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Effect of Amiodarone on Na^+-, K^+-ATPase and Mg^{2+}-ATPase Activities in Rat Brain Synaptosomes*

K. S. PRASADA RAO, S. B. RAO, PH. CAMUS AND H. M. MEHENDALE†

Department of Pharmacology and Toxicology, University of Mississippi Medical Center, Jackson, Mississippi 39216 U.S.A.

Amiodarone hydrochloride is a diiodinated antiarrhythmic agent widely used in the treatment of cardiac disorders. With the increasing use of amiodarone, several untoward effects have been recognized and neuropathy following amiodarone therapy has recently been reported. The present studies were carried out to study the effect of amiodarone on rat brain synaptosomal ATPases in an effort to understand its mechanism of action. Na^+, K^+-ATPase and oligomycin sensitive Mg^{2+} ATPase activities were inhibited by amiodarone in a concentration dependent manner with IC_{50} values of 50 μM and 10 μM respectively. [^{3}H]ouabain binding was also decreased in a concentration dependent manner with an IC_{50} value of 12 μM, and 50 μM amiodarone totally inhibited [^{3}H]ouabain binding. Kinetics of [^{3}H]ouabain binding studies revealed that amiodarone inhibition of [^{3}H]ouabain binding is competitive. K^+-activated *p*-nitrophenyl phosphatase activity showed a maximum inhibition of 32 per cent at 200 μM amiodarone. Synaptosomal ATPase activities did not show any change in rats treated with amiodarone (20 mg kg^{-1} day^{-1}) for 6 weeks, when compared to controls. The treatment period may be short, since the reported neurological abnormalities in patients were observed during 3–5 years of treatment. The present results suggest that amiodarone induced neuropathy may be due to its interference with sodium dependent phosphorylation of Na^+, K^+-ATPase reaction, thereby affecting active ion transport phenomenon and oxidative phosphorylation resulting in low turnover of ATP in the nervous system.

KEY WORDS—Amiodarone neuropathy; Na-, K-ATPase; Mg^2-ATPase; rat brain synaptosomes; *p*-nitrophenyl phosphatase; ion transport in CNS; ATP turnover in CNS; ouabain binding.

INTRODUCTION

Amiodarone, 2-butyl-3-(3′,5′diiodo-4′α-diethylaminoethoxybenzoyl)-benzofuran (Figure 1) is a drug characterized by unique pharmacodynamic and pharmacokinetic properties.[1] It is an extraordinarily potent and versatile antiarrhythmic agent used in many parts of the world[2] and is approaching clinical trials in the U.S.A. Over the years several reports have appeared regarding some neurological symptoms observed in patients treated with amiodarone, including fine tremor of hands, sleep disturbance, proximal muscle weakness, demyelination and axonal degeneration.[3–5] Polyneuropathy was observed following administration of large doses of the drug.[6] Phototoxicity of amiodarone has been well-documented and the involvement of membrane damage and oxidative processes in the phototoxicity mechanism of amiodarone has been reported.[7] It was suggested that oxygen dependent as well as oxygen independent processes contribute to the membrane damage and these were thought to be due mainly to the photochemical alteration of amiodarone in the membrane rather than to that in solution.[7]

The mechanism involved in neurological toxicity of amiodarone has not been elucidated, although efforts have been made in recent years to explain its antiarrhythmic action as due to the affinity for inactivated sodium channels.[8] Myocardial sodium channels are responsible for the propagation of action potential in the specialized conduction tissues of the heart.[8] Similar conclusions with regard to the effect of amiodarone on sodium channels have also been drawn using frog

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†Addressee for correspondence.

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nerve nodes of Ranvier.[9] However the prolongation of duration of the action potential by the drug was an important and independent antiarrhythmic effect.

Ion channels are closely associated with ATPases at the nerve membranes. ATPases are involved both in active ion transport phenomena and in oxidative phosphorylation, and both are important for normal neurological function.[10] Since it appeared likely that amiodarone could inhibit ATPase activities, thereby interfering with energy dependent ion transport phenomena in the nerve tissue, we were interested in testing this possibility. The present studies were designed to study the effect of amiodarone on rat brain synaptosomal ATPases in an effort to establish a basis for its neurotoxic action.

