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INFLUENCE OF CATIONIC AMPHIPHILIC DRUGS ON THE CHARACTERISTICS OF OUABAIN-BINDING TO CARDIAC Na^+/K^+-ATPase

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Abstract—The influence of 12 cationic amphiphilic compounds on the equilibrium and kinetic characteristics of the binding of tritium-labelled ouabain to the lipoprotein Na^+/K^+-ATPase present in a crude membrane suspension of guinea pig myocardium was investigated. The drugs, e.g. local anaesthetic, antiarrhythmic and psychotropic agents, inhibited specific binding of ouabain in a concentration-dependent manner by reducing its affinity without affecting the number of binding sites. In the presence of chlorpromazine, propranolol and dibucaine, the decreased affinity of ouabain was due to both a diminished association rate and an increased dissociation rate, while in the presence of the weakly potent procaine only the association rate of ouabain was found to be reduced. The different potency of the catamphiphilic drugs was well correlated to the degree of their hydrophobicity. Evidence is presented that the protonized form of the drugs is the effective one. Concerning the mode of action, the catamphiphilic drugs are proposed to interact with the phospholipid part of the lipoprotein Na^+/K^+-ATPase, thereby indirectly altering the conformation of the embedded protein moiety and thus reducing the proper fit between ouabain and its receptor.

The binding of tritium-labelled ouabain to cardiac Na^+/K^+-ATPase (EC 3.6.1.3) is a method often applied to characterize the interaction between cardiac glycosides and their receptors [1–3]. In the present study the binding of [^{3}H]ouabain to a membrane suspension of guinea pig myocardium was investigated in the presence of cationic amphiphilic compounds. Since the binding of ouabain to its receptor can be considered as a sensitive indicator for structural alterations of the glycoside-binding conformation of the protein moiety the binding process of ouabain was used as a tool to examine the effects of a number of catamphiphilic drugs (listed in Table 1) on the protein part of the lipoprotein Na^+/K^+-ATPase. It remains undecided, whether the influence on ouabain-binding resulted from a direct interaction with the protein moiety or from an indirect interaction with surrounding phospholipids; the latter mode of action, however, will be discussed as the more probable one. A short report on part of this work has been presented elsewhere [4].

MATERIALS AND METHODS

Preparation of ventricular tissue. Ventricles from guinea pigs (about 400 g body weight), stored at −20°, were thawed, minced and homogenized in a 0.32 M sucrose solution in a volume of 20 ml/g wet weight for 30 sec in a Waring Blendor at low speed, followed by a treatment with six strokes in a Potter–Elvehjem glass homogenizer with a Teflon pestle at medium speed. The supernatant yielded by centrifugation for 10 min at 2000 *g* was recentrifuged for 18 min with 30,000 *g*. The pellets were resuspended in 50 mM Tris–HCl, pH 7.4, in a volume of 4 ml/g w.w., frozen in liquid nitrogen and stored at −20°. All preparation steps were carried out at 4°. Protein content was determined according to Lowry *et al.* [5], using human serum albumin (Behringwerke, Marburg, F.R.G.) as a standard.

Equilibrium binding of [^{3}H]*ouabain.* Tritium-labelled ouabain with a specific activity between 12 and 20 Ci/mmole was obtained from NEN (Dreieichenhain, F.R.G.); the purity of [^{3}H]ouabain was regularly checked by radiochromatography. The binding assay was performed in triplicate in 1.5 ml medium consisting of membrane suspension (0.5–0.7 mg protein/ml), of [^{3}H]ouabain (about 5 nM), and of NaCl (80 mM), $MgCl_2$ (16 mM) and Tris–HCl (50 mM) yielding a final pH of 7.3. For the ouabain-binding studies unlabelled ouabain was added in increasing concentrations up to 10^{-4} M. In order to investigate the influence of different cationic amphiphilic compounds upon [^{3}H]ouabain-binding, increasing concentrations of these drugs were added. The binding reaction was started by ATP-Na_2 (final concentration 2.5 mM) and carried out at 37° for 75 min. The incubation was terminated by cooling the tubes in crushed ice. The incubation vessels consisted of thick-wall polyallomer centrifugation tubes and allowed immediate centrifugation (18 min at 52,000 *g* and 4°) to obtain the membrane-bound radioactivity. The pellet was rinsed 3 times with 1 ml of ice-cold buffer and resuspended in 1.5 ml buffer by sonication (Braun Labsonic 1510). To measure the radioactivity 10 ml of Dimilume 30® (Packard) were added to an aliquot of 1 ml, the samples were counted in a Packard Tricarb 460 C using the external standard method; the counting efficiency was about

