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Association and partitioning of propranolol in model and biological membranes

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Propranolol is a beta-adrenergic receptor blocking agent of considerable importance However, in addition to this specific therapeutic action, it also exerts a nonspecific action on membranes which is a membrane-stabilizing effect similar to that of other anesthetic molecules Membrane stabilization as a result of drug-membrane interaction is thought to be responsible for several of the pharmacological activities of propranolol [1] Previous attempts have been made to understand the mechanism of the propranolol–membrane interaction at the molecular level through *in vitro* experiments with model and biological membranes Thus, Godin *et al* [1] investigated the effects of propranolol on erythrocytes exposed to trinitrobenzene sulfonic acid and enzymes and concluded that reactivities were affected by perturbations in membrane protein as well as the phospholipid components Similar conclusions were drawn when propranolol was reacted with sarcoplasmic reticulum vesicles [2]. On studies with liposome systems, several investigators have reported a lowering of the phase transition temperature of the phospholipid by propranolol addition [3, 4] Also, propranolol has been found to displace Ca^{2+} from binding sites on phospholipid monolayers [5] The apparent partition coefficient of propranolol in negatively-charged liposomes has been found to be several times greater than in neutral liposomes [6, 7] Propranolol has been found to destabilize anionic liposomes at concentrations which provide an antihemolytic action on erythrocytes [8]

The relative importance of the drug–protein and the drug–phospholipid interactions is still not clearly understood for propranolol in its role as a membrane stabilizer Hence, studies of the uptake and partitioning of propranolol in erythrocytes, erythrocyte ghosts, liposomes, and the *n*-octanol–water system were aimed at obtaining additional evidence of the nature of these interactions

Materials and methods

DL-Propranolol hydrochloride was supplied by Ayerst Laboratories, Canada L-α-Dimyristoylphosphatidylcholine, 98% (DMPC) was obtained from the Sigma Chemical Co , St Louis, MO All other chemicals and solvents were reagent grade, and glass-distilled water was used

Studies with erythrocytes Fresh human blood was obtained from the University of Alberta Hospital for each series of experiments Ethylene-diamine-tetraacetic acid (EDTA) (0 12%) had been added as an anticoagulant Before each experiment, the blood was washed three times in isotonic Tris buffer, (15 mM hydroxymethylaminomethane, 50 mM NaCl, 5 mM glucose), pH 7 4, and then diluted to yield a test sample containing 5–7% hematocrit The test sample (2 ml) was diluted with a solution of the drug in Tris buffer (1 ml), divided into two equal portions and transferred to centrifuge tubes (Eppendorf), vortex-mixed, incubated at 37° for 15 min, and then centrifuged at 1500 rpm for 1 min The supernatant fraction was carefully removed, and its absorbance was measured at 288 nm (Pye Unicam SP6-550 spectrophotometer) against buffer as reference Absorbance values were corrected for the absorbance of a blank treated similarly Experiments with several blanks gave the same absorbance value verifying that the amount of absorbing material retained in the supernatant fraction was reasonably constant on all occasions Concentrations of propranolol were determined from a calibration curve and the association or uptake computed accordingly Concentration-dependent studies covered the range 0.1 to 2 3 mM propranolol Results are the averages of quadruplicate experiments

Studies with erythrocyte ghosts Erythrocyte ghosts were prepared from fresh human blood in the following manner Whole blood was centrifuged at 2500 rpm for 5 min and washed three times with normal saline solution The pooled, packed cells were diluted fourteen times with ice-cold isotonic Tris/EDTA solution (1 mM EDTA), mixed for 10 min, and then centrifuged (36,000 *g*, 37° 30 min) (Beckman L8-55 centrifuge) Subsequently, the supernatant fraction was removed, and the pellet was resuspended in Tris/EDTA and centrifuged again (36 000 *g* 37°, 15 min) Following this treatment the pellet was then washed twice in 10 mM Tris buffer and centrifuged as before Finally, the ghost cells were diluted back to the original volume, and this stock suspension was used for the uptake studies Normally, the test sample (4 ml of stock suspension) was diluted with isotonic, buffered drug solution (2 ml), vortex-mixed, incubated for 30 min, and then centrifuged (56,000 *g*, 37° 10 min) The uptake of propranolol by the ghost cells was determined from the residual concentrations of the supernatant fraction Triplicate experiments were run and the results averaged

Studies with liposomes Liposomes were prepared by the method of Bangham *et al* [9] A thin film of DMPC (50 mg) was formed on the wall of a round-bottom flask then dispersed in 5 ml of an isotonic phosphate buffer solution of propranolol at approximately 40°, and vortex-mixed for 10 min, then 3 5 ml was transferred to a centrifuge tube The liposomes were equilibrated at the desired temperature for at least 24 hr and then centrifuged (143,000 *g* 30 min), the concentration of drug in the supernatant fraction was determined spectrophotometrically Under these conditions, the residual phospholipid in the supernatant frac-

tion is negligible [6 10] Experiments were run in duplicate with excellent reproducibility

