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Amiodarone: biochemical evidence for its interaction with myocardial Na^+-K^+-ATPase in guinea pig microsomal preparations

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Amiodarone is a very potent class III antiarrhythmic drug used in the treatment of a broad spectrum of cardiac tachyarrhythmias [1]. The mechanism for its antiarrhythmic effect is probably based on its multiple actions, particularly its inhibition of the fast sodium channels [2], or slow calcium channels [3] or membrane stabilizing effects [4]. However, apart from its well-established antiarrhythmic effect, amiodarone also possesses a number of other properties such as its neurotoxicity [1], or the tendency to induce or enhance cardiac arrhythmias [1, 5, 6], which often causes serious clinical complications. While its inhibition of synaptosomal Na^+-K^+-ATPase has recently been discussed with regard to its neurological side effects [7], the mechanism of its proarrhythmic or arrhythmogenic actions remains to be elucidated. It is known however, that the inhibition of myocardial Na^+-K^+-ATPase (EC 3.6.1.3) is arrhythmogenic in character [8], and it appears to provide adequate explanation of the mechanism of digitalis-induced arrhythmias, for example [9]. Furthermore, it was recently postulated that malfunction of the electrogenic pump activity of the myocardial Na^+-K^+-ATPase may, in general, contribute towards the mechanism of such drug-induced arrhythmias [8]. However, only limited information is currently available in the literature pertaining to studies on interactions between antiarrhythmic agents and this enzyme system. In the present study, we therefore examined the actions of amiodarone on the Mg^{2+}-dependent ATP-hydrolysis by myocardial Na^+-K^+-ATPase, and its mode of interaction with ouabain in this system, in order to assess their potential relevance for some of its cardiac actions.

Materials and Methods

Myocardial Na^+-K^+-ATPase was prepared as described previously [10]. Accordingly, hearts isolated from guinea pigs of either sex weighing 0.6–1.1 kg were homogenized for 10 min in sucrose buffer to give a 15% suspension. The homogenate was centrifuged for 15 min at 14,000 *g* using an RC5C Centrifuge (Sorvall Instruments), and the supernatant was filtered with a Millipore SC 8.0 μm filter (Millipore Corporation, Bedford, MA, U.S.A.). The enzyme was then separated on a TSK Toyopearl HW-55F Gel column (Pierce Chemicals, U.S.A.) equilibrated with KCl buffer (KCl 500 mM, imidazole 10 mM, Na_2·EDTA 1 mM, pH 7.4). The fractions were collected using LKB-2211 Superrac and dialysed in Visking 20/32 dialysis membrane (Serva Finebiochemica, U.S.A.) three times, each time for at least 6 hr using twice 10 mM first, and then once 100 mM imidazole buffer, pH 7.4. All steps were carried out at 4°. Different drug concentrations were then pre-incubated with 10–12 μg protein for 20 min at 37° in 100 mM imidazole buffer containing (in mM) Mg^{2+} 5, Na^+ 100, K^+ 5 and Na_2·EDTA 1. The reaction was initiated by adding 2 mM ATP, and the liberated inorganic phosphate determined spectrophotometrically at 660 nm after 20 min by the method of Eibl and Lands [11]. The Na^+/K^+-stimulated ATPase activity was calculated as the difference between the total and the Mg^{2+}/Na^+-dependent activity.

Protein concentration was determined using Coomassie reagent (Pierce Chemicals). Drugs used were amiodarone (Sanofi) and ouabain (Fluka). All the other reagents were of analytical grade. Statistical significance was calculated by Student's *t*-test using the Statgraphics software package version 3.0 (Graphic Software Systems, Inc., 1988). Significance criteria refer to $P < 0.05$.

Results and Discussion

Both ouabain and amiodarone exhibited concentration-dependent inhibitory actions on myocardial Na^+-K^+-ATPase activity at the range of 0.05 to 100 μM and 0.65 to 90 μM, respectively (Fig. 1). The concentrations required to inhibit the enzyme activity by 50% (IC_{50} values) were 1.93 ± 0.27 μM for ouabain and 8.50 ± 1.87 μM for amiodarone. Thus, the inhibitory potency of amiodarone was comparable to that of ouabain, a specific inhibitor of the enzyme system [9]. The present results therefore demonstrate that amiodarone is a very potent inhibitor of the myocardial Na^+-K^+-ATPase activity.