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Table 1. Investigated drugs; IC_{50} to inhibit specific binding of [^{3}H]ouabain to Na^+/K^+-ATPase; the respective partition coefficients and pK_a values (as compiled by Lüllmann *et al.* [11])

Compound	IC_{50} ($\times 10^5$) (M)	Octanol/water partition coefficient log p	pK_a
2-Aminopyridine	790	0.2	6.95
Phentermine	160	1.9	10.1
Procaine	150	1.9	8.9
Verapamil	90	2.51	9.2
Tetracaine	68	3.73	8.24
Dibucaine	50	4.3	8.83
Propranolol	50	3.14	9.6
Chlorphentermine	49	2.6	9.6
Iprindole	22	4.9	8.2
Imipramine	13	4.62	9.5
Chloroquine	11	4.63	10.1/8.1
Chlorpromazine	8.5	5.33	9.3

IC_{50} values were derived from single dose-effect curves based on triplicate determinations. In the case of 2-aminopyridine the IC_{50} refers to the concentration of the protonized form, which was 15% of the total. For the other drugs, which were almost entirely protonized, the total concentrations are given.

35%. The unspecific binding of [^{3}H]ouabain was determined in the presence of 10^{-4}M unlabelled ouabain and was identical to the binding in the absence of ATP. The specific binding resulted from the difference between total binding and unspecific binding; at 5 nM the latter amounted to about 15% of the total binding.

The concentration-dependent specific binding of ouabain was calculated taking into account the dilution of the specific activity induced by increasing concentrations of unlabelled ouabain. Binding was analysed by Scatchard plots, and could be characterized by the equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}). The free concentration of ouabain was assumed not to be significantly reduced by binding to binding sites, since the concentration of binding sites was less than 10% of the K_D [6].

Kinetics of [^{3}H]*ouabain-binding.* In order to investigate the association and dissociation of [^{3}H]ouabain, a rapid filtration technique was applied to separate the membranes from the incubation medium. The total volume of the incubation medium amounted to 45 ml and was of identical composition as described for equilibrium binding studies except for the concentration of [^{3}H]ouabain which in this case amounted to about 10 nM. The cationic amphiphilic drugs were added in concentrations known to yield half maximum inhibition of [^{3}H]ouabain-binding as determined by equilibrium binding experiments. After a preincubation of 30 min at 37°, specific binding of [^{3}H]ouabain was initiated by addition of ATP (final concentration: 2.5 mM) and followed up for 45 min. Thereafter dissociation of [^{3}H]ouabain was started upon addition of unlabelled ouabain (final concentration: 63 μM), which was in excess to [^{3}H]ouabain by a factor of 6300 ("chase experiment"); the reaction was observed for 45 min (see Fig. 3). At the appropriate points of time 1 ml aliquots of the incubation medium were filtered under suction through Whatman GF/C glass fibre filters followed by rinsing with 2 × 5 ml of ice-cold distilled water; for this procedure less than 15 sec were required. Filters were transferred into scintillation vials, Soluene 350® (Packard) and after 1 hour Dimilume 30® (Packard) were added to allow detection of membrane-bound radioactivity. Unspecific binding of [^{3}H]ouabain was determined at the end of the 30 min preincubation period prior to the addition of ATP and at the end of the dissociation period; it amounted to about 5% of total binding.

Control experiments revealed that the specific binding of ouabain was identical irrespective of whether the techniques of centrifugation or of filtration were applied for separation of the membrane-bound ligand.

The kinetics of [^{3}H]ouabain-binding were assumed to obey the equation [7]

$$\frac{d[RL]}{dt} = k_{+1} \cdot [L] \cdot [R] - k_{-1} \cdot [RL] \qquad (1)$$

where $R = Na^+/K^+$-ATPase, L = [^{3}H]ouabain, RL = bound [^{3}H]ouabain, k_{+1} = association rate constant and k_{-1} = dissociation rate constant.

