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STUDIES ON THE INTERACTION OF PROPRANOLOL WITH ERYTHROCYTE MEMBRANES

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SUMMARY

The interaction of propranolol with erythrocyte membranes at concentrations stabilizing intact erythrocytes against hypotonic hemolysis produced corresponding perturbations in membrane protein and particularly membrane phospholipid components as monitored by increases in the reactivity of membrane amino and sulfhydryl groups towards trinitrobenzenesulfonic acid and 5,5′-dithio-bis-(2-nitro-benzoic acid), respectively. Membrane-propranolol interactions were also analyzed in terms of alterations produced in the kinetic properties of membrane enzymes. These experiments provided evidence that propranolol-induced perturbations were sufficiently generalized as to influence the activity of enzymatic processes associated with both inner and outer membrane surfaces. Configurational changes in membrane phospholipids were implicated in these effects of propranolol, which included alterations in functionally significant membrane-cation interactions. It is suggested that the findings described here may provide a basis for understanding molecular aspects of membrane stabilization in other systems.

INTRODUCTION

The pharmacological properties of propranolol appear to be determined by some combination of β-adrenergic receptor antagonism and membrane stabilization as reflected, for example, in the protection of erythrocytes from hypotonic hemolysis [1]. The β-adrenergic actions of propranolol, which may be distinguished from those related to membrane stabilization on the basis of their stereoselectivity and concentration dependence [2], account for many of the effects of propranolol in vivo [3, 4]. On the other hand, membrane actions may contribute appreciably to the anticonvulsant [5] and antipsychotic [6] effects of propranolol. Finally, there may be instances where a complex interplay between β-blockade and membrane stabilization may be operative, as in the antiarrhythmic properties of propranolol [7, 8].

* Abbreviations: TNBS, trinitrobenzenesulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

Propranolol and other membrane-stabilizing substances have been shown to produce functional alterations in a wide variety of membrane systems which seem to parallel the situation in the erythrocyte membrane. These include such diverse actions as blockade of conduction in nerve fibers [1], inhibition of spreading and adhesion of Sarcoma I cells [9], increased susceptibility of untransformed cells to agglutination by concanavalin A [10], alterations in the permeability of various membranes to sugars and electrolytes [11] and inhibition of serotonin uptake by platelets [12]. The lack of detailed information on the mechanisms whereby membrane stabilizers perturb the structural components of membranes has thus far precluded an interpretation of such observations at a molecular level.

In earlier studies, we have shown that the interaction of a variety of compounds possessing membrane-stabilizing activity with biological membranes, including that of the human erythrocyte, produced marked alterations in the reactivity of membrane amino groups towards trinitrobenzenesulfonic acid (TNBS) [13, 14]. This approach has been further developed and applied here to an analysis of the perturbation by propranolol of erythrocyte membrane protein and lipid components. Vectorial aspects of associated functional alterations have been examined by studying the effects of propranolol on a variety of membrane-enzymatic activities originating in different regions of the membrane.

MATERIALS AND METHODS

Erythrocyte membranes were prepared from outdated human blood by stepwise hypotonic lysis as described previously [15]. Preparations, which contained 3–4 mg protein per ml, were quick frozen and stored at −20 °C prior to use.

Chemical assays. Procedures used to analyze membrane fractions for protein and phospholipid content as well as for the incorporation of trinitrobenzenesulfonic acid into primary membrane amino groups were the same as those employed in our earlier studies [13]. Membrane sulfhydryl groups were assayed colorimetrically using 5,5′-dithio-bis-(2-nitrobenzoic acid) [16].

Enzyme assays. Acetylcholinesterase was assayed spectrophotometrically at room temperature. The 3.0 ml reaction mixture contained 2.6 ml Tris buffer (0.1 M, pH 8.0), 0.1 ml acetylthiocholine (30 mM), 0.1 ml 5,5′-dithio-bis-(2-nitrobenzoic acid) (10 mM, dissolved in 0.1 M Tris buffer, pH 8.0) and 0.1 ml membrane suspension (0.3–0.4 mg/ml). Reactions were initiated with acetylthiocholine and rates were calculated from the absorbance increase at 412 nm during the first 2 min of the reaction.

Nitrophenyl phosphatase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate, as described previously [15]. K^+-stimulated *p*-nitrophenyl phosphatase activities were taken as the difference between activity determined in the presence of 3 mM $MgCl_2$ and 30 mM KCl (total *p*-nitrophenyl phosphatase) and that determined in the absence of KCl (Mg^{2+}-stimulated-*p*-nitrophenyl phosphatase).

$(Ca^{2+}+Mg^{2+})$-ATPase activities were measured using the procedure of Quist and Roufogalis [17] employing an EGTA-Ca^{2+} buffer system to achieve concentrations of free Ca^{2+} in the range of 0.166–330 μM as described by these authors. Reactions were terminated with 1.0 ml cold 8 % silicotungstic acid in 8 % $HClO_4$, a mixture which allows the removal of propranolol from solution by centrifugation

[18] and thereby prevents its interference with the assay for inorganic phosphate by the method of Fiske and SubbaRow [19]. $(Ca^{2+}+Mg^{2+})$-ATPase activity was obtained as the difference between activity measured in the presence of calcium and magnesium and that measured in the absence of calcium.

Antihemolysis studies. The procedure adopted was a modification of that described by Machleidt et al. [20]. 5 ml fresh human blood were withdrawn into a heparinized syringe and the blood was centrifuged at top speed for 5 min using a clinical centrifuge. Erythrocytes were washed three times in 0.9 % NaCl/15 mM Tris · HCl (pH 7.0), using four volumes of buffer per volume of erythrocytes. After the final wash, 2.0 ml packed erythrocytes were suspended in 32 ml of the isotonic buffer, resulting in a final hematocrit of approx. 6 %. The hemolysis test consisted of 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 propranolol or other drugs to be tested. This was followed by a hypotonic challenge with 2.3 ml of 15 mM Tris · HCl buffer (pH 7.0) containing the same concentration of test drug as was present in the previous incubation. Following an incubation for 10 min at room temperature samples were centrifuged at $13\,000 \times g$ for 5 min. The absorbance of supernatants at 540 nm was determined and this value was expressed as percent total hemolysis of 0.2 ml erythrocytes in distilled water. Each experiment was performed in triplicate and results were expressed as mean $\pm$S.E.

