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ACETYLCHOLINESTERASE: A PROBE FOR THE STUDY OF ANTIARRHYTHMIC DRUG-MEMBRANE INTERACTIONS

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Summary

Structural consequences of antiarrhythmic drug interaction with erythrocyte membranes were analyzed in terms of resulting changes in the activity of membrane-associated acetylcholinesterase. When enzyme inhibitory effects of drugs were compared at concentrations producing an equivalent degree of erythrocyte antihemolysis, a number of distinct groupings emerged, indicating that the molecular consequences of drug-membrane interaction are not identical for all agents examined. Differences in drug-induced acetylcholinesterase inhibition in intact erythrocytes, erythrocyte membranes and a brain synaptic membrane preparation emphasized the role of membrane structural organization in determining the functional consequences of antiarrhythmic interaction in any given system. While the inhibitory actions of lidocaine, D-600 and bretylium in intact red cells were not altered by an increased transmembrane chloride gradient, enhanced enzyme inhibition by quinidine and propranolol was observed under these conditions. The diverse perturbational actions of these membrane-stabilizing antiarrhythmics observed here may be indicative of a corresponding degree of complexity in the mechanisms whereby substances modify the potential-dependent properties of excitable tissues.

Introduction

Antiarrhythmic agents constitute a rather heterogeneous group of chemicals [1] which produce a variety of alterations in the electrical properties of myocardial tissues, but which possess in common the ability to correct aberrant cardiac electrical activity arising in a variety of pathological situations. Attempts have been made to group these drugs on the basis of electrophysiological criteria into a number of mechanistically distinct categories [2]. Such

Abbreviation: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

efforts are complicated by the existence of multiple concentration-dependent actions by individual agents [3,4] and by the fact that the effects of some drugs are rate-dependent [5]. Furthermore, the clinical relevance of antiarrhythmic classifications made on the basis of experiments using normal tissues has been questioned since factors such as ischemia are known to alter markedly the response of myocardial tissues to antiarrhythmics [6].

Since the pharmacological actions of antiarrhythmic agents ultimately derive from the ability of these molecules to perturb the lipoprotein structure of cellular and/or intracellular membranes of cardiac and possibly neuronal tissues, we have undertaken model studies to examine the membrane perturbational characteristics of various antiarrhythmics in terms of their effects on human erythrocyte membrane acetylcholinesterase, an enzyme whose functional properties are influenced by membrane lipid composition [7,8]. The erythrocyte would seem to be a particularly appropriate model system to investigate since a correlation appears to exist between the antihemolytic properties of a number of anesthetic molecules and the ability of these substances to alter the functional characteristics of excitable tissues [9]. In this regard, the presence of acetylcholinesterase in plasma membrane fractions derived from nervous tissue has enabled us to directly compare the membrane perturbations produced by antiarrhythmics in these membranes with those in the membrane of the erythrocyte. The extent to which the membrane actions of antiarrhythmics as studied in isolated erythrocyte membranes are relevant to those in the membrane of the intact cell could be assessed, since the localization of acetylcholinesterase on the external aspect of the membrane [10] permitted an analysis of drug effects in both intact cells and hemoglobin-free membranes prepared by hypotonic lysis. Finally, the recent observations of Livne and Bar-Yaakov [11] indicating that the inhibitory effects of the antihemolytic substance linolenoyl sorbitol on erythrocyte acetylcholinesterase are modulated by the magnitude of the transmembrane chloride gradient have prompted us to perform comparable experiments with antiarrhythmics as a possible approach to the potential-dependent properties of these pharmacological agents.

Materials and Methods

Membrane preparations. Erythrocyte membranes were prepared from outdated human blood obtained from the Red Cross Blood Transfusion Service of Vancouver. Erythrocytes from which the buffy coat fraction had been removed during the course of two washes in isotonic saline were subjected to a graded lytic procedure involving progressively hypotonic solutions of sodium chloride with a final wash in 10 mM Tris buffer (pH 7.4) as described previously [12]. The resulting preparations, which contained 3—4 mg membrane protein per ml, were quick-frozen using a solid CO_2/acetone mixture and stored at −20° C until required. Synaptosomal membranes were isolated from guinea-pig brain tissue using the flotation-sedimentation procedure of Jones and Matus [13]. This preparation has been shown to be highly enriched in synaptic plasma membranes [13] and the material used in the present studies was entirely devoid of succinic dehydrogenase activity, indicating that mitochondrial contamination was minimal.

