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EFFECT OF AMIODARONE ON MEMBRANE FLUIDITY AND Na^+/K^+ ATPase ACTIVITY IN RAT-BRAIN SYNAPTIC MEMBRANES

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In rat-brain synaptic membranes at a fixed temperature (37°C), amiodarone dose-dependently inhibits the Na^+/K^+ ATPase activity ($IC_{50} \cong 2.10^{-5}$M) and produces a linear increase in the degree of fluorescence depolarization (P) of 1,6-diphenylhexatriene embedded in the lipid matrix. Amiodarone has no effect on Mg^{++} ATPase and K^+PNPase activity up to 3.10^{-4}M. Studies carried out at different temperatures indicate that 10^{-5}M amiodarone inhibits the Na^+/K^+ ATPase and decreases the lipid fluidity at all the temperatures studied (9 - 40°C). The compound significantly displaces the temperature of transition observed around 20°C in both Na^+/K^+ ATPase activity and lipid fluidity to 24°C with no changes in slopes. The results suggest that part of the selective inhibition of Na^+/K^+ ATPase activity by amiodarone could be due to the effects of the drug on lipid dynamics.

Amiodarone is a potent antianginal and antiarrhythmic drug (1,2). The drug inhibits selectively the Na^+/K^+ ATPase activity in guinea-pig heart particulate fraction (3) ; the inhibition is competitive with respect to ATP ($K_i \cong 65$ µM) and is thus different from that of ouabain which is non-competitive with respect to ATP. Several experimental observations such as complex solubilization properties, including micelle formation (4), low water-solubility (5), high lipid water partition coefficient (6) suggest a significant hydrophobic behaviour of amiodarone. The activity of membranous enzyme in general (7) and of Na^+/K^+ ATPase in particular (8-10) is sensitive to the lipid environment. Due to the properties of the compound, the inhibitory effect induced by amiodarone on Na^+/K^+ ATPase was reexamined in the light of a possible effect of the compound on the lipid matrix.

In the present communication, the effects of amiodarone on both ATPase activity and lipid dynamics were measured in a membranous system in which the relationship between the lipid dynamics and the Na^+/K^+ ATPase activity was well described : the rat-brain synaptic membrane (10-12).

The abbreviations used are:

DPH : 1,6 diphenylhexatriene
P : degree of fluorescence depolarization
EGTA : ethyleneglycol-bis-(2-amino-éthylether)-N,N,N',N'-tetraacetic acid
DMSO : dimethylsulfoxide

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EXPERIMENTAL PROCEDURE

Materials

1,6-diphenylhexatriene (DPH) and ATP-Tris were purchased from Sigma Chemical C°. All other chemicals were reagent grade or better.