Gel filtration of labelled membranes. Membranes (2.0 ml) were labelled with TNBS by incubation at 37 ± 0.5 °C with 10 ml Tris buffer (20 mM, pH 8.0) and 1.0 ml TNBS solution (10 mM, pH 8.0), with or without propranolol or other drugs to be tested, in a final volume of 15 ml for 1 h. Following the addition of 10 ml HCl (1 M), membranes were isolated and washed with Tris buffer by centrifugation at $30\,000 \times g$. The presence of propranolol, even at the highest concentration of 1 mM, was not associated with any decrease in the recovery of membrane material on centrifugation. Pellets were solubilized and membrane lipid and protein components were resolved using a column of Sephadex G-200 (1.6×100 cm) eluted with a mixture of 1 % sodium dodecyl sulfate/0.05 M ammonium bicarbonate/0.02 % NaN_3 as described by Lenard [21]. 50-drop fractions (approx. 1 ml) were collected and flow rates were 3–5 ml per h. Each fraction was analyzed for absorbance at 335 nm, protein and phospholipid as described earlier.

Enzyme treatments. In phospholipase A studies, membranes were treated with heated (10 min at 70 °C) *Naja naja* venom (Sigma). Typically, membranes (2.0 ml, 6–8 mg protein) were incubated for 15 min at 37 ± 0.5 °C with 0.1 ml phospholipase A solution (10 mg/ml), 0.8 ml $CaCl_2$ (20 mM) and 2.0 ml Tris buffer (20 mM, pH 8.0) in a final volume of 5.0 ml. Reactions were terminated by adding 10 ml EDTA (4 mM, pH 7.4) and membranes were isolated and washed once with Tris buffer (20 mM, pH 8.0) by centrifugation. Polyacrylamide gel electrophoretic analysis revealed no evidence of membrane proteolysis following this treatment.

Trypsinization of membranes was carried out by incubating 2.0 ml membrane preparation with 0.2 ml trypsin (Sigma, type III, twice crystallized) solution (0.5 mg/ml) for 15 min at 37 ± 0.5 °C. Following incubation, the reaction mixture was diluted 10-fold and centrifuged at $30\,000 \times g$. Membranes were then washed once with cold double distilled water and isolated by centrifugation.

Materials. Trinitrobenzenesulfonic acid, racemic propranolol · HCl, 5,5'-di-

thio-bis-(2-nitrobenzoic acid) (Nbs_2), trypsin (type III, twice crystallized), phospholipase A (*Naja naja*), ATP (Tris salt), EGTA, imidazole, Tris buffer, *p*-nitrophenyl phosphate (disodium salt) and acetylthiocholine chloride were all purchased from Sigma. $HClO_4$ was obtained from B and A Chemical Co., Canada Ltd., silicotungstic acid from Fisher. The resolved optical isomers of propranolol were generously provided by Ayerst Laboratories. Outdated blood (O positive) was obtained from the Red Cross blood transfusion service of Vancouver.

RESULTS

Kinetics of TNBS incorporation

The interaction of propranolol with erythrocyte membranes at concentrations producing antihemolysis of intact erythrocytes, produced a progressive increase in TNBS incorporation (Fig. 1) associated with a progressive decrease in Arrhenius activation energy (Fig. 2). Prior membrane trypsinization delayed but did not prevent the decrease in activation energy by propranolol. On the other hand, in membranes treated with phospholipase A, following an initial sharp decrease in activation energy at the lowest propranolol concentration, increasing concentrations now produced an increase, rather than a further decrease, in activation energy. TNBS incorporation

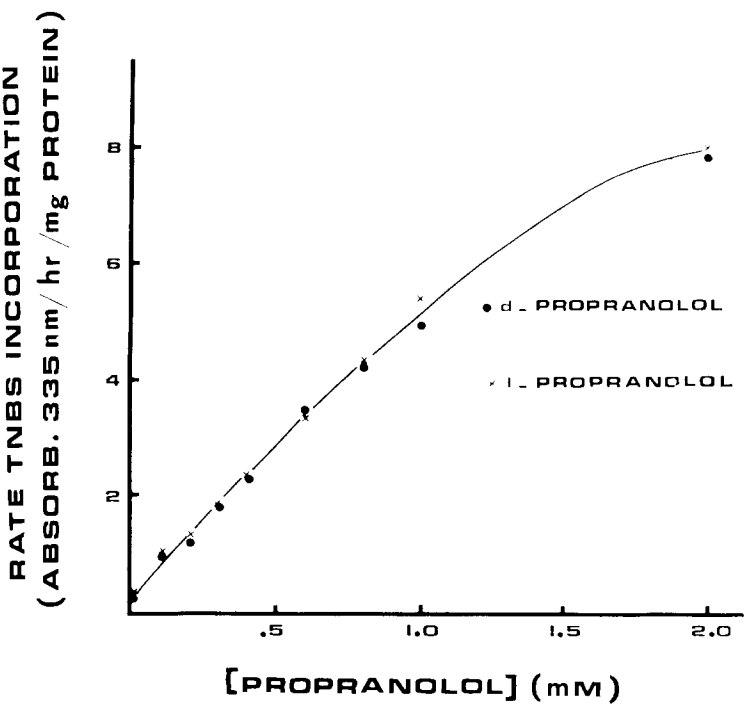

Fig. 1. Stimulatory effects of propranolol optical isomers on the rate of TNBS incorporation by erythrocyte membranes. TNBS incorporation was measured at 37±0.5 °C, using reaction times of 30 min in the absence of propranolol and 5 min in the presence of propranolol.