MATERIALS AND METHODS

Animals and Chemicals

Male Sprague-Dawley rats (300–400 g each) were obtained from an in-house colony traceable to Charles River Breeding Laboratories, Wilmington, MA., and were maintained in the central animal facilities until used. Animals were provided with water and a commercial diet *ad libitum*, and were maintained away from any known contaminants. Amiodarone hydrochloride (> 98 per cent purity) was obtained from Clin-Midy Groupe Sanofi, France. The radioactive [^{3}H]ouabain (20·4mCi mg^{-1}) was purchased from New England Nuclear Corporation, Boston, MA and [^{14}C]-amiodarone (50 mCi mmol^{-1}) was a gift from Labaz Pharmaceutical S.A., Brussels, Belgium. All the other chemicals used in the enzyme assays were purchased from Sigma Chemical Co., St. Louis, MO.

In vitro *studies*

Solutions of the desired concentrations of amiodarone were prepared in ethanol. Test solutions (1 µl) were added to the reaction mixture to yield the desired final concentrations. The same amount of alcohol without amiodarone was added to controls. Alcohol (1 µl) had no effect on ATPases. The concentrations of amiodarone used to study its effect on different parameters are mentioned separately in the results section.

In vivo *studies*

A 2 per cent amiodarone HCl solution was prepared in distilled water. Rats were divided into batches of two each in a cage and they were treated intraperitoneally (i.p.) with 20 mg amiodarone kg body weight^{-1} day^{-1} up to 6 weeks. The control animals were given i.p. 0·3 ml of distilled water. Since amiodarone treated rats show a decrease in food intake and water consumption during treatment, pair-fed control rats receiving the same amount of food and water as that of amiodarone treated rats were maintained. The rats were killed after 1, 2, 4 and 6 weeks of treatment and synaptosomes were prepared from the whole brains.

Preparation of Synaptosomes

The whole brains of rats were removed after decapitation and homogenized in nine volumes of ice-cold 0·32 M sucrose solution (pH 7·5) containing 10 mM imidazole and 1 mM EDTA (homogenizing medium). Synaptosomes were prepared using a slightly modified procedure of Cotman and Matthews.[11] Briefly, the homogenate is centrifuged at 750 *g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 17 000 *g* for 20 min and the pellet was suspended in homogenizing medium and again centrifuged at 17 000 *g* for 20 min. The pellet was suspended in 10 ml of 0·32 M sucrose solution and layered on a two-step discontinuous Ficoll-sucrose gradient, consisting of 13 per cent (w/v) and 7·5 per cent (w/v) Ficoll in 0·32 M sucrose. After centrifugation in a Beckman L5-65 ultracentrifuge at 65 000 *g* for 45 min, the synaptosomal fraction was obtained at the interface of the 7·5–13 per cent Ficoll-sucrose layer. The synaptosome band was removed, diluted with nine volumes of sucrose solution and centrifuged at 17 000 *g* for 30 min. After centrifugation, the pellet was suspended in homogenizing medium, divided into small aliquots, quick frozen in liquid nitrogen and stored at −80°C until used.

After preparation of synaptosomes using a Ficoll-sucrose gradient, a small fraction was fixed for electron microscopy to check its nature and purity. Fixation was performed by layering the sample over an equal volume of glutaraldehyde (final concentration of 2·5 per cent in 0·1 M

phosphate buffer, pH 7·4). The synaptosomes were centrifuged through the fixation medium after standing for 2 h. Postfixation was in 2 per cent osmium tetroxide in 0·1 M sodium cacodylate buffer, pH 7·4 for 30 min. Staining *en bloc* was performed with 2 per cent uranyl acetate for 30 min. After dehydration of the synaptosomal pellet in an alcohol gradient, it was embedded in Spurr's low viscosity medium. Sections at a setting of 70–80 nm were taken with a diamond knife on an LKB Ultratome. Micrographs were taken with a Zeiss EM 10 and are presented in Figure 2. Numerous synaptosomes were seen as vesicular structures and the preparation is comparatively pure as reported by Cotman and Matthews.[11]