Methods

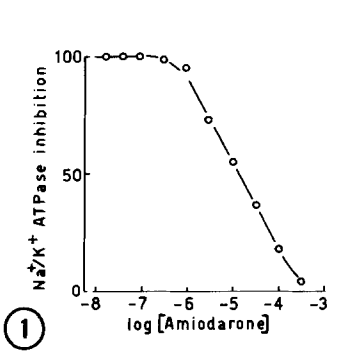
Rat-brain synaptic membranes were prepared according to Gray and Whittaker (13) ATPase activity was assayed according to Bers (14). The free inorganic phosphate was determined according to the colorimetric method of Fiske and Subbarow (15). The diluted synaptic membranes were first preincubated 30 min at 4°C with the desired amount of drug or the solvent (DMSO) in a medium containing 100 mM NaCl, 15 mM KCl, 1 mM EGTA, 4 mM $MgCl_2$, 50 mM Tris HCl (total ATPase) or 1 mM EGTA, 4 mM $MgCl_2$ and 50 mM Tris HCl (Mg^{++} ATPase) final concentration. The reaction was initiated by the addition of 100 µl of a solution of ATP 50 mM in prewarmed tubes. The final volume was 1 ml and the pH was adjusted to 7.4. After 4,8,12 and 16 minutes, the reaction was stopped by addition of 2 ml of a solution of 2 % of ascorbic acid, 10 % trichloracetic acid. 1 ml of the suspension was assayed for inorganic phosphate. Para-nitrophenyl phosphatase activity was measured as described by Tashima et al. (16). Protein content was determined according to Lowry et al (17) using bovine serum albumine as the standard. The labelling of the synaptosome suspensions was performed with 1,6-diphenyl-1,3,5-hexatriene (DPH) according to Shinitzky (18). DPH stock solution (2.10^{-3}M in tetrahydrofuran) was diluted 1000 times in a Tris HCl buffer (62.5 mM, pH 7,4) with vigorous stirring just before use. One volume of the diluted DPH dispersion was added to 1 volume of the synaptosome suspension. The mixture was incubated 60 min at 37°C in presence of the desired amount of drug or of the solvent. The suspension was centrifuged at 4.000 g for 10 min. The supernatant was collected, centrifuged (30 min at 70.000 g). The pellet was homogeneized in the above buffer at the final concentration of 0.2 mg proteins/ml. Excited-state lifetimes (τ) of DPH were performed using a SLM 4800 Spectrofluorimeter (SLM Instruments Champaign, IL). Steady state measurements of the degree of fluorescence depolarization (P) of DPH were performed on an Elscint Microviscosimeter (Haifa, Israël). Statistical differences were determined by Student's t test.

RESULTS

Amiodarone inhibits concentration-dependently the activity of Na^+/K^+ ATPase in rat-brain synaptic membranes with a threshold concentration of 1 µM and a concentration inhibiting enzyme activity by 50 % (IC_{50}) of $\cong 2.10^{-5}$M (figure 1). The results are expressed in percent of the enzyme activity in the absence of amiodarone. The total, Na^+/K^+ and Mg^{++} ATPase activities are respectively of the order of 40,30 and 10 µM ATP hydrolysed/h/mg proteins in the synaptic membranes used. In the range of concentration investigated, amiodarone has no effect on the Mg^{++} dependent ATPase which represents 25 % of the total ATPase activity.

The lipid dynamics was appreciated by the degree of fluorescence depolarization P of DPH incorporated in the lipid acyl chains. At 37°C, in the absence of amiodarone, P had a value of 0.217 (figure 2). Incubation with increasing amounts of amiodarone induces a increase of P. According to the Perrin equation

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{\tau.RT}{\eta}\right)$$



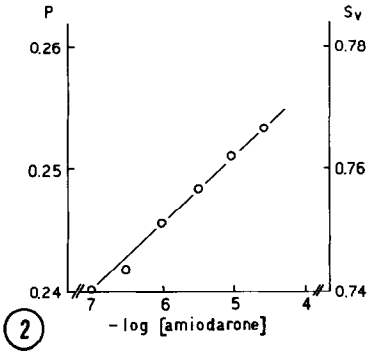


Fig. 1 : Dose-response curve of the inhibition by amiodarone of Na^+/K^+ ATPase activity in rat brain synaptic membranes. The results are the mean of 4 experiments. Temperature : 37°C.

Fig. 2 : Dose-response curve of the effect of amiodarone on the degree of fluorescence depolarization (P) of DPH (left ordinate) and on the derived lipid order parameter (S_v) (right ordinate) in rat brain synaptic membranes. Temperature : 37°C. The results are the mean of 4 experiments.

where P is the measured fluorescence depolarization, P_o is the limiting value of P, τ is the fluorescence lifetime, V is the effective molar volume of the probe and η is the viscosity of the medium. The movements of the probe within the medium depend on both τ and η. It is generally assumed that τ is constant at one temperature and thus P is directly related to η. However, the presence of a third compound can modify τ if this compound acts as a quencher. A direct measurement of τ in the absence or presence of quencher enables this possibility to be verified. At 37°C, values of τ of 10.6 $\pm$ 0.8 and 10.3 $\pm$ 0.9 nsec were obtained respectively in the absence and presence of 2.10^{-5}M amiodarone suggesting that the compound has no appreciable effect on the fluorescence lifetime of DPH. The increase in P can be interpreted in terms of reduction of DPH mobility and of decrease in membrane fluidity.