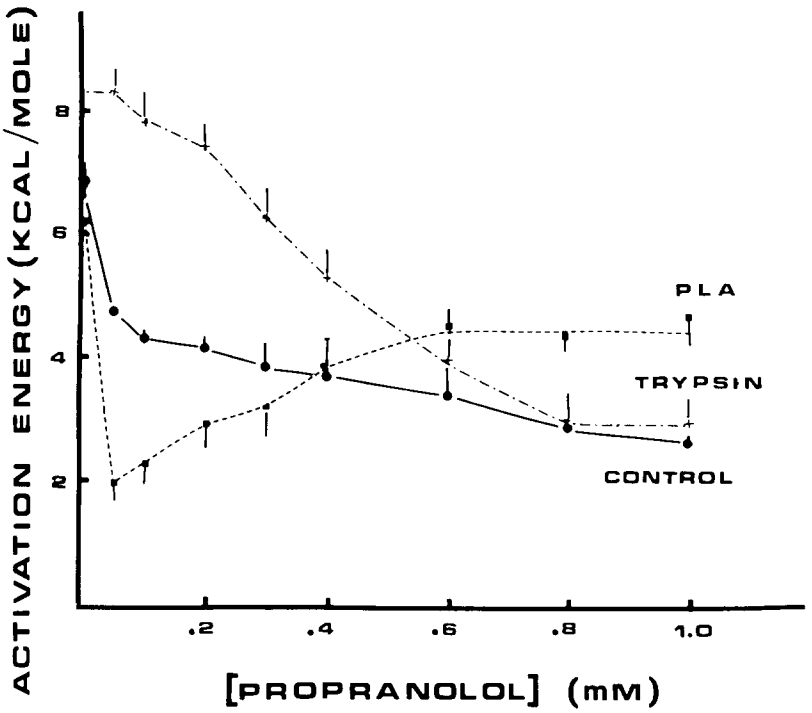

Fig. 2. Effects of propranolol on the activation energy for TNBS incorporation by control, phospholipase A- and trypsin-treated erythrocyte membranes. Enzyme treatments of membranes were carried out as described in Materials and Methods and rates of TNBS incorporation were determined in the temperature range between 15 and 45 ± 0.5 °C, using reaction times of 5 min (in the presence of propranolol) or 30 min (in the absence of propranolol). Arrhenius plots of ln rate versus the reciprocal of the absolute temperature were constructed and activation energies were evaluated from the slope of the best fitting line as determined with a Compucorp 140 (Statistician) calculator. The activation energies shown in each case represent the mean ± S.E. for three different membrane preparations.

in the absence of propranolol was minimally affected by these treatments. These experiments suggested a critical role of membrane protein and especially phospholipid components in determining the structural consequences of membrane-propranolol interaction.

Labelling studies with TNBS

Structural alterations induced by propranolol were analyzed further by isolating labelled membranes and separating trinitrophenylated protein and lipid moieties by gel filtration. The time course of TNBS incorporation into membranes in the presence of increasing concentrations of propranolol is shown in Fig. 3. Parallel effects of increasing propranolol concentration on TNBS incorporation were apparent at all times examined and 1-h incubations were chosen for the labelling studies described below. Following labelling and solubilization of membranes, labelled protein and lipid components were resolved by Sephadex G-200 gel filtration as described by Lenard [21] and correspond to peaks I and II, respectively, in Fig. 4. This diagram

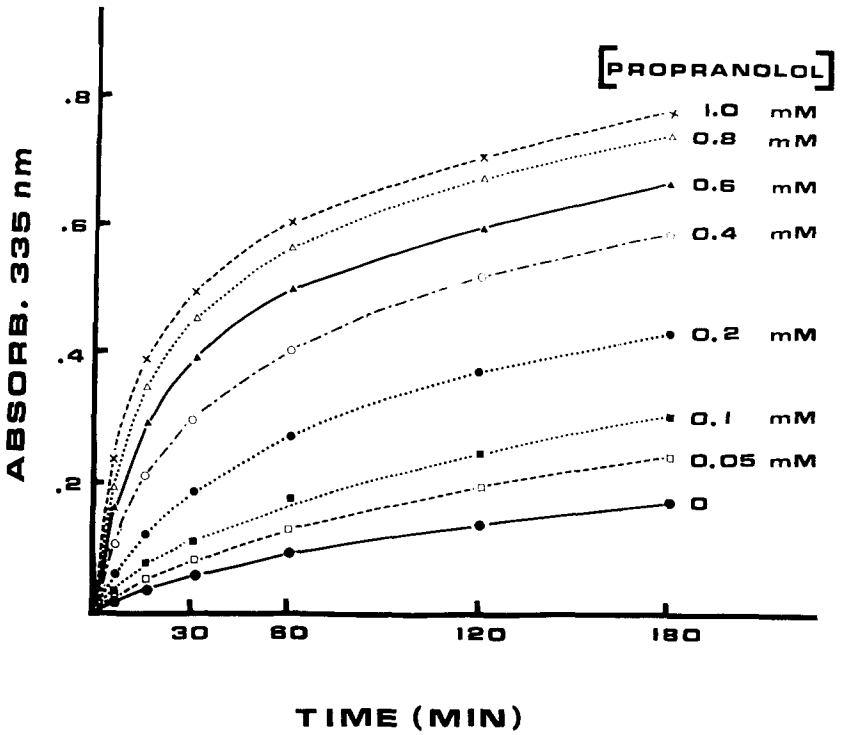

Fig. 3. Time course of TNBS incorporation into erythrocyte membranes in the absence and presence of propranolol. Membranes (0.2 ml) were incubated with TNBS and propranolol at the concentrations indicated (as in Fig. 1). Reactions were terminated with 10 % sodium dodecyl sulfate/1 M HCl and the absorbance of solutions at 335 nm determined.

illustrates the modest stimulation by propranolol of TNBS incorporation into membrane proteins (peak I) and the marked stimulation of incorporation into phospholipids (peak II). Regression analysis indicated a strong correlation between protein and lipid labelling stimulated by propranolol ($r = 0.969$), suggesting the existence of a structural association between these two components. Further, antihemolysis by propranolol in intact cells correlated better with the ratio of lipid to protein propranolol-stimulated TNBS labelling in isolated membranes ($r = 0.957$) than with the labelling of either protein ($r = 0.838$) or lipid ($r = 0.866$) components individually (Fig. 5). Membrane proteins, therefore, while constituting a minor component of TNBS labelling relative to phospholipids, may contribute significantly to propranolol-induced antihemolysis.

Modification of membrane sulfhydryls by Nbs_2

The protein perturbational effects of propranolol were monitored independently in terms of alterations produced in the reaction of membrane sulfhydryl groups with 5,5'-dithio-bis-(2-nitrobenzoic acid) (Nbs_2). Sulfhydryl groups were chosen for study because the hemolytic behaviour of erythrocytes is critically dependent on the integrity of these groups [22]. The results shown in Fig. 6 suggest the presence of at