Determination of ATPase Activity

The synaptosomal ATPase activities were measured using endpoint phosphate analyses.[12] A 1 ml reaction mixture was used and contained in final concentration: 5·0 mM ATP, 5·0 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 135 mM imidazole–HCl buffer (pH 7·5), and 30–50 μg of enzyme protein. The reaction rate was proportional to the amount of protein used in this study. The total cationic ligand-stimulated ATPase activity was measured with Na^+, K^+ and Mg^{2+} present in the reaction mixtures. Mg^{2+}-ATPase was measured in the presence of 1 mM ouabain, a specific inhibitor of Na^+, K^+-ATPase. Thus, delineation of the (Na^+, K^+)-activated component of ATPase was obtained by difference between total ATPase and Mg^{2+}-ATPase. Mg^{2+}-ATPase activity was further delineated into oligomycin-sensitive (O.S.) and -insensitive (O.I.) Mg^{2+}-ATPases, using 5×10^{-6} M oligomycin in the reaction mixture. The incubation was at 37°C for 20 min and the reaction was stopped by the addition of 0·1 ml of 50 per cent TCA. Samples were then assayed for inorganic phosphate using the method of Lowry and Lopez.[12] Enzyme activity was expressed as micromoles of inorganic phosphate per milligram of protein per hour. The effect of amiodarone was assessed by preincubating the enzyme with different concentrations of test compound, before the reaction was started with ATP. Ethanol (1 μl) was added to control reaction mixtures. Protein was determined by the method of Lowry *et al.*[13] using bovine serum albumin as the standard.

Analysis of p*-Nitrophenyl Phosphatase Activity*

K^+-stimulated phosphatase activity (PNPPase) of brain synaptosomal enzyme preparation was measured using methods described by Ahmed and Judah,[14] and Albers and Koval.[15] Hydrolysis of the substrate *p*-nitrophenyl phosphate (PNPP) was measured in the presence of 5 mM $MgCl_2$, 10 mM KCl, 5 mM PNPP, 100 mM Tris–HCl buffer (pH 7·4), and 30–50 μg of synaptosomal protein at 38°C in final volume of 1·0 ml. Incubation time was 20 min, after which trichloracetic acid was added at a final concentration of 5 per cent (w/v) to stop the reaction. Aliquots of the cleared reaction supernatants then were diluted with 1·0 M Tris (pH 10·4) and the optical density was determined at 400 nm against a suitable blank. Mg^{2+}-activated PNPPase was assayed in the presence of 5 mM Mg^{2+} and subtracted from the total (K^+ and Mg^{2+}) PNPPase to get K^+ activated PNPPase. The K^+-stimulated PNPPase activity was expressed as μ moles of *p*-nitrophenol per milligram protein per hour. Amiodarone was added to the reaction mixtures as previously described.

[^{3}H]ouabain Binding to Synaptosomes

The binding of [^{3}H]ouabain to rat brain synaptosomes was determined by using a slightly modified method of Schwartz *et al.*[16] The reaction mixture of 2·0 ml contained: 5 mM ATP, 5 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris–HCl (pH 7·4) and 40–50 μg synaptosomal protein. The reaction mixture was incubated for 15 min at 37°C after the addition of [^{3}H]ouabain (0·1 μCi). After incubation, the reaction mixture was filtered through a 0·45 μm millipore filter and washed with buffer. The filters were transferred directly to vials containing 10 ml Aquasol^R, which were placed in a liquid scintillation counter. Unlabelled ouabain at 1 mM concentration was used for determining the non-specific binding. This was subtracted from the total binding to obtain the precise amount of [^{3}H]ouabain bound to synaptosome. Different concentrations of amiodarone were added to the reaction mixture as described previously.