The variations of lipid fluidity (fig. 2) and of the enzyme inhibition (fig. 1) induced by amiodarone are related by a linear regression ($y = - 13.25 + 5.30\ x$) with a coefficient of correlation (r) of 0.99 ($P < .001$).

In the absence of amiodarone, the Arrhenius plot of the Na^+/K^+ ATPase activity shows a break around 20°C with a linear evolution of the enzymic activity above and below the break (fig. 3). The mean value of the break temperature and of the energies of activation are given in Table 1. They are in agreement with reported data (10-12). Addition of 10^{-5}M amiodarone inhibits enzyme activity throughout the range of temperature studied (9-40°C) (fig. 3).

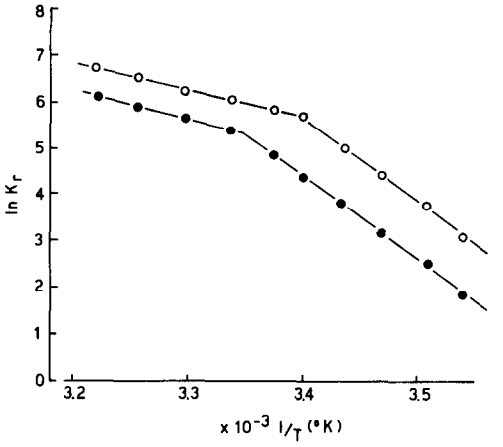


Fig. 3 : Arrhenius plot of the Na^+/K^+ ATPase activity in the absence (o) and in the presence (•) of 10^{-5} M amiodarone in rat brain synaptic membranes. The results represent the mean of 3-4 experiments.

There is no significant change in the energies of activation (E_a), but the break is significantly displaced by 3.7°C to the high temperature range (Table 1). As a result of the absence of effect on E_a, the percent of Na^+/K^+ ATPase inhibition which is 47 % in the high temperature range (corresponding to the results obtained at 37°C, figure 1) increases below the break and is 67 % in the low temperature range (figure 3).

Table 1

Characteristics of Arrhenius plots of rat-brain synaptic membranes. Na^+/K^+ ATPase activity and lipid dynamics in the absence and presence of 10^{-5} M amiodarone

Experimental conditions	Na^+/K^+ ATPase			Lipid dynamics		
	T_t	E_a		T_t	E_a	
		$T < T_t$	$T > T_t$		$T < T_t$	$T > T_t$
Control	20.5 ± 0.8	19.3 ± 0.5	11.1 ± 2.0	19.0 ± 11.8	10.3 ± 1.2	13.6 ± 1.3
10^{-5} M amiodarone	24.2 ± 0.3	20.0 ± 1.7	12.1 ± 2	23.1 ± 0.7	10.1 ± 1.6	13.9 ± 1.0
	P < 0.001			P < .01		

T_t : temperature of transition (°C)

E_a : activation energy (kcal/mol)

The results are the mean ± SEM of 3-4 separate experiments.

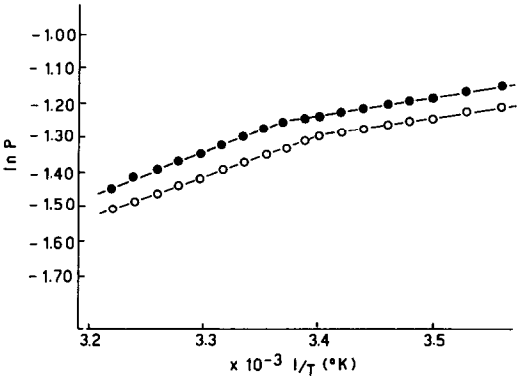


Fig. 4 : Arrhenius plot of the degree of fluorescence depolarization (P) of DPH in the absence (o) and in the presence (•) of 10^{-5}M amiodarone in rat brain synaptic membranes. The results represent the mean of 3-4 experiments.