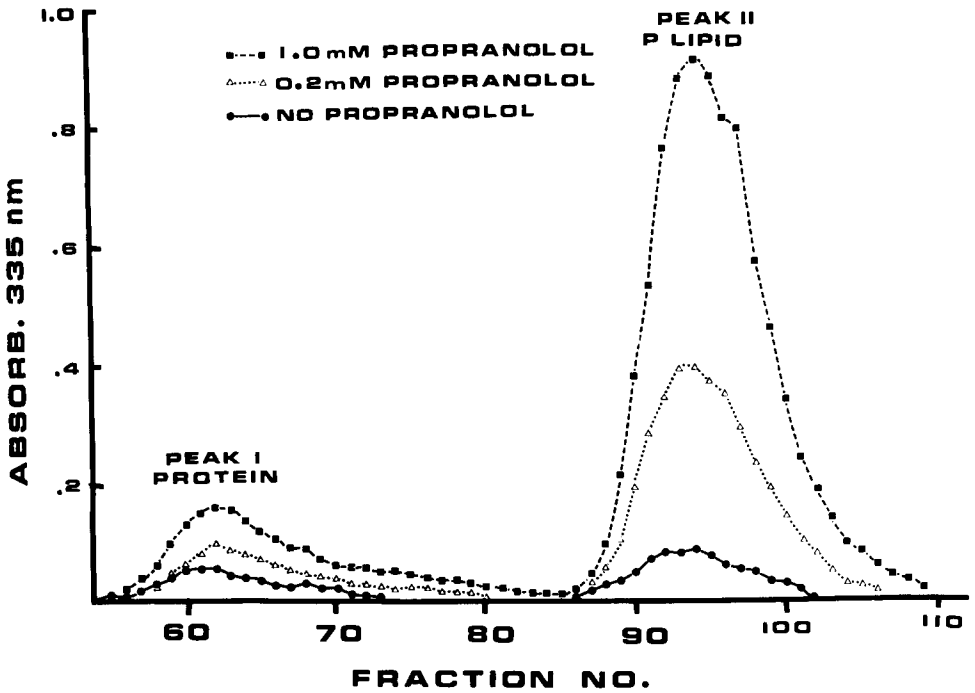

Fig. 4. Labelling of erythrocyte membranes by TNBS in the absence and presence of propranolol. Aliquots (2 ml) of membrane suspension were labelled with TNBS for 1 h, dialyzed and solubilized in sodium dodecyl sulfate. Aliquots (1 ml) were applied to a 1.6 × 100 cm Sephadex G-200 column and elution was performed with 1 % sodium dodecyl sulfate/0.05 M ammonium bicarbonate/0.02 % NaN_3. 50-drop fractions were analyzed and the absorbance of each at 335 nm determined spectrophotometrically.

least two classes of sulfhydryls with one class undergoing modification in the range where antihemolysis by propranolol increases sharply with concentration (up to 0.4 mM) (Fig. 5) and another class undergoing modification at concentrations of propranolol corresponding to the plateau region of the antihemolysis curve. These observations are again consistent with an association of membrane protein structural perturbations with antihemolysis induced by propranolol and further suggest that the decrease in antihemolysis in the plateau region results from progressive membrane disruption by propranolol, as reflected in the marked exposure of sulfhydryl sites at highest drug concentrations.

Enzyme inhibition studies

The activity of erythrocyte acetylcholinesterase, an enzyme located on the external surface of the membrane [23], underwent progressive inhibition by increasing concentrations of propranolol (Fig. 7, upper). A very similar course of inactivation was found with Mg^{2+}-dependent K^+-stimulated *p*-nitrophenyl phosphatase, a ouabain-sensitive process presumed to reflect the terminal K^+-dependent dephosphorylation step of ATP hydrolysis [24] and which is dependent upon the structural integrity of the membrane for optimal activity [25]. The basal Mg^{2+}-dependent

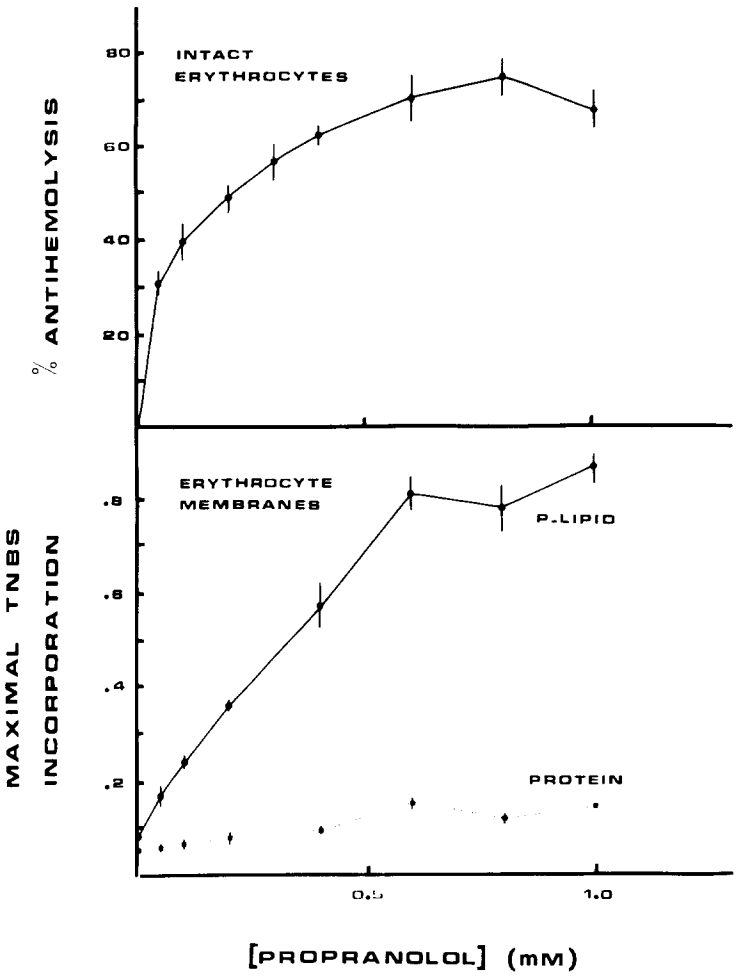

Fig. 5. Upper. Concentration dependence of propranolol-induced antihemolysis of intact erythrocytes. Each point represents the mean ± S.E. of two sets of triplicate experiments using two different blood samples. Lower. Propranolol-stimulated incorporation of TNBS into protein and phospholipid components of the membrane. Each point represents the mean ± S.E. of experiments with three different membrane preparations.

hydrolysis of *p*-nitrophenyl phosphate was considerably less sensitive to inhibition by propranolol than the K^+-stimulated component (Fig. 7, lower). High concentrations of sucrose (0.5 M), shown previously to antagonize the inhibition of calcium transport in cardiac sarcoplasmic reticulum induced by propranolol [26], significantly decreased the inhibition by propranolol of K^+-stimulated *p*-nitrophenyl phosphatase activity (by 15 % at 1 mM propranolol) without exerting any effect on the inhibition of basal Mg^{2+}-stimulated *p*-nitrophenyl phosphatase. Further, the activation energy characterizing K^+-*p*-nitrophenyl phosphatase was altered in the presence of propranolol (1 mM) and this effect was abolished by sucrose (Table I). The activation energy for Mg^{2+}-*p*-nitrophenyl phosphatase, on the other hand, was not affected by propran-