^{14}C-Amiodarone Binding to Synaptosomes

These studies were performed using the same

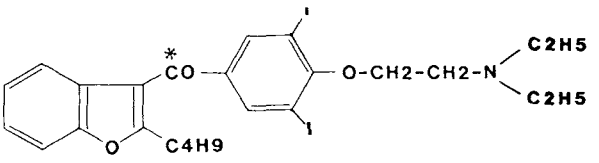

Figure 1. Structural formula of amiodarone [2-butyl-3-(3′,5′-diiodo-4′-α-diethylaminoethoxybenzoyl)-benzofuran]. *Indicates location of [^{14}C] label on the compound used in these studies.

reaction mixture and conditions as mentioned for [^{3}H]oubain binding. ^{14}C-amiodarone binding to synaptosomes was studied in the presence and absence of ATP to test whether it requires energy.

Kinetic Analysis

Kinetic analysis of amiodarone effect on [^{3}H]ouabain binding to synaptosomes was undertaken to determine the nature of the inhibition.

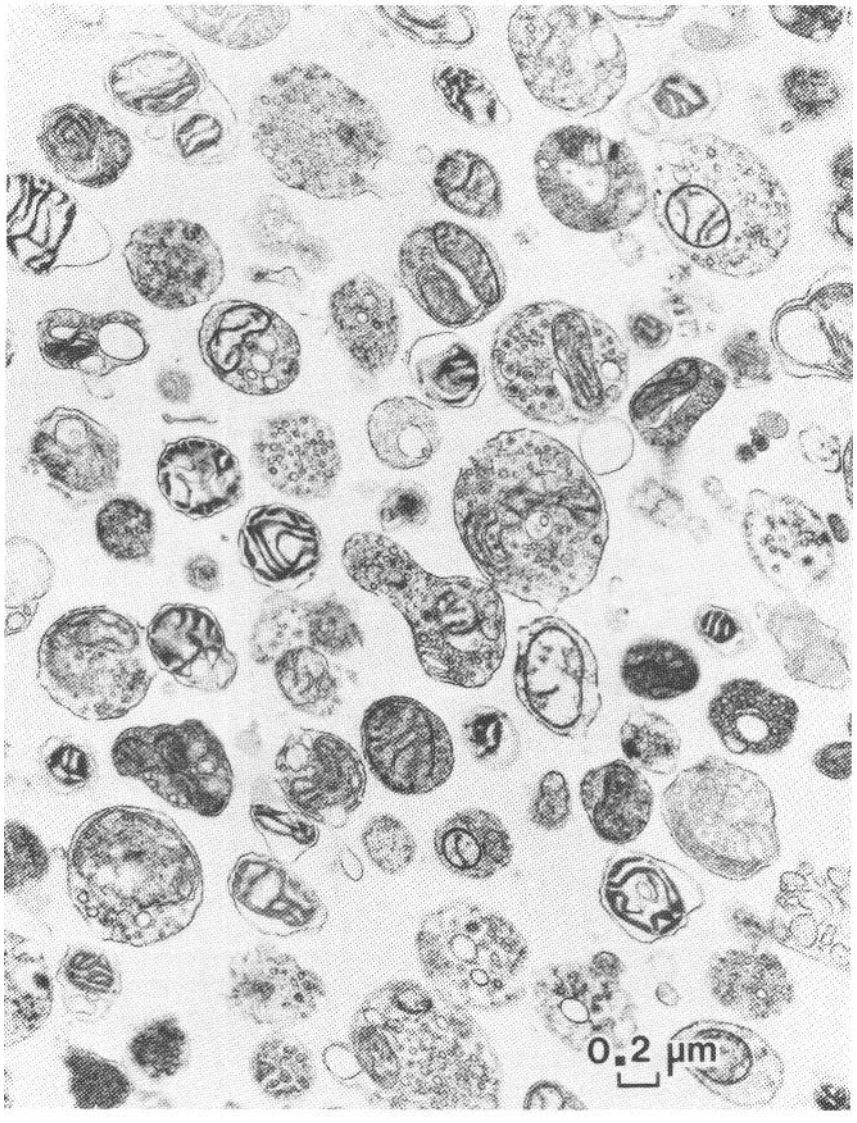

Figure 2. Electron micrograph of rat brain synaptosome fraction from the 7·5–13 per cent band of the Ficoll-sucrose gradient used in the present studies. Numerous synaptosomes are seen. The details of fixing and staining are given in the methods section. × 40 000.