For the initial phase of association the dissociation process can be neglected, such that (1) can be reduced to

$$\frac{d[RL]}{dt} = k_{+1} \cdot [L] \cdot [R]. \qquad (2)$$

L was considered to be identical to the concentration of [^{3}H]ouabain added, since B_{max} was smaller than $\frac{1}{10} \cdot K_D$ [6]. R equalled B_{max}, since (a) the concentration of [^{3}H]ouabain was about $\frac{1}{15} \cdot K_D$ and (b) only the initial process of association was used for analysis. The initial association rate was estimated by the slope of the line connecting the origin with the 45 sec value (see inset Fig. 3). This association velocity divided by the [^{3}H]ouabain-concentration and by B_{max}, the latter being determined by equilibrium binding experiments, yielded the rate constant of association k_{+1}. The rate constant of dissociation k_{-1} was calculated from the half life time of disso-

Table 2. Association rate constant k_{+1}, dissociation rate constant k_{-1}, calculated equilibrium dissociation constant K_D of [^{3}H]ouabain-binding in the presence of selected catamphiphilic compounds at IC_{50}-concentrations

Compound	k_{+1} ($\times 10^{-4}$) ($M^{-1} \cdot sec^{-1}$)	k_{-1} ($\times 10^3$) (sec^{-1})	K_D ($\times 10^7$) (M)
Control (N = 7)	2.5 ± 0.6 *	3.9 ± 0.5 n.s.	1.6
Procaine (N = 5)	1.0 ± 0.1	3.6 ± 0.5	3.6
Chlorpromazine (N = 5)	1.5 ± 0.3 *	5.6 ± 0.9 *	3.7
Dibucaine (N = 2)	1.6 ± 0.3	5.7 ± 0.8	3.6
Propranolol (N = 2)	1.5 ± 0.5	5.5 ± 0.5	3.7

Presented are the mean values ± S.D. and N = number of experiments. The control values and the data obtained in the presence of procaine and chlorpromazine were compared using Student's *t*-test; *: $P < 0.01$; n.s.: not significant.

ciation $t_{1/2}$ as evaluated from a plot log ouabain bound vs time ($k_{-1} = \ln 2/t_{1/2}$).

RESULTS

Binding of ouabain under control conditions

Specific ouabain-binding under control conditions slightly varied for different preparations yielding a dissociation constant K_D of 1.2–1.7 × 10^{-7} M dependent on the different membrane preparations. The maximum number of binding sites was found to range between 4.0 and 6.0 pmoles/mg protein and thus agreed well with values reported in literature [8, 9]. The kinetic data of ouabain-binding are given in Table 2; they are similar to values communicated by De Pover and Godfraind [10]. During the 75 min of incubation ATP was almost entirely hydrolyzed by Mg^{2+}-ATPase present in the membrane-suspension.

Addition of ADP instead of ATP resulted in a similar equilibrium binding of ouabain (P_i could not substitute for ATP). The association rate of [^{3}H]ouabain, however, decreased by 30%, when initiated by addition of ADP instead of ATP; this procedure thus leads to an underestimation of k_{+1}. Preincubation of the membranes with ATP for 75 min prior to addition of [^{3}H]ouabain yielded an association rate identical to the ATP-induced association. These findings suggest that the ouabain-binding enzyme conformation is formed by a phosphorylation reaction, which if induced by ATP is not rate-limiting for ouabain-binding. Since K^+ was omitted from the incubation medium, it is reasonable to assume that all Na^+/K^+-ATPase molecules remained in the phosphorylated conformation, because the K^+-induced termination of the conformational cycle was prevented (for details about ouabain-binding to Na^+/K^+-ATPase see [1]).