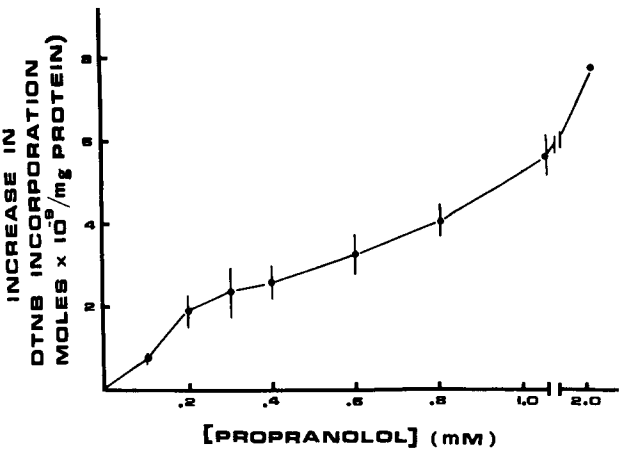

Fig. 6. Effect of propranolol on the labelling of erythrocyte membrane sulfhydryl groups by Nbs_2. Reactions were carried out for 30 min at 37 ± 0.5 °C in a 3.0 ml final volume containiug 0.2 ml membrane suspension, 1.0 ml imidazole · HCl (0.15 M, pH 7.4), 0.1 ml Nbs_2 (0.3 mM) and propranolol as indicated. Following centrifugation, the absorbance of supernatants at 412 nm was used to calculate the number of sulfhydryls modified using a molar extinction coefficient of $1.36 \cdot 10^4$ [16]. Results were expressed as increments of Nbs_2 incorporation over control (in absence of propranolol) and each point represents the mean $\pm$ S.E. of experiments using 12 different membrane preparations.

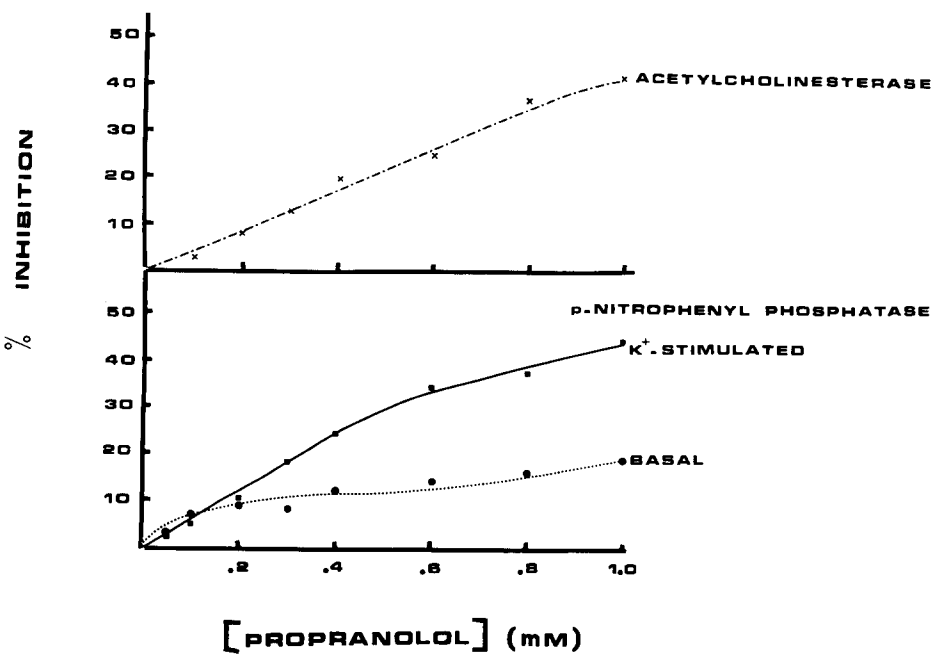

Fig. 7. Inhibitory effects of propranolol on the activity of three membrane enzymes, acetylcholinesterase, basal (Mg^{2+}-stimulated) *p*-nitrophenyl phosphatase and K^+-*p*-nitrophenyl phosphatase. Each enzyme was assayed in a 3.0 ml incubation mixture with or without propranolol as described in Materials and Methods. Each point represents the mean from experiments using a minimum of three different membrane preparations.

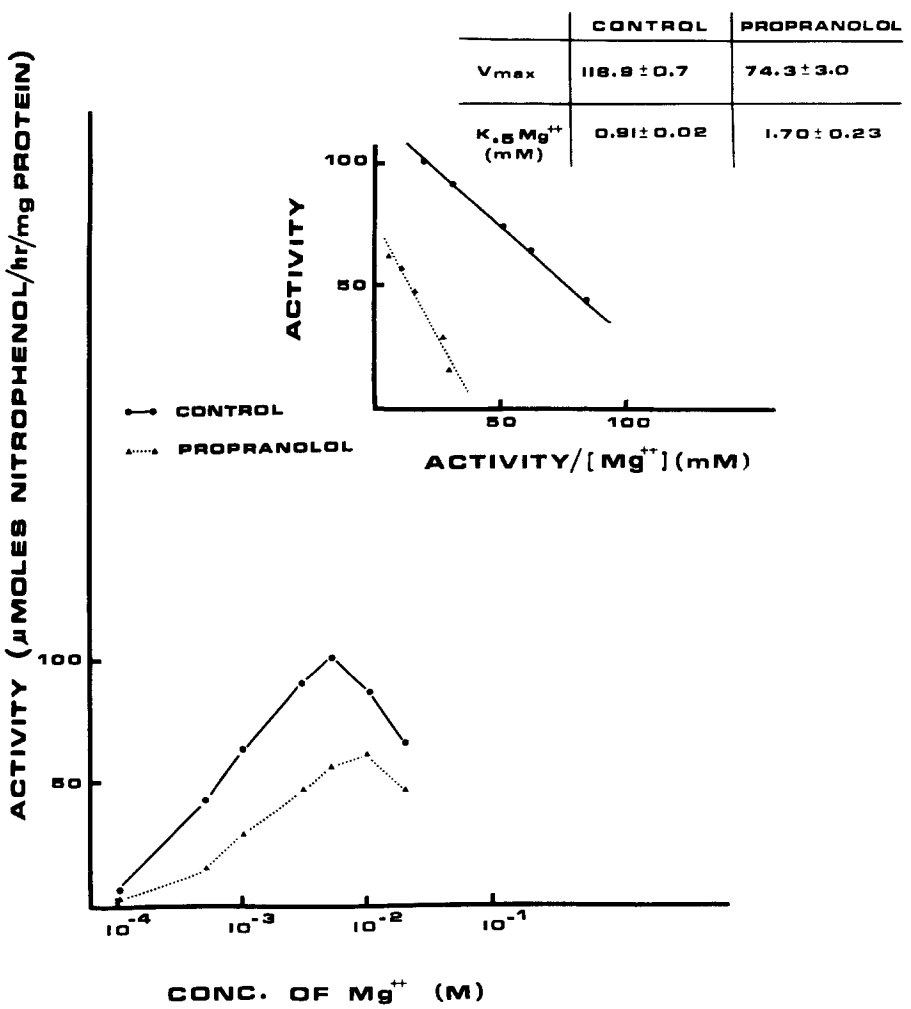

Fig. 8. Mg^{2+} dependence of K^+-stimulated *p*-nitrophenyl phosphatase in the absence or presence of propranolol (1 mM). Inset: Eadie plots of the same data where *y* intercept $= V$ and slope $= -K_{0.5}$ Mg^{2+}. Assays were performed exactly as described in Materials and Methods, except that the final concentration of $MgCl_2$ in the 3.0 ml incubation mixture was varied from 0.1 to 20.0 mM. Each point represents the average of experiments with three different membrane preparations and mean values ± S.E. of the kinetic quantities are summarized in the accompanying table.