Double reciprocal plots of kinetic data were constructed according to Lineweaver and Burk.[17] Data were subjected to regression analysis and regression lines were plotted for best straight line fit. K_D and B_{max} values were calculated from the graph (Figure 7).

Expression of Results

Each point in the graph is the mean ± SE of three or more different synaptosomal preparations and each preparation was assayed in triplicate. Data were analyzed by Student's *t*-test to assess the differences between control and experimental values. The significance is represented as asterisks in the figures ($*P < 0{\cdot}05$; $**P < 0{\cdot}001$).

RESULTS

In Vitro *Effects of Amiodarone on ATPase System*

Amiodarone inhibited Na^+, K^+-ATPase and O.S Mg^{2+}-ATPase activities in a concentration dependent manner, with no significant effect on O.I. Mg^{2+}-ATPase (Figure 3). O.S. Mg^{2+}-ATPase (76 per cent inhibition at 100 μM) was more sensitive than Na^+, K^+-ATPase (48 per cent inhibition at 100 μM) to the inhibitory action of amiodarone. K^+-activated *p*-nitrophenyl phosphatase (K^+-PNPPase) activity was also inhibited by amiodarone but the inhibition was significant only at amiodarone concentrations of 100 μM and higher (Figure 4). Inhibition of K^+-PNPPase at 100 μM (27 per cent) was significantly less than that observed for O.S Mg^{2+} and Na^+, K^+-ATPases.

In parallel with ATPase inhibition, amiodarone also inhibited [^{3}H]ouabain binding to synaptosomes in a concentration dependent manner with an IC_{50} value of 12 μM. A complete inhibition was observed at 50 μM amiodarone (Figure 5). Kinetic studies of [^{3}H]ouabain binding were performed in the presence and absence of amiodarone by varying [^{3}H]ouabain concentrations from 0·5 to 8 nM (Figure 6). Double reciprocal plots of [^{3}H]ouabain binding to synaptosomes demonstrated an increase in the K_D values from 8 to 20 mM without a change in the B_{max} values (10 pmol [^{3}H]ouabain mg protein h^{-1}) in the presence of 12·5 μM amiodarone (Figure 7).

To assess the dissociation of bound ouabain by amiodarone, the synaptosomes were preincubated for 15 min with amiodarone before the

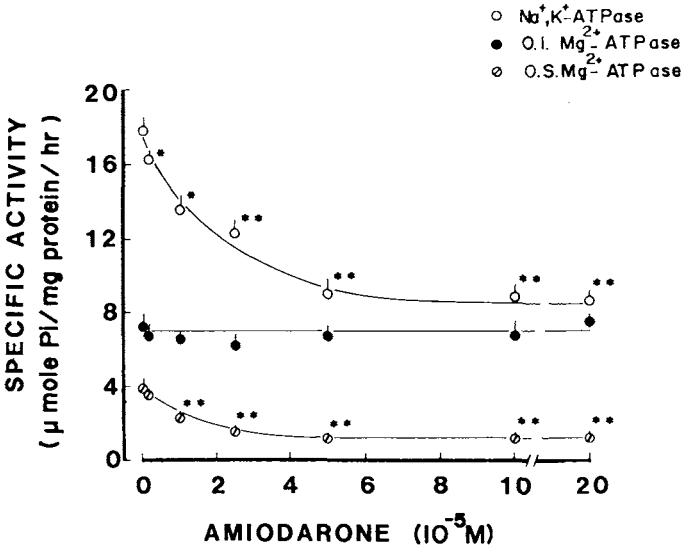

Figure 3. Inhibition of rat brain synaptosomal ATPases *in vitro* by amiodarone. Amiodarone at 10 μM and above significantly inhibited Na^+, K^+- (○) and O.S. Mg^{2+}-ATPases (⊘) with no significant effect on O.I. Mg^{2+}-ATPase (●). Asterisks indicate the signifance ($*P < 0{\cdot}05$; $**P < 0{\cdot}001$).