Influence of cationic amphiphilic drugs upon the equilibrium binding of ouabain

The drugs investigated belong to different groups of pharmacological agents such as local anaesthetics, β-blockers, psychotropic drugs; they are listed in Table 1 in the order of their efficacies. Table 1 also provides information on their octanol/water partition coefficients and their pK_a. All compounds inhibited [^{3}H]ouabain-binding in a concentration-dependent manner. As an example the dose–response curves for three drugs, i.e. imipramine, tetracaine and phentermine, are depicted in Fig. 1. They display more or less parallel shifts and exert maximum effects, i.e. total inhibition of specific [^{3}H]ouabain-binding. All other drugs yielded the same type of curves, thus allowing to characterize the efficacy by IC_{50}-values (drug concentration inhibiting [^{3}H]ouabain-binding by 50%). However, in a few cases, poor solubility prevented the application of concentrations sufficient to produce total inhibition of ouabain-binding (i.e. chlorpromazine, iprindole and verapamil; the latter allowing only 42% inhibition, necessitating an extrapolation of the IC_{50}).

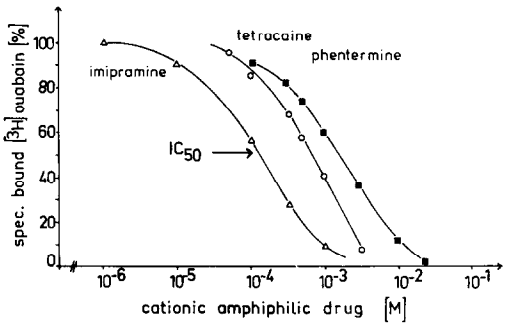

Fig. 1. Inhibition of [^{3}H]ouabain-binding to Na^+/K^+-ATPase present in a membrane suspension of guinea pig myocardium (concentration of [^{3}H]ouabain: 5 nM, concentration of binding sites: about 3 nM) by imipramine (△), tetracaine (○) and phentermine (■). Mean values of a triplicate determination are presented. The parallel curves allowed a characterization of the efficacy of the drugs by the concentration that inhibited binding by 50% (IC_{50}).

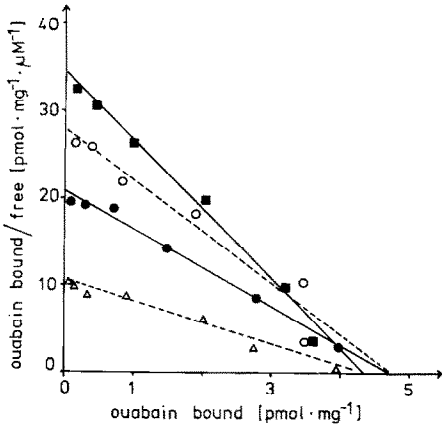

Fig. 2. Scatchard analysis of the specific binding of ouabain in the presence of various concentrations of propranolol. Control (■——■), propranolol 2×10^{-4} M (○ – – ○), 3.2×10^{-4} M (●——●), 1×10^{-3} M (△ – – △). Presented are mean values of a triplicate determination. The decreasing slopes indicate a reduction of the affinity of ouabain-binding; the lines intersect the abscissa within a close range suggesting that the maximum number of binding sites remains unaffected.

The IC_{50}-values are also presented in Table 1; they range between 8.5×10^{-5} M in the case of chlorpromazine and 7.9×10^{-3} M in the case of 2-aminopyridine (protonized form). The IC_{50}-values were derived from single dose-effect curves based on triplicate determinations. The reliability of the dose-effect curves was established in subsequent experiments, in which selected concentrations of the catamphiphilic drugs evoked the expected effects. A reduction of [^{3}H]ouabain-binding by amphiphilic compounds can principally be the result of either a decrease of the maximum number of binding sites or a diminished affinity to the binding sites. In order to distinguish between these two possibilities, concentration-dependent binding of ouabain was measured in the presence of selected concentrations of an amphiphilic drug and analysed by means of Scatchard plots. A typical example is given in Fig. 2 for three concentrations of propranolol; the Scatchard analysis revealed that propranolol reduced the affinity but did not influence the maximum number of binding sites. Chlorpromazine, chloroquine, imipramine, dibucaine and procaine were similarly analysed and behaved in the same way.