olol or sucrose, whether alone or in combination. Propranolol-induced inhibition of acetylcholinesterase was similarly unaffected by the presence of sucrose.

The functionally relevant binding sites for Mg^{2+} and K^+ in the *p*-nitrophenyl phosphatase reaction are believed to be located on the inner and outer surfaces of the membrane, respectively [27]. A detailed analysis of the influence of propranolol on the activation of this enzyme by Mg^{2+} and K^+ was therefore undertaken as an approach to vectorial aspects of propranolol-induced membrane structural alterations.

The Mg^{2+} dependence of K^+-stimulated and basal activities in the absence

TABLE I

ACTIVATION ENERGIES (E_a) FOR BASAL (Mg^{2+}-STIMULATED) AND K^+-STIMULATED *p*-NITROPHENYL PHOSPHATASE IN THE ABSENCE OR PRESENCE OF PROPRANOLOL (1 mM) AND SUCROSE (0.5 M) ALONE OR IN COMBINATION

Enzyme assays were performed as described in Materials and Methods, except that incubation temperature was varied in the range 15–45 ± 0.5 °C. Arrhenius plots of ln (specific activity) versus reciprocal absolute temperature were constructed and slopes of the resulting regression lines (obtained using a Compucorp 140 (Statistician) calculator) were multiplied by the gas constant (R) to obtain E_a in kcal/mol. Each value represents the mean ± S.E. of three experiments, each using a different membrane preparation.

E_a (kcal/mol)

	Basal (Mg^{2+}-stimulated) *p*-nitrophenyl phosphatase		K^+-stimulated *p*-nitrophenyl phosphatase	
	No sucrose	Sucrose	No sucrose	Sucrose
Control	17.9 ± 0.6	18.5 ± 0.4	15.7 ± 0.4	13.9 ± 0.4
Propranolol	17.6 ± 0.3	17.1 ± 0.1	10.2 ± 0.1	13.1 ± 0.6

and presence of propranolol (1 mM) are shown in Figs. 8 and 9 (note differences in the ordinates of these two figures). The lack of appreciable effect of propranolol on the Mg^{2+} dependence of the basal activity is apparent. In contrast, the ability of Mg^{2+} to stimulate the K^+-stimulated activity is impaired, as reflected in a decreased V of the enzyme and an increased $K_{0.5}$ for Mg^{2+}, i.e. the inhibition was of a mixed type. Similarly, when K^+ stimulation of *p*-nitrophenyl phosphatase was examined, a pattern of mixed inhibition by propranolol emerged with V decreased and $K_{0.5}$ for K^+ increased (Fig. 10). Thus, the membrane structural alterations produced by propranolol are sufficiently generalized as to influence the functional properties of both inner and outer membrane surfaces.

Effects on (Ca^{2+} + Mg^{2+})-ATPase

Propranolol and other β-adrenergic antagonists possessing membrane-stabilizing effects can interfere with certain Ca^{2+} transport processes, presumably as a result of competition at the level of membrane phospholipids [28]. The erythrocyte membrane contains Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase activity which is believed to be involved in the active extrusion of Ca^{2+} from the cell [29]. The stimulation of this ATPase activity by Ca^{2+} is complex (Fig. 11) and Eadie plot analysis (data not shown) indicated the presence of two distinct classes of calcium binding sites, probably located on two distinct classes of ATPase molecules [17]. The susceptibility of the (Ca^{2+} + Mg^{2+})-ATPase system to propranolol inhibition (Fig. 11) was low, being comparable to that of basal *p*-nitrophenyl phosphatase activity (inhibition of approx. 20 % at 1 mM propranolol) but very much less than that of K^+-stimulated *p*-nitrophenyl phosphatase or acetylcholinesterase activities (which were both inhibited by about 50 % at 1 mM propranolol). Propranolol produced a greater decrease in the ATPase activity at high concentrations of Ca^{2+} as compared with the activity at the lowest Ca^{2+} concentrations. The greater susceptibility of the