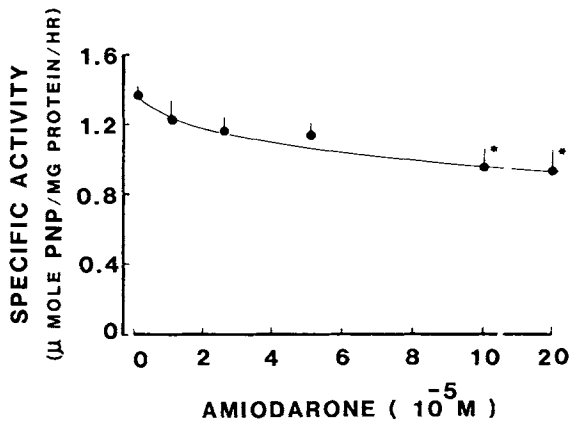

Figure 4. Inhibition of K^+-activated *p*-nitrophenyl phosphatase by amiodarone in rat brain synaptosomes. Significant inhibition was achieved only at amiodarone concentrations of 100 μM and higher ($^*P < 0{\cdot}05$).

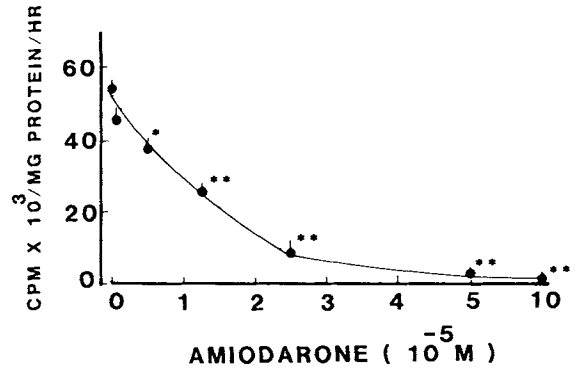

Figure 5. Effect of amiodarone on [^{3}H]ouabain binding to rat brain synaptosomes. Asterisks represent the significance ($^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$).

addition of [^{3}H]ouabain in one experiment. In another, [^{3}H]ouabain was added first and after incubation for about 15 min, amiodarone was added. Amiodarone inhibited [^{3}H]ouabain binding in a dose dependent manner, in both the experiments (Figure 8).

Since amiodarone altered the ATPase activities and inhibited [^{3}H]ouabain binding to synaptosomes competitively and since this occurs as a result of amiodarone binding to this enzyme complex, studies were further extended towards [^{14}C]-amiodarone binding to synaptosomes. (Figure 9). These studies were carried out in the presence and absence of ATP. In both the cases, [^{14}C]-amiodarone was bound to synaptosomes. However, the binding was slightly higher in the presence of ATP, suggesting that [^{14}C]-amiodarone binding may be facilitated by ATP (Figure 9).

In vivo *Effects of Amiodarone on the ATPase System*

Synaptosomal Na^+, K^+-ATPase, O.S. and O.I. Mg^{2+}-ATPase activities were determined in rats treated with 20 mg amiodarone kg^{-1} day^{-1} after 1, 2, 4, and 6 weeks of treatment and are presented in Table 1. The activity levels of Na^+, K^+- and Mg^{2+}-ATPases were not significantly altered in the rats treated with amiodarone when

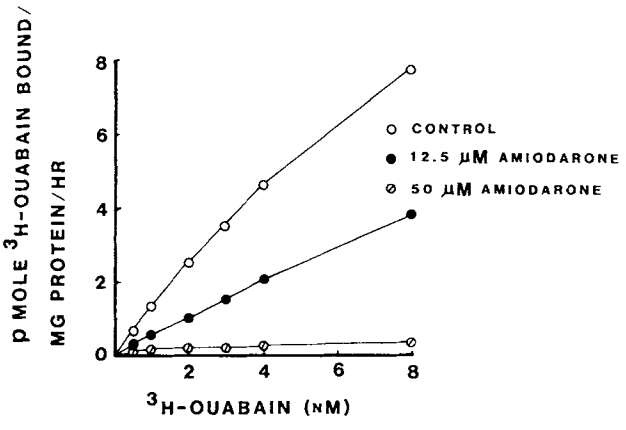

Figure 6. Amiodarone effect on [^{3}H]ouabain binding to rat brain synaptosomes.