Acetylcholinesterase assay. For experiments involving intact erythrocytes, fresh human blood was obtained by venous puncture using heparin as anticoagulant. Following centrifugation, the plasma was removed and the erythrocytes were washed twice with isotonic saline followed by one wash with 0.1 M phosphate buffer (pH 8.0). Enzyme assays were performed on erythrocyte suspensions prepared by diluting packed red cells 1 : 200 (by volume) in 0.1 M phosphate buffer (pH 8.0). A pH of 8.0 was chosen for these enzyme assays in the presence of antiarrhythmics in order to duplicate as closely as possible conditions employed in concurrent studies of the structural basis of the observed enzyme effects of these drugs wherein the influence of these substances on the incorporation of trinitrobenzenesulfonic acid into membrane protein and phospholipid components [14] was investigated.

Acetylcholinesterase activity was assayed spectrophotometrically by measuring the conversion of acetylthiocholine to thiocholine using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [15]. The reaction mixture contained 1.0 ml phosphate buffer (0.2 M, pH 8.0), 1.0 ml solution of drug (dissolved in double-distilled water) to be tested, 0.1 ml 10 mM DTNB (in 0.1 M phosphate buffer, pH 8.0), 0.6 ml phosphate buffer (0.1 M, pH 8.0) and 0.2 ml erythrocyte suspension (prepared as described above). The reaction was initiated with 0.1 ml acetylthiocholine chloride (30 mM) and absorbance readings at 412 nm were recorded every 30 s. Enzyme activities in the absence and presence of drug were calculated from the absorbance increase at 412 nm during the first 5 min of the reaction. Non-enzymatic hydrolysis of substrate was corrected for in all assays.

Erythrocyte membranes and brain synaptosomal membranes were also analysed for acetylcholinesterase activity in the absence or presence of drugs. These membrane preparations were subjected to the same washing procedure as described above for intact erythrocytes and dilutions of 1 : 200 and 1 : 8 (in 0.1 M phosphate buffer, pH 8.0) for erythrocyte membranes and synaptosomal membranes, respectively, were assayed for enzyme activity exactly as described above.

In the experiments where the influence of the transmembrane chloride gradient on drug inhibition of acetylcholinesterase in intact cells was examined, the two isotonic saline washes were omitted and instead erythrocytes were washed three times with 0.1 M phosphate buffer, pH 8.0. The final reaction mixture for the acetylcholinesterase assay was as described above, except that the 1.0 ml phosphate buffer (0.2 M, pH 8.0) contained either 0.45 M NaCl or 0.3 M Na_2SO_4 to give final salt concentrations of 150 and 100 mM, respectively, as described by Livne and Bar-Yaakov [11]. The transmembrane potential of human erythrocytes suspended in 150 mM sodium chloride medium, calculated using the Nernst equation and assuming an intracellular chloride concentration of 104 mM [16], is approx. −9 mV. The transmembrane potential of intact red cells suspended in a medium where Cl^- has been replaced by an essentially impermeant anion such as SO_4^{2-} will reverse in sign and assume a positive value (calculated theoretical maximum approx. +120 mV, assuming no compensatory ion movements take place) [16]. While the absolute magnitude of the resulting transmembrane potential under our experimental conditions cannot be precisely specified without performing detailed ion flux measure-

ments, it is clear that the transmembrane potential characteristics of erythrocytes are markedly altered upon transfer from high chloride to low chloride media.

Antihemolysis studies. The procedure employed to test the effects of antiarrhythmics on the stability of erythrocytes to hypotonic lysis has been described in detail previously [14]. Briefly, the method involved an initial 15-min incubation at room temperature of 0.2 ml erythrocyte suspension in isotonic buffer (0.9% NaCl/15 mM Tris · HCl, pH 7.0) containing various concentrations of antiarrhythmics. This was followed by a hypotonic challenge using 15 mM Tris · HCl buffer (pH 7.0) containing the same concentration of drug as was present in the previous incubation. The extent of hemolysis was estimated from the absorbance of supernatants at 540 nm and was expressed as a percent of total hemolysis in distilled water.

Materials. The antiarrhythmic drugs used in these studies were quinidine-hydrochloride (K and K Laboratories), propranolol-hydrochloride (Sigma), practolol (free base, Ayerst Laboratories), bretylium-tosylate (Burroughs Wellcome Ltd.), QX-572-hydrochloride (Astra Pharmaceuticals), D-600-hydrochloride (Knoll AG Chemische Fabriken) and lidocaine (free base, K and K Laboratories). The acetylcholinesterase substrate acetylthiocholine chloride and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma.