Influence of cationic amphiphilic drugs upon the kinetics of [^{3}H]*ouabain-binding*

Since the reduction of the affinity of ouabain to its binding site could have been the result of either a diminished rate of association or an enhanced rate of dissociation, the influence of four selected catamphiphilic compounds on the ouabain-binding kinetics was investigated. For this purpose, chlorpromazine as the most effective drug, propranol and dibucaine with intermediate and procaine with low effectivity were chosen and applied in concentrations known to yield half maximum binding of [^{3}H]ouabain. Experimental curves obtained with chlorpromazine and procaine are shown in Fig. 3. All four drugs clearly reduced the association rate constant of ouabain, whereas the dissociation rate constant was increased by chlorpromazine, dibucaine, and propranolol. In contrast procaine did not accelerate the dissociation (Table 2).

When addition of [^{3}H]ouabain was used to start the association reaction to a membrane suspension preincubated with ATP and the catamphiphilic drug, association rate constants were found to be identical to those obtained when starting the reaction with ATP. Hence, the retarded association of [^{3}H]ouabain in the presence of the amphiphilic drugs observed after a start with ATP was not due to a diminished rate of formation of the ouabain-binding enzyme conformation.

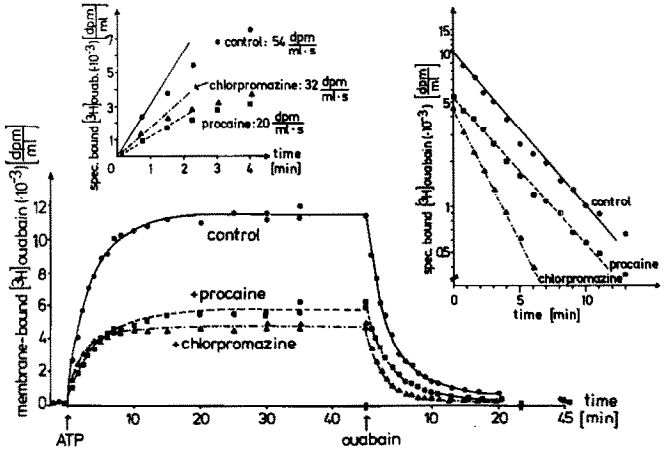

Fig. 3. Time course of the total binding of [^{3}H]ouabain (10 nM) to cardiac cell membranes. Indicated are the start of the association by addition of ATP (2.5 mM) and the initiation of the dissociation by an excess of unlabelled ouabain (63 μM). Control (●——●), procaine (1.5 mM) present (■ – – ■), chlorpromazine (85 μM) present (▲ · – · ▲). The points given are individual data obtained in representative experiments. The evaluation of the initial association rate and the analysis of the dissociation process are depicted in the insets showing the time-dependent specific binding of [^{3}H]ouabain.

From the equilibrium experiments it could be taken that a reduction of [^{3}H]ouabain-binding by 50% was due to an equivalent reduction of the affinity; when the association and dissociation rate constants obtained in kinetic experiments were used to calculate the affinity a reduction by about 50% was found as had to be expected. This proves the validity of the kinetic experiments.

DISCUSSION

The investigated amphiphilic compounds demonstrated rather different potencies to inhibit specific ouabain-binding to the Na^+/K^+-ATPase. As an attempt to obtain some insight into the underlying mechanism the efficacies were correlated with the hydrophobicity of the molecules obtained from octanol/water partition coefficients. As shown in Fig. 4 increasing hydrophobicity is related to enhanced potency. All compounds included in the study except for 2-aminopyridine were almost entirely charged at the pH of the incubation medium of 7.3. The addition of the catamphiphilic drugs influenced this pH only slightly, i.e. for ±0.1 units, except for 2-aminopyridine, which induced a pH of 7.7 at its IC_{50}-concentration; at pH 7.7 ouabain-binding was not reduced under control conditions. 2-Aminopyridine with a pK_a of 6.95 was only protonized by 15%. As indicated by the two symbols in Fig. 4 the IC_{50} referring to the protonized concentration fits the correlation. This suggests that a positive charge is the prerequisite for the effect while the hydrophobicity determines the efficacy. The same conclusion was reached by Lüllmann *et al.* [11], when the ability of cationic amphiphilic compounds to replace $^{45}Ca^{2+}$ from phospholipid monolayers was studied.