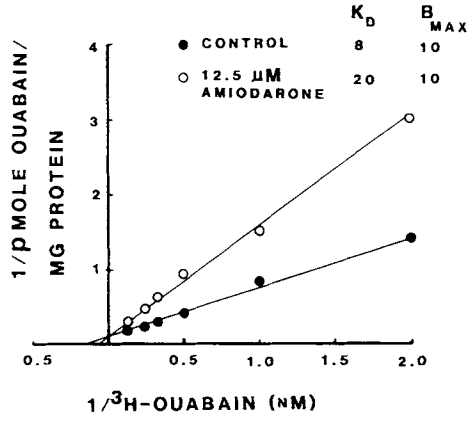

Figure 7. Double reciprocal plots of [^{3}H]ouabain binding to rat brain synaptosomes in the absence (●) and presence (○) of 12·5 μM amiodarone. K_D (mM) and B_{max} (μ moles [^{3}H]ouabain mg protein^{-1}) values were obtained from the data plotted in the figure.

Table 1. Activity levels of synaptosomal ATPases (μmol Pi mg protein^{-1} h^{-1}) in the rats treated with 20 mg amiodarone kg^{-1} day^{-1} up to 6 weeks

Specific ATPase measured	Vehicle treated control	Amiodarone treatment (weeks of treatment)			
		1	2	4	6
Na^+, K^+-ATPase	18·78±0·52	17·12±0·89	18·49±0·48	18·89±1·10	17·54±0·93
O.S. Mg^{2+} ATPase	5·36±0·10	4·87±0·23	4·62±0·42	4·98±0·23	4·94±0·49
O.I. Mg^{2+}-ATPase	7·69±0·23	7·39±0·69	7·21±0·41	7·42±0·39	6·81±0·35

Each value is the Mean ± SE of four or more different preparations and each preparation was assayed in triplicate.

compared to the respective pair-fed controls (Table 1).

DISCUSSION

The results of the present study indicate that amiodarone is an inhibitor of synaptosomal ATPases *in vitro*, since this drug significantly inhibited Na^+, K^+-ATPase and O.S. Mg^{2+}-ATPase. Similar observations on the inhibition of the ATPase system by a number of halogenated hydrocarbons and other toxins have been reported earlier in different species.[18–20] Na^+, K^+-ATPase has been shown to be the biochemical manifestation of the Na^+ pump and has also been linked to the transport of various amino acids including several neurotransmitters.[21, 22] It is evident from the literature that ionic movement

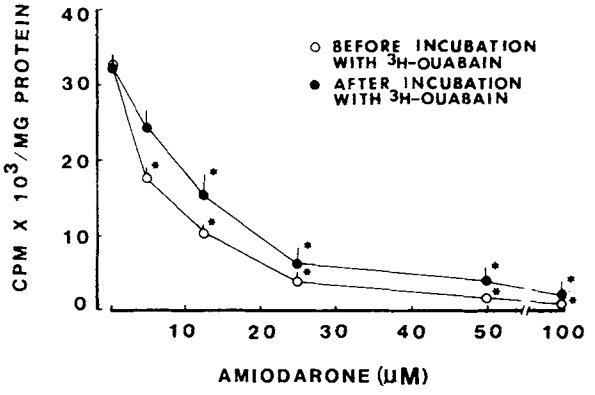

Figure 8. Effect of amiodarone on [^{3}H]ouabain binding to rat brain synaptosomes before (○) and after (●) incubation (15 min) of synaptosomes with [^{3}H]ouabain. Asterisks indicate the significance ($^*P < 0·05$).