Results

Antiarrhythmics were shown to cause progressive inhibition of erythrocyte acetylcholinesterase at concentrations producing stabilization of these cells against hypotonic hemolysis. When inhibitory effects at each drug concentration were expressed relative to the corresponding degree of erythrocyte antihemolysis, the various antiarrhythmics examined fell into a number of distinct categories (Fig. 1). This finding suggested the possibility that differences may

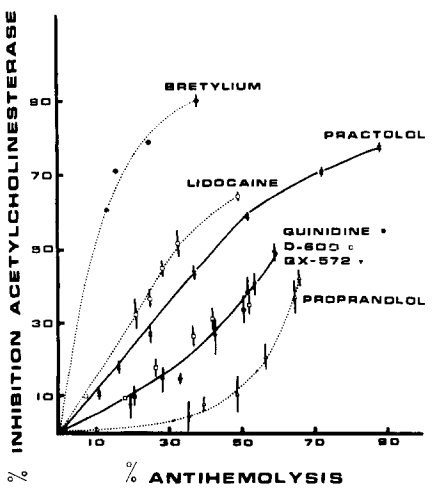

Fig. 1. Inhibition of intact erythrocyte acetylcholinesterase activity by various antiarrhythmics relative to their antihemolytic properties. Each point represents the mean ± S.E. of experiments using three different samples of blood.

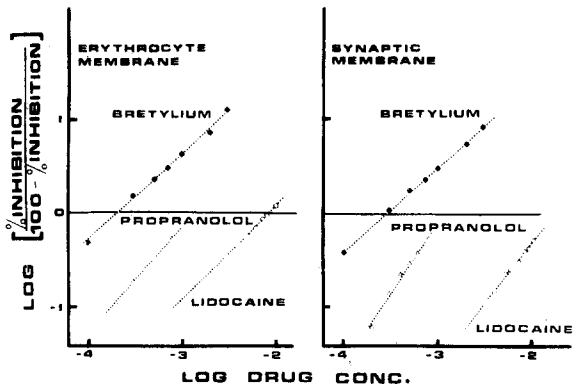

Fig. 2. Hill plot analysis of the inhibition of erythrocyte membrane and synaptic membrane acetylcholinesterase by antiarrhythmics. Each point represents the mean ± S.E. of experiments using three different erythrocyte membrane preparations and two different synaptic membrane preparations.

exist in the molecular basis of enzyme inhibition induced by these various agents.

The inhibitory effects of antiarrhythmics on the acetylcholinesterase of intact erythrocytes, erythrocyte membranes and brain synaptic membranes were compared using Hill plot analysis, as illustrated for bretylium, propranolol and lidocaine in Fig. 2. The slopes of these linear relationships provide a measure of the cooperativity and/or the stoichiometry characterizing the inhibitory process [17] and the antilog of the x-intercept represents the $K_{0.5}$ value of the inhibitor, that is, the concentration required to produce 50% inhibition of acetylcholinesterase activity. The results of these studies are summarized in Table I. No marked differences between the actions of these agents in the intact erythrocyte and its isolated membrane were apparent, although for a few

TABLE I

INHIBITORY EFFECTS OF ANTIARRHYTHYMICS ON THE ACETYLCHOLINESTERASE ACTIVITY OF INTACT ERYTHROCYTES, ERYTHROCYTE MEMBRANES AND BRAIN SYNAPTIC MEMBRANES

The data for erythrocyte and synaptic membranes were obtained using a minium of two different membrane preparations and the experiments with intact erythrocytes were performed in triplicate on a minimum of three different blood samples. In each case, inhibition data were plotted as shown in Fig. 2 and inhibitory constants evaluated from regression lines determined using a Compucorp (140) statistical caluclator. Data are expressed as mean ± S.E.

Drug	Erythrocyte		Erythrocyte membranes		Synaptic membranes	
	$K_{0.5}$ (mM)	Hill slope	$K_{0.5}$ (mM)	Hill slope	$K_{0.5}$ (mM)	Hill slope
Bretylium	0.22 ± 0.03	1.19 ± 0.13	0.21 ± 0.00	0.92 ± 0.00	0.28 ± 0.01	0.87 ± 0.02
Quinidine	1.84 ± 0.28	0.92 ± 0.06	1.84 ± 0.09	0.97 ± 0.00	5.00 ± 0.31	0.96 ± 0.01
Lidocaine	6.05 ± 0.40	1.15 ± 0.04	8.50 ± 0.04	1.02 ± 0.09	22.70 ± 0.00	0.95 ± 0.12
D-600	0.90 ± 0.07	0.72 ± 0.03	0.61 ± 0.00	1.00 ± 0.03	0.67 ± 0.02	1.24 ± 0.04
Practolol	0.70 ± 0.03	0.84 ± 0.03	0.99 ± 0.05	1.03 ± 0.03	1.58 ± 0.03	1.01 ± 0.19
Propranolol	1.10 ± 0.11	1.06 ± 0.17	1.40 ± 0.08	1.13 ± 0.01	1.14 ± 0.06	1.54 ± 0.04
QX-572	0.73 ± 0.06	1.11 ± 0.13	0.76 ± 0.03	0.87 ± 0.10	1.73 ± 0.06	1.63 ± 0.13