The Scatchard plot indicates a reduced affinity between ouabain and its binding site in the presence of cationic amphiphilic compounds; this might imply a competitive type of antagonism. However, the kinetic experiments revealed not only a reduced association but also an increased dissociation rate of ouabain. This renders a competition of the catamphiphilic drugs for the binding sites unlikely, because under this aspects the competitor would reduce the probability of the agonist to hit an unoccupied receptor but would not influence the dissociation of the agonist–receptor complex. Thus the reduced affinity has to be explained by an indirect influence of catamphiphilic drugs on the ouabain binding site. Since the Na^+/K^+-ATPase is a lipoprotein (cf. [1, 2] for reviews), either an interaction with the protein moiety or the surrounding phospholipids has to be discussed. While little is known of the influence of cationic amphiphilic drugs on protein conformations, their interaction with phospholipid-layers has been thoroughly investigated. As studied by means of NMR, electron spin resonance or microcalorimetric methods, these compounds interfere with the molecular array of phospholipids (e.g. Refs 12–16). On the other hand it has been shown that negatively charged phospholipids, such as phosphatidylserine or phosphatidylinositol, being closely associated with the protein moiety of the Na^+/K^+-ATPase, are important for its enzymatic function [17–23]. Additionally, a strong interdependence between the enzymatic function of the protein part and the structural arrangement of the adjacent lipids has been demonstrated; Grisham and Barnett [24] found a dependence of the ATPase activity on the fluidity of the attached phospholipids and Kimelberg and Papahadjopoulos [25] reported on the relationship between the discontinuity of the Arrhenius plot of the enzymatic function and the transition temperature of different phospholipids used to reactivate the enzyme. Also the binding of ouabain was shown to be considerably enhanced by restitution with phosphatidylserine [26]. Charnock *et al.* [27] found the association rate of [^{3}H]ouabain to Na^+/K^+-ATPase to be temperature-dependent; a thermal transition, which occurred at 25°, was not detectable, when the membranes were pretreated with detergents or phospholipase A.

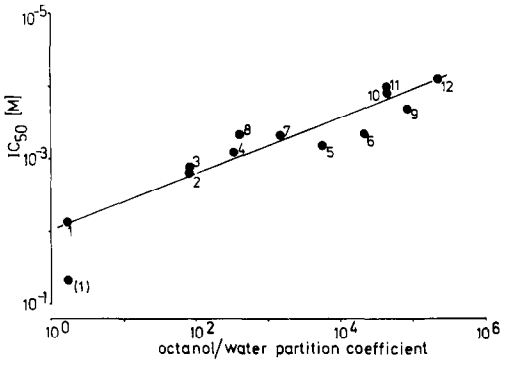

Fig. 4. Relationship between the efficacies of the catamphiphilic drugs (IC_{50}-values, ordinate), and their hydrophobicity (octanol/water partition coefficients, abscissa). The numbers characterizing the compounds are identical to those used in Table 1. Since the compounds are protonized to more than 90% under the conditions the actual concentrations are not corrected for the non-protonized fraction (pK_a see Table 1), except for 2-aminopyridine: 1 corrected, (1) not corrected. The correlation coefficient is $r = 0.94$.

Since there exists such a close interdependence between the lipid portion and the protein moiety of the Na^+/K^+-ATPase, we assume that the primary site of action of the catamphiphilic drugs is located in the lipid portion of the membranes. This is supported by the finding that the rank-order of efficacy of the catamphiphilic compounds found in the present study is in good agreement with that obtained by measuring the direct interaction between these drugs and phospholipid monolayers [11]. Although several publications reports on an inhibition of the Na^+/K^+-ATPase activity by some cationic amphiphilic agents (for a compilation see [28]) a reduced enzyme activity cannot account for the decreased ouabain binding, since the absence of potassium excluded enzymatic transport activity in the present experiments; in contrast the enzyme probably persisted in the ouabain-binding conformation (see above). In analogy to models proposed for the mode of action of local and general anaesthetics, which explain the alterations of specific membrane proteins as a result of drug–lipid interaction [28, 29], it may be assumed that the alterations induced by inter-

calation of the cationic amphiphilic molecules between the phospholipids are transferred to the embedded protein part of the Na^+/K^+-ATPase, affecting its conformation and thus impairing the proper fit between ouabain and its binding site. A different depth of penetration into the hydrophobic interior of the membrane might account for the different effects of chlorpromazine and procaine on the association and dissociation rate constants of ouabain.

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