As depicted in Fig. 2, incubation of the enzyme in presence of both ouabain and amiodarone produced additive effects at amiodarone concentrations below 1.0 μM. However, the combined effects became significantly less additive in character with increasing amiodarone concentrations above 1.0 μM. Thus for example, while single concentrations of ouabain inhibited the enzyme activity by approximately 55% at 2.5 μM or 65% at 5.0 μM, the addition of 2.5 μM ouabain reduced the amount of amiodarone required for total inhibition from 90 μM to approximately 50 μM. Similarly, about 40 μM of amiodarone were required to attain 100% inhibition in the presence of 5.0 μM ouabain. On the other hand, less than

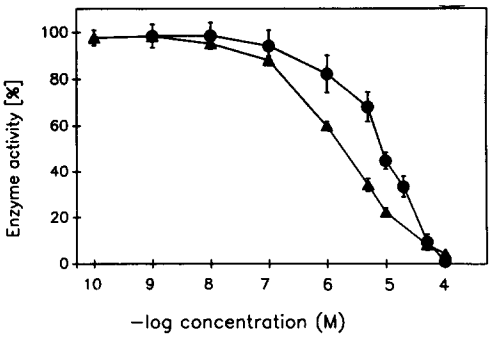

Fig. 1. Influence of amiodarone (●) and ouabain (▲) on guinea pig myocardial Na^+-K^+-ATPase activity. The average baseline activity of the enzyme preparation (100%) was 24.7 ± 5.4 μM P_i/mg protein/hr. Plotted are the mean values ± SE of 8 and 7 individual determinations, respectively.

10 μM and 1.0 μM of amiodarone would be required in combination with 2.5 and 5.0 μM ouabain to produce additive effects of 100% inhibition. In particular, it is noteworthy that the concentrations of amiodarone required for total inhibition in the presence of either 2.5 or 5.0 μM ouabain is about four to five times higher than the ones that would attain the same in an additive manner. Accordingly, although the combination of the two drugs seemed to produce additive effects at very low concentrations, it exerted a negative influence on the total of the individual drug actions with increasing concentrations. This type of trend is probably indicative of a competitive mode of interaction, suggesting therefore that similar binding sites of Na^+-K^+-ATPase might be involved in effecting the inhibitory actions of both amiodarone and cardiotonic steroids. The suggestion finds support in the recent findings that concomitant administration of amiodarone and digoxin also results in elevated digoxin serum levels [1].

Assuming therefore that amiodarone does inhibit the myocardial Na^+-K^+-ATPase activity in a fashion similar to that of cardiotonic steroids, it is reasonable to suggest that this action may be responsible for some of the cardiac actions it shares with these agents, such as the potential to induce or enhance cardiac arrhythmias. Just as in the case of the cardiac glycosides, the inhibition by amiodarone of

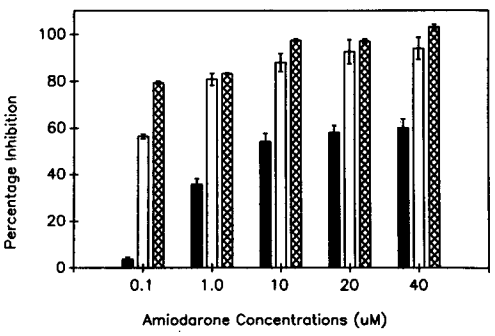

Fig. 2. Influence of different concentrations of amiodarone alone (■), amiodarone + 2.5 μM ouabain (□) and amiodarone + 5.0 μM (⊠) on guinea pig myocardial Na^+-K^+-ATPase activity in presence of 2.5 and 5.0 μM ouabain. The average baseline activity of the enzyme (100%) was 26.5 ± 7.8 μM P_i/mg protein/hr. Plotted are the mean values ± SE of 7–9 individual determinations.

the Mg^{2+}-dependent ATP-hydrolysis activity of the myocardial Na^+-K^+-ATPase may lead to malfunctioning of the electrogenic pump activity of the enzyme. This usually results in upsetting of cardiac ionic balance, particularly those of K^+ and Na^+. Such a disturbance in ionic gradients may induce or enhance arrhythmias during treatment with cardiac drugs which inhibit Na^+-K^+-ATPase activity [8]. The recent findings of Aomine [12] is particularly interesting in this regard. The author established that amiodarone inhibits the active sodium extrusion, which elicited the electrogenic pump activity of the myocardial Na^+-K^+-ATPase, and suggested that this is probably a result of the inhibition of the enzyme activity by amiodarone. The present findings appear therefore to provide evidence for such a hypothesis.

We may therefore conclude at this stage that physiological concentrations of amiodarone do inhibit the myocardial ouabain-sensitive ATP-hydrolysis by the Na^+-K^+-ATPase. This inhibitory action is probably pertinent to some of the cardiac properties shared by both amiodarone and cardiotonic steroids, such as their common tendency to induce cardiac arrhythmias.