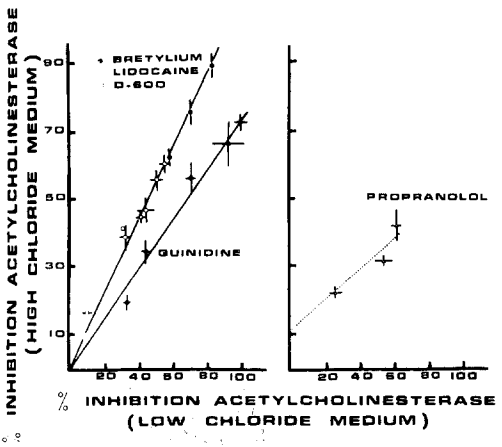

Fig. 3. Effect of transmembrane chloride gradient on the inhibition of intact erythrocyte acetylcholinsterase activity by antiarrhythmics. Each point represents the mean ± S.E. of experiments using a minimum of two different samples of blood.

drugs, namely lidocaine and practolol, $K_{0.5}$ values for enzyme inhibition were somewhat greater for the membranes than for the intact cells. Considerably greater differences in $K_{0.5}$ values were noted when erythrocyte membranes and brain synaptic membranes were compared (Table I). This was particularly so in the case of quinidine, lidocaine, practolol and QX-572, suggesting that the membrane environments where these drugs interact are not identical in the two systems studied. Examination of Hill plot slopes for inhibition revealed an apparent lack of cooperativity or of stoichiometry greater than one in the inhibitory effects of most antiarrhythmics so that Hill coefficients of unity were observed in most cases [17]. Two exceptions were propranolol and QX-572, whose interaction with synaptic membranes (but not erythrocyte membranes) was characterized by a slope significantly greater than one.

As an approach to studying the influence of transmembrane potential on the ability of antiarrhythmics to interact with and modify the structure of erythrocyte membranes as reflected in the inhibition by these agents of erythrocyte acetylcholinesterase, enzyme activity in the presence of a number of antiarrhythmics was assayed in a high chloride medium or in a low chloride medium (with sulfate ion replacing chloride). The inhibition of acetylcholinesterase by most of the drugs tested was independent of the nature of the medium (Fig. 3). However, the inhibitory properties of both quinidine and propranolol were enhanced in low chloride media (Fig. 3). These latter findings paralleled the results of Livne and Bar-Yaakov [11] who demonstrated that inhibition of erythrocyte acetylcholinesterase by linolenoyl sorbitol was enhanced as the magnitude of the transmembrane chloride gradient increased. The fact that the membrane actions of antiarrhythmics in the present model system vary in their dependence on transmembrane potential may point to possible differences in the molecular basis by which these compounds modify the functional properties of electrically excitable tissues.

Discussion

The erythrocyte membrane has proven to be a convenient model system with which to investigate the molecular consequences of drug-membrane interaction. The fact that substances such as propranolol induce alterations in the structural and functional characteristics of erythrocyte membranes [14] at concentrations producing hypotonic stabilization of intact erythrocytes [9,14] and electrical stabilization of nerves [18,19] suggests that an analysis of drug-induced perturbations in isolated erythrocyte membranes might provide meaningful information regarding the mechanisms by which pharmacological agents alter the functional properties of excitable tissues. It, therefore, becomes important to ascertain whether or not molecular aspects of drug action studied in isolated membranes are relevant to the situation in the intact cell. In this regard, Aloni and Livne [20] have recently shown that the interaction of the antihemolytic substance linolenoyl sorbitol with the membrane of the intact erythrocyte causes a concentration-dependent inhibition of acetylcholinesterase which is not observed in isolated erythrocyte membranes or in solubilized preparations of the enzyme. The experiments in the present study have shown that this dependence of acetylcholinesterase inactivation by antihemolytic agents on cellular integrity is not a general phenomenon (Fig. 1, Table I). Thus, the inhibitory effects of bretylium, quinidine and QX-572 on erythrocyte acetylcholinesterase of intact cells were virtually identical to those in isolated membranes. For other drugs, namely lidocaine, D-600 and practolol, small but significant differences in the concentrations of drugs producing 50% inhibition ($K_{0.5}$ values) in ghosts versus intact cells were noted. These differences may arise from alterations in membrane architecture produced during hypotonic lysis [21,22].