Partition coefficient determinations Partition coefficients of propranolol in erythrocytes, ghosts and liposomes were each calculated from the results of the uptake experiments on the basis of equivalent phospholipid Thus, the phospholipid content of erythrocytes and ghosts was determined from phosphate analysis [11] of erythrocytes and DMPC Erythrocytes and ghosts were assumed to possess equivalent amounts of phospholipid at 3 33% cell concentrations of each An average of 15 93 μmoles phosphate/ml equivalent to 21 42 μmoles phospholipid/ml was found (N = 4) Liposome studies were normally conducted at 10 mg/ml (equivalent to 14 4 μmoles DMPC/ml) Apparent molar partition coefficients (K') of propranolol were calculated according to

$$K' = \frac{C_m}{C_u} \quad (1)$$

where C_m and C_u are the concentrations in the membrane (mole/mole phospholipid) and aqueous phases (mole/mole water), respectively

Partition coefficients of propranolol in the *n*-octanol–aqueous isotonic phosphate buffer system (pH 7 4) were also determined at 37° Aliquots of aqueous, buffered stock solution of propranolol (5 ml) were combined with *n*-octanol (0 5 ml) each solvent mutually saturated with the other, in a 25-ml round-bottom flask and equilibrated in a shaker water-bath Subsequently, the aqueous phase was removed by pipet and analyzed spectrophotometrically for propranolol Concentrations in the *n*-octanol phase were determined from the mass balance equation, and values of K' were calculated according to equation 1 but substituting C_m with C_o (mole/mole *n*-octanol)

Antihemolysis experiments The stabilization of the erythrocyte membrane against hemolysis by hypotonic osmotic shock using propranolol was measured at 37° at various concentrations of drug according to the procedure reported by Florence and Rahman [12] The degree of hemolysis was indicated by the extent of release of hemoglobin as detected spectrophotometrically at 540 nm Results are expressed as the relative hemolysis (%) defined as the ratio of supernatant absorbances of propranolol-containing cells to absorbances of controls

Results and discussion

The action of propranolol as a membrane-stabilizing agent of human erythrocytes subjected to hemolysis by hypotonic osmotic shock is clearly demonstrated in Fig 1 Addition of propranolol to yield final concentrations varying from 10^{-5} to 10^{-3} M resulted in a gradual increase

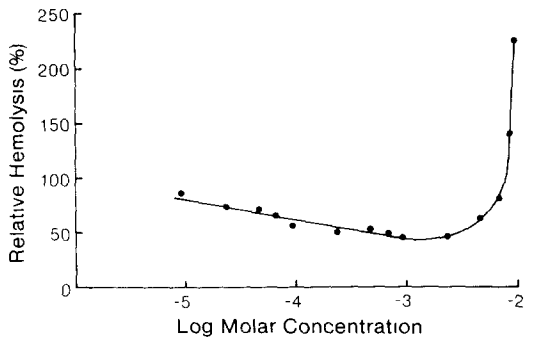

Fig 1 Relative hemolysis of human erythrocytes as a function of propranolol concentration Washed cells were subjected to hypotonic osmotic shock and the extent of hemolysis was determined from absorbances of hemoglobin released in solution at 540 nm

Table 1 Association (C_m) of propranolol with human erythrocytes, erythrocyte ghosts, and dimyristoylphosphatidylcholine liposomes at pH 7 4 and 37°

Propranolol concentration (mM)	C_m (mole/mole phospholipid)		
	Erythrocytes	Ghosts	Liposomes
0 10	0 06	0 03	0 01
0 20	0 11	0 05	0 01
0 30	0 17	0 11	0 02
0 43	0 19	0 12	0 02
0 60	0 20	0 14	0 03
1 2	0 47	0 30	
2 3	1 0	0 63	0 11

in stability of the erythrocytes, reaching a maximum amount of protection of approximately 54%. This compares with 37 5% when steroids are incorporated [12] and about 80% when the solute is *tert*-butylphenol [13] However at concentrations above 2 5 mM, propranolol reversed its effect on the erythrocyte membrane and destabilized it A similar biphasic behavior of propranolol on erythrocytes has been reported [8], and it appears to parallel the action of other anesthetic-like molecules with various membranes [1] A comparison of the association or uptake (C_m) of propranolol in erythrocytes, ghosts, and liposomes at different concentrations of propranolol is shown in Table 1 Values of C_m in erythrocytes and ghosts rose only slightly over the range of 0 10 to 0 60 mM propranolol concentration but rose more sharply at higher concentrations, corresponding approximately to the concentration range over which erythrocyte membrane stabilization and then destabilization occurred In contrast, the uptake of propranolol in liposomes rose very gradually and uniformly over the entire concentration range but values of C_m were about an order of magnitude smaller than in the erythrocytes The uptake in ghosts was approximately 35% smaller up to 0 60 mM propranolol and 46% smaller at higher concentrations than that observed in erythrocytes The interaction of propranolol with membranes has been found by others [1, 6, 8] to involve electrostatic interactions between the cation and negatively-charged binding sites on the cell surface, which may be protein, phospholipid or both, in addition to a strong hydrophobic effect which perturbs the arrangement of molecules in the bilayer structure of the membrane [2, 4, 7] Thus, a reduced protein content of ghost cells compared to intact erythrocytes may account for the lower uptake of propranolol in the former whereas, in addition to protein, the occurrence of acidic phospholipids in erythrocytes and ghosts, which are absent in the liposomes, may largely account for the differences in C_m between cell membranes and liposomes