Arrhenius plots of the degree of fluorescence depolarization (P) show a break around 19°C in the control membranes (figure 4). This transition temperature separates two domains where the evolution of P is directly proportional to the variation of temperature. In the presence of 10^{-5}M amiodarone, an increase of P is observed throughout the range of temperature studied. As for the effect on Na^+/K^+ ATPase activity, there is no change in E_a but the break of temperature is significantly displaced by 3.6°C to the high temperature range (Table 1). The mean values of the break temperatures and of the energies of activation are given in Table 1.

DISCUSSION

Amiodarone inhibits specifically the Na^+/K^+ ATPase activity in rat-brain synaptic membranes. The inhibition was further investigated by measurements of the K^+ dependent ouabain-sensitive P-nitrophenylphosphatase activity. However, amiodarone up to 10^{-4}M has no effect on this enzymic activity (data not shown) suggesting that the compound does not exert its inhibitory effect on the dephosphorylation step which is a part of the sequence of ATP hydrolysis by Na^+/K^+ ATPase (16).

The Na^+/K^+ ATPase inhibition is paralleled by modifications of the lipid dynamics observed both as a function of concentration (at a fixed temperature) and as a function of temperature. As the activity of Na^+/K^+ ATPase is thought to be controlled at least in part by the lipid environment (8-12), our data suggest that the inhibition of Na^+/K^+ ATPase by amiodarone may be due to the effect of the drug on the lipid dynamics.

In favour of this suggestion is the lack of effect of the compound on both Mg^{++} ATPase and K^+-P-nitrophenylphosphatase. In comparative studies, in fact the activity of these enzymes has been shown to be less sensitive to lipid perturbation than the activity of Na^+/K^+ ATPase (20,21).

The mode of inhibition by amiodarone is probably different from the commonly reported data in that the compound does not fluidify the lipid matrix as usually observed (11) but on the contrary decreases lipid fluidity. Such a solidifying effect is observed with long chain alcohols (22,23), fatty carboxylic acids (23) and with cholesterol (18). These compounds are thought to increase order in the hydrocarbon core of the bilayer through their position and localization in the bilayer which promote interactions with the hydrocarbon chains of the lipids. It may be expected that amiodarone displays the same kind of interactions in the lipid matrix. Through their observations that the depolarizing rotations of DPH are anisotropic in a lipid bilayer, Jähnig (24) and Van Blitterswijk et al (25) improve the usefulness of P measurement by relating it to the lipid order parameter S_v where v indicates the mean position of DPH along the fatty acid chains, through the equation $r_\infty = 2/5\ S_v^2$ where r_∞, a non-zero limiting fluorescence anisotropy when the probe is immobilized, is given by $r_\infty = 9/8\ r - 1/20$. r is given by the relation $r = \frac{2P}{3-P}$. The application of these equations to our data clearly indicates that amiodarone increases the lipid order in the lipid matrix of rat-brain synaptic membranes (figure 2). This could account for the inhibitory effect induced by the compound, as the increase in membrane lipid acyl chain order parameter results in rate limitation of Na^+/K^+ ATPase (26). Beside this effect, amiodarone could possibly inhibit the enzymic activity by other mechanisms such as a direct interaction with the polypeptidic chain at the interface between the lipids and the protein, modifications of surface charges as amiodarone is an amine derivative mainly ionized at pH 7.4 or electrostatic interactions with some acidic phospholipids essential for the Na^+/K^+ ATPase activity (11).

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