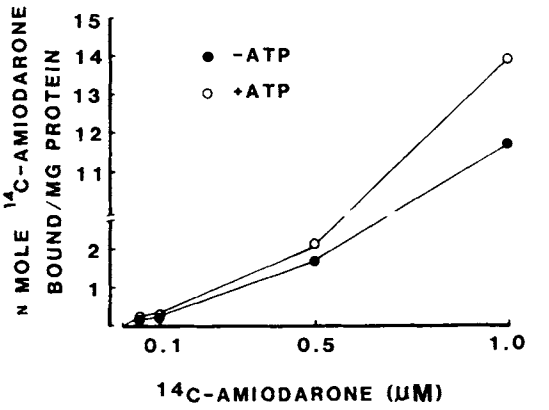

Figure 9. [^{14}C]-amiodarone binding to rat brain synaptosomes in the presence (○) and absence (●) of ATP.

of Na^+ and K^+ has a major role in neuronal function. O.S. Mg^{2+}-ATPase is involved in oxidative ATP production. ATP thus produced is utilized by the Na^+, K^+-ATPase for its active transport in addition to serving as energy source for various other metabolic processes in brain.[23] Inhibition of synaptosomal ATPase activities by amiodarone indicates its interference with these two phenomena.

Studies were extended to characterize amiodarone inhibitory action. Na^+, K^+-ATPase is a cylic reaction which involves phosphorylation in the presence of Na^+ and dephosphorylation in the presence of K^+. K^+-activated PNPPase is involved in the dephosphorylation of a phosphoryl–enzyme complex. K^+-PNPPase activity was also inhibited by amiodarone, but to a lesser extent than Na^+, K^+-ATPase. This suggests that

amiodarone inhibition of Na^+, K^+-ATPase may be due to its interference with Na^+-dependent phosphorylation. This is supported by the earlier observations of Mason *et al.*[8] who reported that amiodarone depresses the sodium current, not by binding to open channels, but by selective block of inactivated sodium channels.

Ouabain, a cardiac glycoside, is a specific inhibitor of Na^+, K^+-ATPase. [^{3}H]ouabain binding to synaptosomes was also inhibited significantly, confirming that amiodarone is an inhibitor of Na^+, K^+-ATPase. The kinetic studies on [^{3}H]ouabain binding revealed that amiodarone inhibition is of competitive type. Amiodarone was also found to dissociate ouabain bound to the synaptosomes. These results suggest that amiodarone has a higher affinity than ouabain and may interfere with the receptor site(s) for ouabain on Na^+, K^+-ATPase. This conclusion is further strengthened by [^{14}C]-amiodarone binding studies where a significant binding of amiodarone to synaptosomes was observed.

However, the activity levels of synaptosomal ATPases were not altered significantly in rats treated with 20 mg amiodarone kg^{-1} day^{-1} for 6 weeks. The possible reason for this discrepancy may be that the treatment period was not long enough to allow sufficient accumulation of the drug in the brain. The reported neurological abnormalities in patients occurred during 3–5 years of amiodarone treatment[4,24,25] indicating that much longer periods of exposure may be associated with the neurological problems. Also, Plomp *et al.*[26] reported very low levels of amiodarone in the brain when rats were treated for 10 days with a total dose varying from 250–500 mg $kg.^{-1}$ They explained that the high plasma protein binding (97 per cent) capacity[27] and the large molecular weight (M.W. 645) of amiodarone might have resulted in a poor diffusion through the blood–brain barrier even though the drug is highly lipophilic. Kannan *et al.*[28] reported that only a trace amount of amiodarone was accumulated in the brain when rabbits were treated i.p. with 20 mg amiodarone kg^{-1} for 6 weeks, a treatment protocol identical to the one used in our study in rats. Similar distribution behavior of amiodarone in the rat was reported by Riva *et al.*[29] at 16 h after a single intravenous dose of 50 mg kg^{-1} of amiodarone. Repeated administration of amiodarone for a long period might lead to the accumulation of amiodarone in amounts sufficient to cause these abnormalities, since this drug has a very long half life.

From the present study, it can be inferred that neuropathy induced by amiodarone may be due to its interference with sodium dependent phosphorylation of Na^+, K^+-ATPase, thereby affecting active ion transport, and also oxidative phosphorylation resulting in a low energy state in the nervous system. However, a direct demonstration of this effect in animal models requires long-term treatment of animals in accordance with the long-term treatments received by the patients.

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