Results of the measurements of partition coefficients (K') in erythrocytes, ghosts, liposomes, and the *n*-octanol-buffer system are depicted in Fig 2 These clearly show that the *n*-octanol–buffer system although it generally behaves as a distribution system that differentiates between the relative hydrophobic tendencies of solutes did not reflect the interactions of propranolol with cell membranes which gave rise to variations in the K' at low concentrations of propranolol The similar concentration-dependence of log K' up to about 0 6 mM in erythrocytes, ghosts and liposomes suggests that the interaction of propranolol caused a perturbation in the cell membrane and a possible change in conformation of membrane molecules which led to a decreased capacity for propranolol However, it is apparent that the effect of membrane perturbation on log K' varied with the different compositions of the cell membranes Protein anchored in the erythrocyte membrane could minimize the perturbation of the phospholipid bilayers by propranolol [6] Erythrocyte ghosts which are

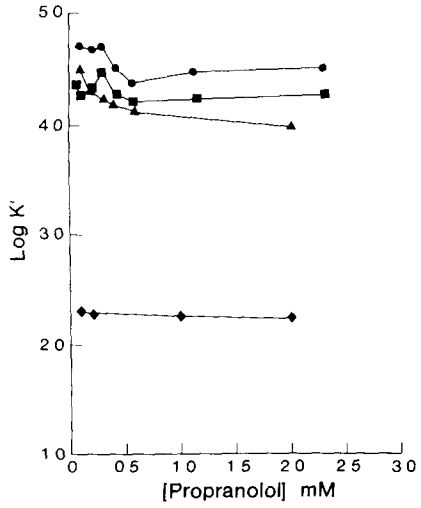

Fig 2 Log molar apparent partition coefficients (K') of propranolol as a function of propranolol concentration in model and biological membranes· (●) erythrocytes, (■) erythrocyte ghosts, (▲) DMPC liposomes, and (◆) *n*-octanol–aqueous buffer solution Temperature 37°, pH 7 4

partially protein-depleted, underwent greater membrane perturbation and possibly increased exposure of acidic phospholipids to propranolol and electrostatic binding which could account for the slight rise in log K' in the low propranolol concentration region The approximate constance of log K' at higher concentrations implies that the environment for partitioning was similar in all three cell membrane systems with respect to bilayer structure and characteristics

In summary, studies of the uptake and partition coefficient of propranolol in erythrocytes, ghosts, and liposomes demonstrate subtle changes occurring in the lipid matrix up to about 0 6 mM propranolol These changes were mediated by protein and acidic phospholipids In erythrocytes, interaction between propranolol and protein appeared to improve the stability of the cell membrane before sufficient propranolol had partitioned into the lipid matrix to cause extensive disruption In ghost cells, perturbation of the membrane, likely through interaction of propranolol with acidic phospholipids, caused fluctuation in log K' but otherwise log K' exhibited behavior similar to that in erythrocytes, although uptake and partitioning of propranolol was less The uptake of propranolol was an order of magnitude less in liposomes compared to erythrocytes, but the uniform concentration dependence of log K' and the osmotic fragility of liposomes in the presence of propranolol [8] indicate that the hydrophobic interaction plays a major role in creating membrane disturbances [14] The *n*-octanol-buffer system does not serve as a suitable model in this instance to describe the non-specific membrane-stabilizing activity of propranolol

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Effects of alloxan on *S*-adenosylmethionine metabolism in the rat liver

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In the rat liver up to 40% of the phosphatidylcholine is synthesised by the transmethylation pathway being *S*-adenosyl-l-methionine (AdoMet)* the methyl donor [1] This pathway is modulated by a variety of hormones and by the levels of AdoMet and *S*-Adenosyl-l-homocysteine (AdoHcy) (reviewed in [2]) AdoHcy is a competitive inhibitor of the transmethylation reaction Therefore, the ratio AdoMet/AdoHcy must be carefully controlled to maintain the cellular necessities of phosphatidylcholine In the liver of alloxan-diabetic rats the content of phosphatidylcholine is lower than in control animals [3, 4] The activity of the enzyme phospholipid methyltransferase is also reduced in liver microsomes of alloxan-diabetic rats [5] We have measured the levels of AdoMet and AdoHcy and the synthesis of phosphatidylcholine by the transmethylation pathway in hepatocytes isolated from alloxan-

* Abbreviations used AdoMet, *S*-adenosyl-L-methionine AdoHcy, *S*-adenosyl-L-homocysteine, AdoMet-synthetase, *S*-adenosyl-L-methionine synthetase