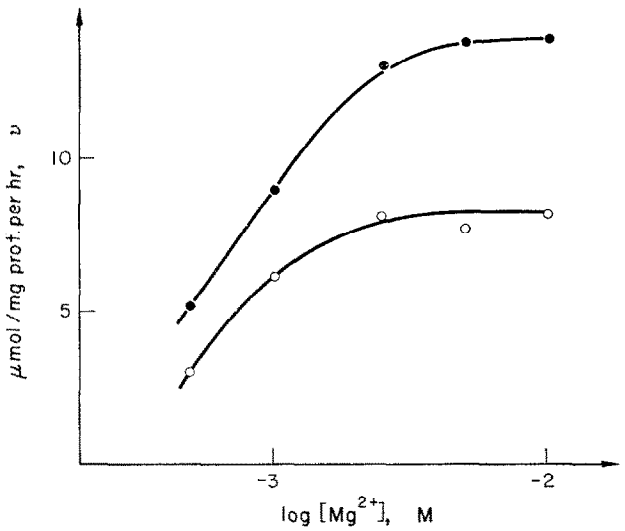

FIG. 3. Effect of increasing Mg^{2+} concentrations on guinea pig heart Na^+, K^+-activated ATPase inhibited by 53 μM amiodarone. ● controls, ○ amiodarone.

TABLE 3. KINETICS OF AMIODARONE INHIBITION OF Na^+, K^+-ACTIVATED ATPase, WITH VARYING CONCENTRATIONS OF ACTIVATING IONS

Ion	Conc. range (mM)	K_a (mM)	V	Amiodarone (μm)	K_{a_i} (mM)	V_i	K_i (μM)
Mg^{2+}	0·25–2·5	0·82	14·3	53	0·82	8·3	78
	0·25–2·5	0·83	14·3	69	0·79	7·4	68
	35–125	50·0	23·3	44	48·5	13·7	65
Na^+	37–125	53·8	38·5	44	56·2	25·0	65
	45–125	54·4	27·8	44	58·2	17·9	71
	0·1–0·8	0·17	16·1	50	0·15	7·9	55
	0·1–0·4	0·15	12·5	50	0·16	6·6	56
K^+	0·1–0·8	0·16	7·2	50	0·20	3·7	51
	0·1–0·8	0·17	8·7	50	0·20	4·7	48

V and V_i as μmole/P_i liberated/milligram protein/hr.
Measured at 37° with 5 mM ATP; final concentrations were, except when stated otherwise, 5 mM for Mg^{2+}, 125 mM for Na^+ and 15 mM for K^+.

Na^+ ions. Na^+ in excess is unable to overcome the inhibition of the enzyme brought about by amiodarone. K_a values for Na^+ did not change in the presence of the drug and K_i was about the same as that stated above. Table 3 gives the results of three experiments and Fig. 5 depicts one of them.

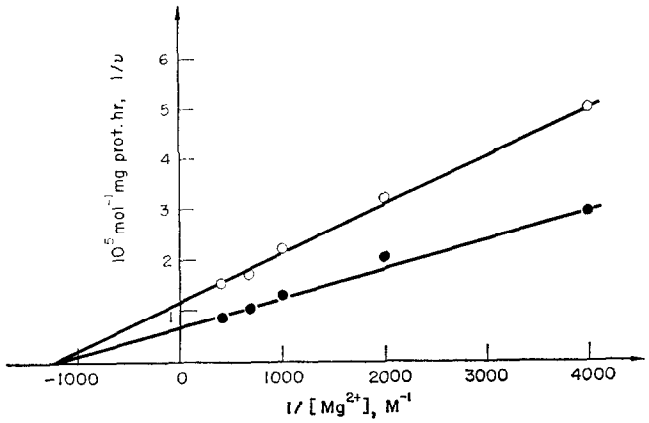

FIG. 4. Lineweaver–Burk plot of the inhibition by 53 μM amiodarone of guinea pig heart Na^+, K^+-activated ATPase as a function of Mg^{2+} concentration. ● Controls, ○ amiodarone.

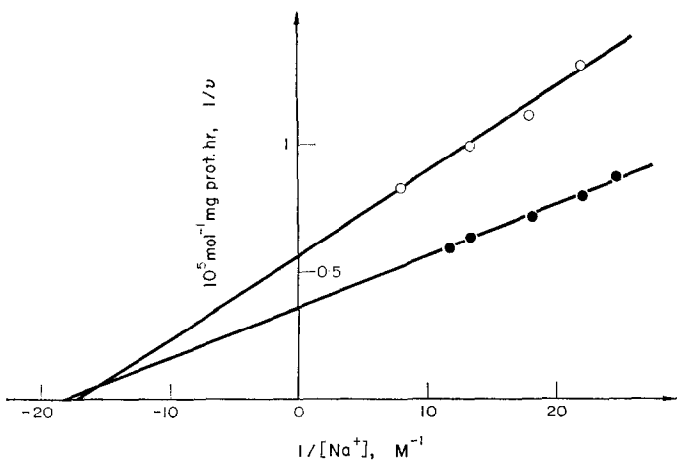

FIG. 5. Lineweaver–Burk plot of the inhibition by 44 μM amiodarone of guinea pig heart Na^+, K^+ activated ATPase as a function of Na^+ concentration. ● Controls, ○ amiodarone.