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Characterization of liver microsomal cytochrome P450 from rats treated with muscone (3-methylcyclopentadecanone)

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Muscone (3-methylcyclopentadecanone) has been used recently in the treatment of coronary disease in China [1]. It is also a component in many perfumes and a product usually made from the musk glands of male musk deer. Previous studies have demonstrated a phenobarbital (PB)-type induction pattern of the hepatic drug metabolizing enzymes after muscone treatment [1, 2]. In addition we have shown that the serum dimethadione (DMO)/trimethadione (TMO) ratios at 2 hr after oral administration of TMO as a model compound to estimate hepatic drug-oxidizing capacity were significantly increased in the rats treated with muscone *in vivo* [2]. Recently, we indicated that four forms of cytochrome P450 (P450), P450 PB-1 (P450 IIIA2), P450 PB-2 (P450 IIC6), P450 PB-4 (P450 IIB1) and P450 PB-5 (P450 IIB2), were inducible in rat hepatic microsomes with PB and that testosterone 6β- and 6β-hydroxylation activities which are mainly catalysed by P450 PB-1 and P450 PB-4, respectively, were also inducible [3, 4]. Furthermore the amount of P450 UT-2 (P450 IIC11) decrease by starvation and PB-treatment together with decrease of testosterone 2α-hydroxylation activity [4, 5], and P450 DM (P450 IIE1) was induced by starvation or treatment with ketone [5, 6].

In this study, we investigated changes in testosterone hydroxylation activities of hepatic microsomes and alterations of amounts of P450s, P450 PB-1, PB-2, PB-4, PB-5, UT-2, and DM by treatment with muscone to confirm that muscone, one of the ketone, is a PB-type inducer or a ketone-type inducer.

Materials and Methods

Chemicals. Muscone, PB and streptozotocin (STZ) were purchased from Ogawa & Co. Ltd (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan) and the Sigma Chemical Co. (St Louis, MO, U.S.A.), respectively.

Animals and dosing. Adult male Wistar rats weighing 200–240 g were obtained from Doken (Ibaraki, Japan). Muscone was dissolved in corn oil and administered by intraperitoneal (i.p.) injection at a dose of 150 mg/kg for 3 days. The rats in the control group received corn oil only for 3 days. PB (80 mg/kg) dissolved in saline was also given by i.p. injection for 3 days. Diabetes was induced in rats by intravenous (i.v.) injection with STZ (65 mg/kg) freshly dissolved in 0.01 M citrate. The rats were killed at 6 weeks after induction of diabetes.

Biochemical analyses. Preparation of microsomes [2], purification of P450 and preparation of antibody were performed by methods described previously [6–8]. Characterization of antibodies used in this study was described elsewhere [5]. The immunoblotting and immunochemical quantitation [7], and the measurement of testosterone hydroxylase activity were also performed as described previously [9, 10]. The designations given to the rat hepatic P450s described in this study can be related to the standardized gene designation [11].

Results and Discussion

Typical levels of P450 UT-2, PB-1, PB-2, PB-4/5 and DM in muscone- and PB-treated microsomes after Western blotting are shown in Table 1. P450 PB-1, PB-2, and PB-4/5 were induced 1.1-, 1.9- and 13.8-fold with muscone and 1.5-, 1.9- and 46.5-fold with PB, respectively. On the other hand, P450 UT-2 was present in the hepatic microsomes of muscone and PB-treated rats at lower levels. The P450 DM levels with muscone was not changed but reduced 0.6-fold with PB. Imaoka *et al.* [4] have reported

Table 1. Alteration in the content of cytochrome P450 isozymes of rat hepatic microsomes

	Content of cytochrome P450				
Microsomes	UT-2	P450 PB-1	P450 PB-2	P450 PB-4/5	P450 DM
UT	406.0 ± 6.4	145.0 ± 7.8	105.0 ± 7.1	14.0 ± 5.0	68.5 ± 9.2
MS	358.5 ± 47.4	163.5 ± 0.7	195.0 ± 25.5*	192.5 ± 7.8†	68.3 ± 21.2
PB	292.0 ± 46.7	213.5 ± 16.3*	198.5 ± 21.9*	650.5 ± 123.7*	43.3 ± 7.1*

Hepatic microsomes (0.5–2 μg of protein) were analysed by Western blotting. Levels of cytochrome P450s were assayed by densitometry of nitrocellulose immunoblotted from SDS–polyacrylamide gel. Measurement was done with duplicates of two to four different preparations of microsomes. The values are expressed as mean ± SD of pmol of cytochrome P450/mg of microsomal protein. Total cytochrome P450 was measured by the reduced-CO spectral method [3].

UT, untreated; MS, muscone-treated; PB, phenobarbital-treated; DM, streptozotocin-treated.

* $P < 0.05$, † $P < 0.01$.