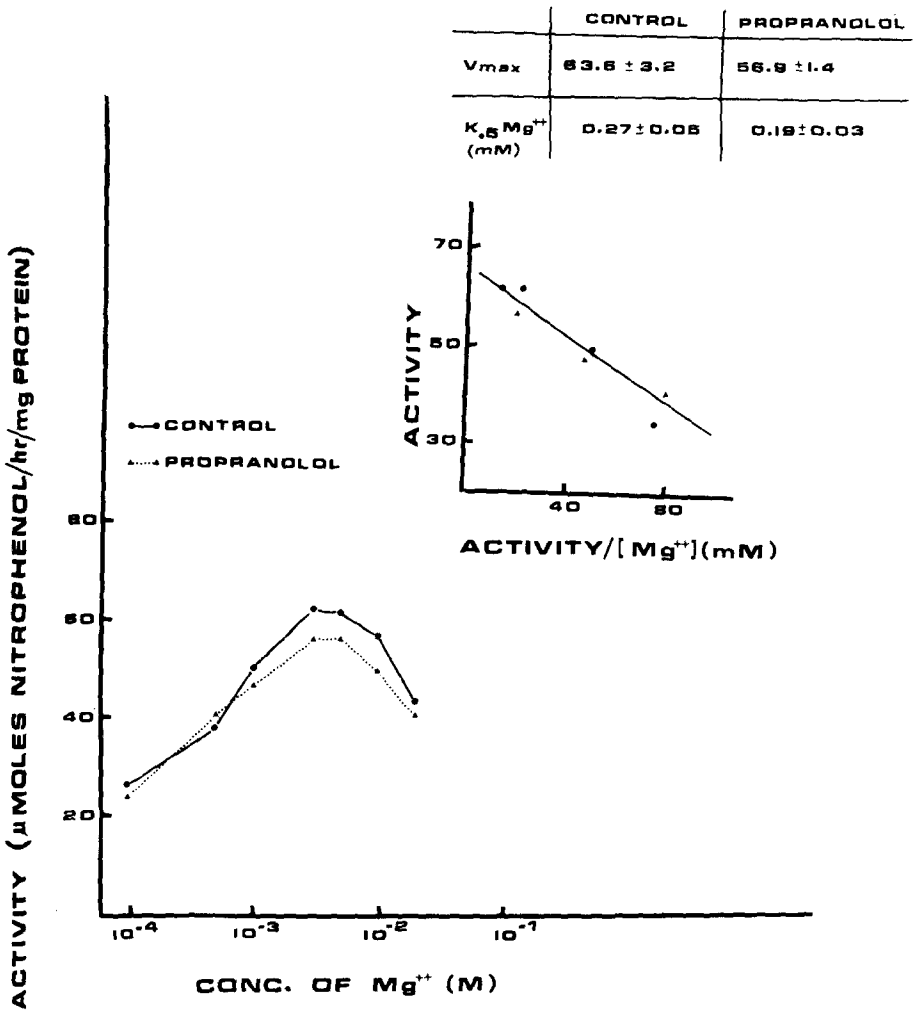

Fig. 9. Mg^{2+} dependence of basal (Mg^{2+}-stimulated) *p*-nitrophenyl phosphatase in the absence or presence of propranolol (1 mM). Inset: Eadie plot of the same data where *y* intercept = *V* and slope = $-K_{0.5}$ Mg^{2+}. Note differences in ordinate of Figs. 8 and 9. Each point represents the average of experiments with three different membrane preparations and mean values ± S.E. of the kinetic quantities are summarized in the accompanying table.

"low affinity" activity to propranolol inhibition is interesting in view of recent data [17] indicating that this enzyme activity is critically dependent on membrane structural integrity and interaction with membrane phospholipids, in contrast to the "high affinity" enzyme which seems to occupy a more peripheral location in the membrane.

	CONTROL	PROPRANOLOL
V_{max}	99.5 ± 2.7	57.0 ± 3.4
$K_{0.5}$ K^+ (mM)	3.81 ± 0.28	8.19 ± 0.85

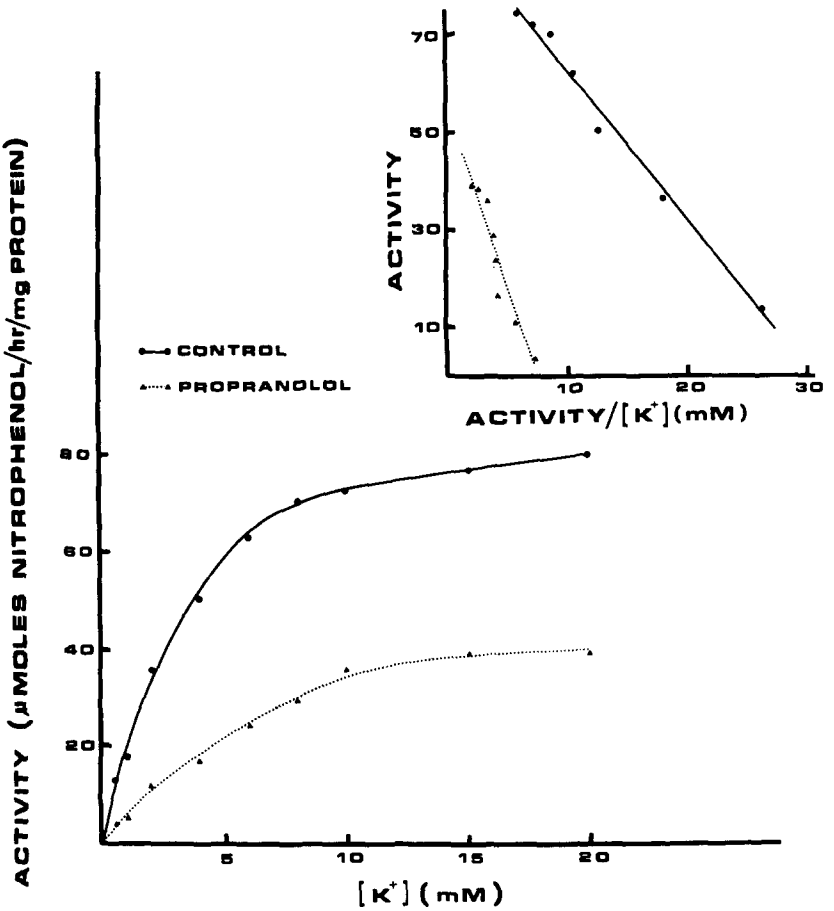

Fig. 10. K^+ dependence of K^+-stimulated *p*-nitrophenyl phosphatase in the absence or presence of propranolol (1 mM). Inset: Eadie plot of the same data where *y* intercept = *V* and slope = $-K_{0.5}$ K^+. Assays were performed exactly as described in Materials and Methods, except that the final concentration of KCl in the 3.0 ml incubation mixture was varied from 0.5 to 20.0 mM. Each point represents the average of experiments with four different membrane preparations and mean values ± S.E. of the kinetic quantities are summarized in the accompanying table.

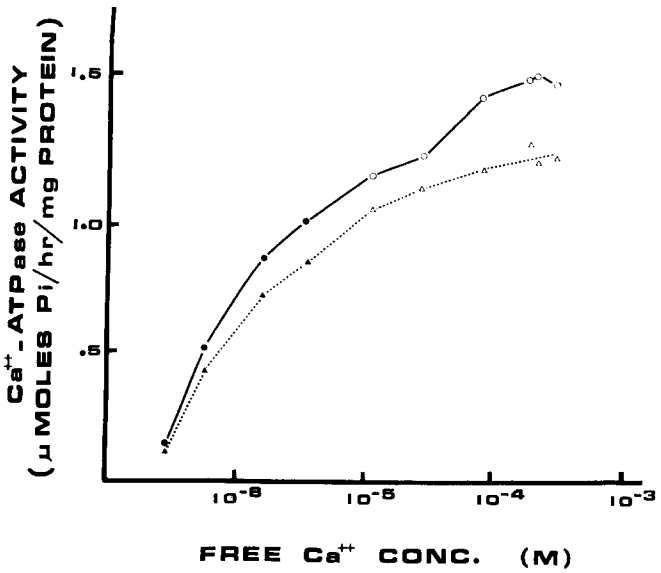

Fig. 11. Ca^{2+} dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity in the absence or presence of propranolol (1 mM). Membranes were always assayed less than 24 h after preparation. The mixture contained 0.2 ml membrane suspension, ATP (2 mM), $MgCl_2$ (6.4 mM), NaCl (66 mM), EGTA (0.1 mM), Tris buffer (55 mM, pH 7.4) and $CaCl_2$ to produce concentrations of free calcium in the range 0.3–300 μM. Each point represents the mean of experiments performed with five different membrane preparations.