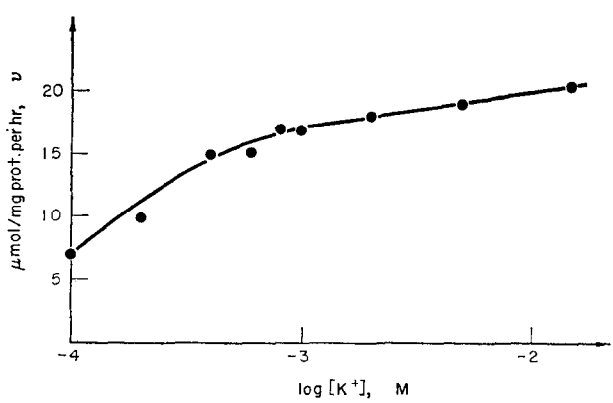

FIG. 6. Effect of increasing K^+ concentrations on guinea pig heart Na^+, K^+-activated ATPase

K^+ ions. The kinetics of the ATPase activation by K^+ are complicated by the fact that increasing concentrations of the ion in the presence of a fixed Na^+ concentration lead to a slowly increasing maximum velocity (see Fig. 6). It could be shown, however, that K^+ ions cannot overcome amiodarone inhibition of Na^+, K^+-activated ATPase. K_a values for K^+ were not significantly different from K_{a_i} ones and K_i was the same as that reported above. Table 3 contains the results of four experiments and Fig. 7 depicts one of them.

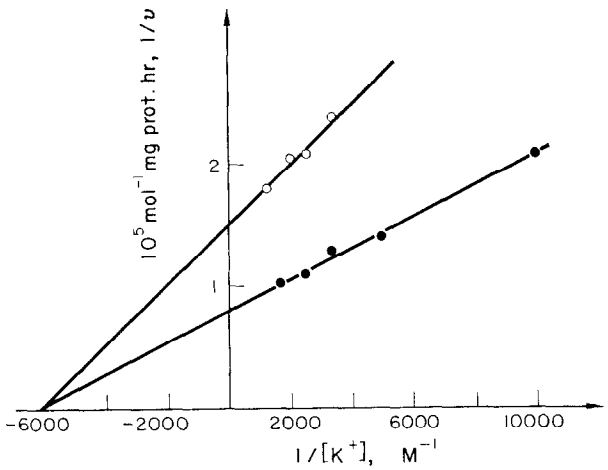

FIG. 7. Lineweaver–Burk plot of the inhibition by 50 μM amiodarone of guinea pig heart Na^+, K^+-activated ATPase as a function of K^+ concentration. ● Controls, ○ amiodarone.

DISCUSSION

In spite of the absence of any similitude in the structures of amiodarone (Fig. 8) and ATP, evidence has been obtained in this work that amiodarone behaves like a competitive inhibitor of guinea pig heart Na^+, K^+-activated ATPase. The drug competes with ATP but not with any of the three activating ions Na^+, K^+ and Mg^{2+}. On the other hand, no inhibition of the Mg^{2+}-activated ATPase occurred at amiodarone concentrations up to 0·1 mM.

The effect of amiodarone differs strikingly from that of the cardiac glycoside ouabain which has been shown to be a non-competitive inhibitor of heart Na^+, K^+-activated ATPase in various species.[12] Ouabain seems to interact with the phosphorylated Na^+-activated enzyme to form a complex that cannot be split by K^+ ions.[15] Furthermore, data of Table 1 indicate that when the enzyme is incubated with both drugs, amiodarone does not react at the ouabain sensitive site.

It is not yet clear whether the inotropic properties of a drug are correlated with its action on Na^+, K^+-dependent ATPase. Evidence has been presented that the cardiac glycosides modify rather the sarcotubular transport of calcium ions,[16,17] but an indirect effect on this mechanism may also be considered as the consequence of the competition between Ca^{2+} and Na^+ ions for transportation across the cell membranes or for fixation on specific intracellular cation stores.[18–21] Amiodarone has no inotropic properties, therefore, the results reported here are not in favour of a correlation between cardiotonic action and reduced Na^+, K^+-dependent ATPase activity.

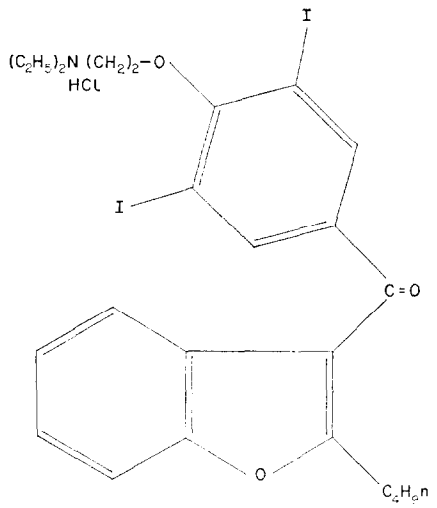

FIG. 8. Amiodarone.