Expression of acetylcholinesterase inhibition by antiarrhythmics relative to their corresponding antihemolytic effects (Fig. 1) illustrates that for a given degree of membrane occupation by drug [9] the structural consequences of this interaction vary with the structure of the agent in question. The acetylcholinesterase of erythrocyte membranes has been shown to be a lipoprotein [23] whose properties are influenced by changes in the lipid environment of the membrane [7,8]. Studies currently being conducted in our laboratory have shown that antiarrhythmics differ markedly in their ability to perturb erythrocyte membrane structure, as reflected in the effects of these agents on the trinitrophenylation of protein and phospholipid amino groups by trinitrobenzenesulfonic acid (Godin, D.V. et al., unpublished observations). Bretylium was minimally disruptive in this regard and this finding when taken with the pronounced and virtually identical inhibitory effects of this compound on the membrane acetylcholinesterase of both erythrocytes and synaptosomes (Table I) may indicate a relatively specific action of this quaternary molecule on the enzyme, possibly at the anionic site normally involved in binding the cationic nitrogen of choline ester substrates. It would seem likely that the diverse effects of the other antiarrhythmics on membrane acetylcholinesterase activity are governed by the differing abilities of these agents to interact with and modify protein or phospholipid structural components of the membrane. Further experimentation will be required before a detailed analysis of the molec-

ular basis of these inhibitory effects is possible. However, our experiments are important in emphasizing that lipid-soluble amphipathic substances, which have generally been assumed to protect erythrocytes against hypotonic lysis and stabilize electrically excitable membranes by a comparable mechanism [9], do not exert identical perturbational actions on the erythrocyte membrane. While these findings in erythrocytes suggest a corresponding degree of complexity in the direct membrane actions of antiarrhythmics on myocardial tissues, extrapolation of results obtained with erythrocytes to the situation in more complex excitable membrane systems must be undertaken with considerable caution. This is apparent from the differences in $K_{0.5}$ values and Hill coefficients for cholinesterase inhibition noted for some drugs when erythrocyte and synaptic membrane preparations were compared directly (Table I). Since membrane partition coefficients for drugs in erythrocyte and synaptosomal membranes are generally equivalent [24] and the molecular properties of cholinesterases in erythrocytes comparable to those derived from excitable tissues [25], these differences point to the importance of membrane structural organization in determining the functional consequences of drug interaction in a particular membrane system.

Erythrocytes have been shown to possess significant diffusion potentials either by using fluorescent cyanine dyes whose distribution across the membrane is potential-sensitive [16] or by means of direct microelectrode measurements, in the case of salamander giant red blood cells [26]. In human erythrocytes the magnitude of this potential, which is largely determined by the distribution of Cl^- and is approx. −9 mV [16], has been shown to govern the inhibition of erythrocyte acetylcholinesterase by linolenoyl sorbitol [11]. In contrast, the present studies have shown that enzyme inhibition by bretylium, D-600 and lidocaine is independent of the chloride gradient across the erythrocyte membrane (Fig. 3). The inhibitory effects of quinidine and propranolol, on the other hand, were enhanced in the presence of an increased transmembrane chloride gradient. Although it is known that the interaction of propranolol with intact erythrocytes causes an increase in the membrane permeability to K^+ [27], an action which might modify the potential difference across the membranes of erythrocytes suspended in a low chloride medium, this action does not seem to be shared by quinidine, which rather tends to decrease membrane permeability to K^+ [28,29]. The greater inhibitory effects of propranolol and quinidine in low chloride media might be the result of alterations in the configuration of membrane structural components induced by the modified transmembrane potential, which alterations could modify the ability of quinidine and propranolol to interact with and/or pertub the structure of the erythrocyte membrane. Future experiments will attempt to elucidate the molecular basis of this proposed potential-dependent membrane configurational change using covalent bond-forming group-specific chromophoric probes as described earlier in our studies of drug-induced alterations in erythrocyte membranes [14]. It is our hope to eventually extend these studies to an investigation of membrane structural changes accompanying depolarization of excitable tissues using isolated synaptosomes, which undergo depolarization in vitro in the presence of veratridine or high concentrations of potassium [30,31], as model. This system might also provide a means of examining the

effects of membrane-active drugs on these potential-dependent membrane phenomena.

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