DISCUSSION

The functional alterations produced in biological membranes by propranolol and other membrane stabilizers [30] are presumed to result from changes in the configurational state of membrane structural components induced by these substances. Our studies here represent an attempt to analyze the molecular basis of such proposed membrane perturbations using the human erythrocyte as a model membrane system.

Propranolol interaction with erythrocyte membranes produces a concentration-dependent increase in the incorporation of TNBS into membrane amino groups (Fig. 1) associated with a progressive decrease in the activation energy for TNBS incorporation (Fig. 2). A comparison of the properties of (+)- and (−)-isomers of propranolol was made since the (+)-isomer has been reported to be more active with respect to certain cardiovascular [31] and erythrocyte [32] effects than the (−)-isomer. However, TNBS incorporation did not reveal such differences and the stimulatory effects of both isomers, like their membrane-stabilizing properties [2], were equal (Fig. 1).

The involvement of protein and particularly phospholipid components in propranolol-induced membrane perturbations appeared likely both from the influence of trypsinization and phospholipase A treatment on changes in activation energy for TNBS incorporation produced by propranolol (Fig. 2) and from the increased labelling of protein and especially phospholipid amino groups by TNBS in the presence of increasing concentrations of propranolol (Fig. 4). This increase in phos-

pholipid labelling is consistent with the well-known ability of membrane stabilizers and local anesthetics to interact with phospholipids in biological membranes or in protein-free artificial membrane systems [33] and further shows that such interaction perturbs the structural state of these lipids. Although the exact role of membrane proteins in membrane stabilization has yet to be clarified, the fact that an approx. 10-fold discrepancy exists between the volume of occupation and the extent of membrane expansion produced by membrane stabilizers in erythrocyte membranes but not in protein-free lipid systems [30, 34] indicates that proteins are important in the process of membrane stabilization. Propranolol-induced perturbations in protein amino (Figs. 4 and 5) or sulfhydryl (Fig. 6) groups correlated well with propranolol-induced phospholipid alterations, suggesting interdependence of protein and lipid structural alterations. Although caution must be exercised in attempting to extrapolate from the effects of propranolol in erythrocyte ghosts to those in intact erythrocytes, regression analysis of the stabilizing effects of propranolol in intact erythrocytes and the perturbational effects of propranolol on proteins as well as lipids of isolated membranes suggests that membrane proteins contribute significantly to the stabilizing action of propranolol.

The inhibitory effects of propranolol on enzymatic processes arising from either outer (acetylcholinesterase, K^+ stimulation of *p*-nitrophenyl phosphatase) or inner (Mg^{2+} stimulation of *p*-nitrophenyl phosphatase) surfaces of the membrane (Figs. 7–10) were indicative of generalized membrane perturbations. Regression analysis indicated a high degree of correlation (r values > 0.95) between propranolol-induced labelling of membrane phospholipids by TNBS and the inhibition of these enzyme activities by propranolol. This suggests that enzyme inhibition is associated with an alteration by propranolol in the structural state of membrane phospholipids, since fluidity changes in membrane lipids have been shown to influence the properties of these enzymes in rat erythrocyte membranes [35]. The lesser susceptibility of ($Ca^{2+}+Mg^{2+}$)-ATPase to inhibition by propranolol (1 mM) (Fig. 11) as compared with ($K^+ + Mg^{2+}$)-*p*-nitrophenyl phosphatase (Fig. 7) (and ($Na^+ + K^+$)-stimulated Mg^{2+}-ATPase which behaved identically, results not shown) suggested a lack of structural and/or functional interdependence of these two ATP-hydrolyzing systems in the membrane, a suggestion borne out by the demonstration that these ($Na^+ + K^+$)- and Ca^{2+}-stimulated activities are associated with chromatographically distinguishable phosphorylated intermediates [36].

The impairment by propranolol of Mg^{2+}-activation of K^+-stimulated *p*-nitrophenyl phosphatase (Fig. 8) is interesting in the light of the recent suggestion by Goldman and Albers [37] that phosphatidylethanolamine, which is the phospholipid preferentially modified by TNBS in the presence of propranolol (results not included), may act as a modulator controlling the affinity of this enzyme for Mg^{2+}. Further, the observation that K^+ activation and Mg^{2+} activation of *p*-nitrophenyl phosphatase, which occur at outer and inner surfaces of the membrane, respectively [38], were impaired in a similar manner by propranolol (doubling of their respective $K_{0.5}$ values and comparable decreases in V, Figs. 8 and 10), may point to a role of amino phospholipids in mediating functional communication between these spatially separated cation binding sites. Alteration of this proposed communication may explain the change in activation energy produced by propranolol in ($Mg^{2+}+K^+$)-*p*-nitrophenyl phosphatase but not in the basal (K^+-independent) activity (Table I). The

ability of propranolol to modify divalent cation-membrane interactions likely also determines its inhibitory effects on the $(Ca^{2+}+Mg^{2+})$-ATPase (Fig. 11), as has been shown to be the case in the Ca^{2+}-transporting system of cardiac sarcoplasmic reticulum [39]. The preferential effects of propranolol on the "low affinity" activity in the erythrocyte (Fig. 11) strengthens the recent proposal that this activity is more relevant to active Ca^{2+} extrusion than the "high affinity" activity [17].

The studies described here provide a basis for analyzing the effects of membrane stabilizers in systems other than the erythrocyte. The observation that non-steroidal anti-inflammatory drugs increase sulfhydryl-disulfide exchange between membrane proteins and DTNB in vitro [40] may indicate that the proposed stabilizing action of these drugs on lysosomal membranes bears some molecular analogy to that produced by propranolol in erythrocytes where progressive exposure of membrane sulfhydryls was observed (Fig. 6). Similarly, propranolol has been shown to increase K^+ efflux from intact erythrocytes [41], an effect which appears mediated by drug-induced displacement of membrane Ca^{2+}. This property of propranolol, which could be relevant to its effects on cardiac pacemaker activity [42], may well be another manifestation of phospholipid structural alterations analogous to those observed in the present study. We are currently applying the approaches developed here to a study of membranes from excitable tissues in order to determine the extent to which the molecular characteristics of antihemolytic stabilization in erythrocytes resemble those associated with the electrical stabilization of excitable tissues.

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