Correlation between Na^+, K^+-dependent ATPase activity and shape of action potential is also poorly understood. It has been shown, e.g.[22] that digitoxin and digoxin increase the action potential duration, whereas ouabain decreases it to a slight extent. These effects depend however on experimental conditions, principally on calcium concentrations.[23] On the other hand, quinidine is known to prolong the duration of the repolarisation phase of heart muscle action potentials, and it was shown [24] that the drug inhibits 50 per cent of toad cardiac Na^+, K^+-activated ATPase at about 30 μM. Even if action potential modifications are brought about by the inhibition of Na^+, K^+-dependent ATPase, no correlation seems to appear between these features and the effects of drugs on heart rhythm.

It should be pointed out, on the other hand, that 10–30 min after i.v. injection of 10 mg/kg ^{125}I-labelled amiodarone into rats, heart muscle contains about 0·04 μmole drug per gram fresh tissue, and that 30–50 μg iodine/gram fresh cardiac tissue have been found in humans after medical treatment with amiodarone;[25] these values correspond to about 0·05 mM amiodarone. Even if a part of the iodine present is under an inactive form, it may be assumed that enough drug is present to inhibit the enzyme *in vivo*.

REFERENCES

1. R. CHARLIER, *Antianginal Drugs, Handbook of Experimental Pharmacology*, Vol. 31. pp. 255–288, Springer, Berlin (1971).
2. R. CHARLIER, G. DELAUNOIS and J. BAUTHIER, *Arzneim. Forsch.* **22,** 545 (1972).
3. B. N. SINGH and E. M. VAUGHAN-WILLIAMS, *Br. J. Pharmac.* **39,** 657 (1970).
4. I. M. GLYNN, *Pharmac. Rev.* **16,** 381 (1964).
5. H. DRANSFELD, J. LIPINSKI and E. BORSCH-GALETKE, *Naunyn-Schmiedebergs. Arch. Pharmak.* **270,** 335 (1971).
6. K. KASPEREK, V. SILLER and H. J. KNIERIEM, *Z. Ges. exp. Med.* **150,** 316 (1969).
7. T. AKERA, F. S. LARSEN and T. M. BRODY, *J. Pharmac. exp. Ther.* **170,** 17 (1969).
8. E. S. BAGINSKI, P. P. FOA and J. B. ZAK, *Clin. chim. Acta*, **15,** 155 (1967).
9. O. H. LOWRY, N. J. ROSENBROUGH, A. L. PARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
10. J. V. AUDITORE, *Arch. int. Pharmacodyn.* **156,** 22 (1965).

11. J. BOURGOIGNIE, S. KLAHR, J. YATES, L. GUERRA and N. S. BRICKER, *Am. J. Physiol.* **217,** 1496 (1969).
12. J. C. SKOU, *Physiol. Rev.* **45,** 596 (1965).
13. H. A. POTTER, J. S. CHARNOCK and L. J. OPIT, *Austr. J. exp. Biol. Med. Sci.* **44,** 503 (1966).
14. C. LIEBECQ, in *Fundamentals of Biochemical Pharmacology* (Ed. Z. M. BACQ) pp. 59–86, Pergamon Press, Oxford (1971).
15. T. AKERA and T. M. BRODY, *J. Pharmac. exp. Ther.* **176,** 545 (1971).
16. K. S. LEE and S. J. CHOI, *J. Pharmac. exp. Ther.* **153,** 114 (1966).
17. S. FUJINO, S. KAWAGISHI, N. EGUCHI and M. TANAKA, *Jap. J. Pharmac.* **21,** 423 (1971).
18. H. R. BESCH JR., J. C. ALLEN, G. GLICK and A. SCHWARZ, *J. Pharmac. exp. Ther.* **171,** 1 (1970).
19. K. S. LEE and W. KLAUS, *Pharmac. Rev.* **23,** 193 (1971).
20. K. S. LEE, S. A. HONG and D. H. KANG, *J. Pharmac. exp. Ther.* **172,** 180 (1970).
21. H. R. BESCH JR and A. SCHWARZ, *J. Molec. cell. Cardiol.* **1,** 195 (1970).
22. M. ITO, P. B. HOLLANDER, B. H. MARKS and S. DUTTA, *J. Pharmac. exp. Ther.* **172,** 188 (1970).
23. P. J. DEMPSEY, Z. MCCALLUM, K. M. KENT and T. COOPER, *J. Pharmac. exp. Ther.* **177,** 78 (1971).
24. K. G. KENNEDY and W. G. NAYLER, *Biochim. biophys. Acta* **110,** 174 (1965).
25. J. BROEKHUYSEN, R. LARUEL and R. SION, *Arch. int. Pharmacodyn.* **177,** 340 (1969).