Acknowledgements—We thank the Upjohn Co. for authentic prostaglandins, Ms. R. Harris for secretarial assistance, and Mr. J. Saady and G. Nelson for technical assistance. Supported by NIH HL 18932 and NS 12587.

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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1784–1787, 1982.
Printed in Great Britain.

0006-2952/82/091784-04 $03.00/0

Promotion of membrane resealing by local anesthetics

(*Received* 27 *June* 1981; *accepted* 14 *October* 1981)

The spontaneous repair of membrane defects induced by mechanical trauma or osmotic shock is an important but little-understood ability of red cells. Most observations have been incidental to studies of red cell ghost membranes in which lysed and resealed ghosts were used to study various structural and transport features of the membrane. We have reported the detailed temperature dependence of resealing [1, 2] and found that the curve is sigmoidal, suggesting that resealing is a highly cooperative process. This temperature dependence could be dependent on a phase transition in a particular lipid region of the membrane [3]. To investigate this possibility, we have studied the effects of various membrane fluidizing compounds on resealing. We found that cationic amphiphiles increased resealing, while other fluidity-altering agents did not affect resealing.

Materials and methods

Ghost preparation and measurement of resealing using hemoglobin as a marker have been described [1, 2]. Briefly, mixtures of unsealed ghosts and hemoglobin are held at 0° for 10 min to permit hemoglobin to equilibrate with the ghost interior volume. The mixture is then brought to the desired final temperature and salt concentration simultaneously. After appropriate incubation times, resealing is stopped by adding a large volume of cold isotonic buffer, and trapped hemoglobin is determined. To assay drug effects, the compounds were added to the ghost–hemoglobin mixture during the preequilibration period at 0°. The volatile anesthetics were tested in sealed tubes to minimize evaporative loss, but we did not directly assay these compounds [4]. All compounds were obtained from the Sigma Chemical Co., St. Louis, MO, with the following exceptions: ether and chloroform (Mallinckrodt, St. Louis, MO) and halothane (Ayerst, New York, NY). For cholesterol depletion of red cells, the methods of Gottlieb [5] were used. Ghosts were prepared and resealing experiments were as described. The amounts of cholesterol [6] and phospholipid [7] were determined by standard methods.

Results

Anesthetics. The local anesthetics, dibucaine and tetracaine, were able to induce red cell ghost resealing at low temperatures. A typical result for dibucaine is shown in Fig. 1. Since amphipathic compounds are believed to fluidize lipid bilayers or to induce phase changes, it seemed possible that these results were related to changes in lipid bilayer properties. To test this, the extent of resealing after 1 hr at 10° was measured for ten amphipathic compounds. This temperature was chosen since resealing is ordinarily very slow at 10°, but, as Fig. 1 shows, dibucaine, and anesthetic was able to cause resealing at 10°. The compounds were chosen because studies are available of their fluidizing effects on bilayers and, in some cases, on erythrocyte membranes. Figure 2 shows typical results; the data are summarized in Table 1.

The effects of cationic amphiphiles on resealing were biphasic, i.e. as concentrations were increased, the percentage of ghosts resealed went through a maximum and declined (Fig. 2). This is similar to the effects of these compounds on erythrocyte osmotic fragility [8], where low concentrations of amphiphiles protect red cells against osmotic hemolysis, but higher levels promote hemolysis. Comparison between the concentrations of the drugs needed for 50% anti-hemolysis [8] and the concentrations

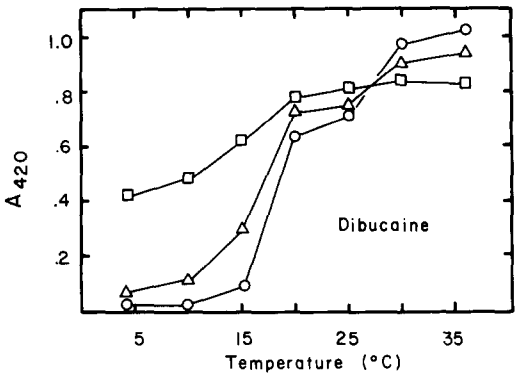


Fig. 1. Effect of dibucaine on the cooperativity of erythrocyte ghost resealing. Washed ghosts were resealed for 1 hr in 75 mM NaCl, 5 mM Tris–Cl, 1 mM EDTA, pH 7.4, at the indicated temperatures, and the absorbance (A_{420}) of the trapped hemoglobin was assayed as described. Dibucaine was present at zero mM (○), 0.2 mM (△), or 2.0 mM (□).

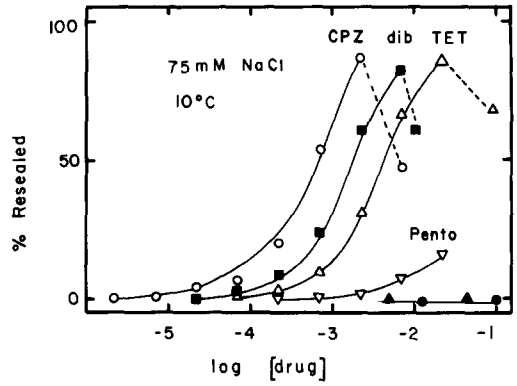


Fig. 2. Effect of some amphipathic compounds on ghost resealing. The percent resealed was determined after 1 hr at 10° in 5 mM Tris–Cl, pH 7.4, 75 mM NaCl. The 100% value was hemoglobin trapped by control ghosts after 1 hr at 37°, which leads to nearly quantitative resealing [2]. Key: (○) chlorpromazine, (■) dibucaine, (△) tetracaine, (▽) pentobarbital, (▲) benzyl alcohol, and (●) ethanol. The dotted line shows that greater than optimal drug concentrations diminished resealing.

which induced resealing shows correlations within classes, i.e. the order of effectiveness of the amine compounds is similar for anti-hemolysis and resealing (Table 1 and Fig. 3). There is one exception, however: pentobarbital is effective in preventing hemolysis [8], but is ineffective in resealing membranes. For the amine compounds, the most effective class of resealing agent, the concentrations required to promote resealing were usually about ten times greater than those needed for 50% anti-hemolysis. Resealing of ghosts was promoted by concentrations of amphiphile that lyse erythrocytes.

Cholesterol. Alteration of cholesterol content has long been known to alter fluidity in bilayers [9] and red cell membranes [10]. We lowered the cholesterol content of erythrocytes by incubation with cholesterol-depleted serum [5, 11] and found no effect on the temperature dependence of resealing (Fig. 4). This experiment was also done with membranes depleted of cholesterol by incubation with liposomes [12], with variable results. We suspect that liposomes prepared from sonicated phospholipids contain compounds that affect resealing rates (e.g. lysolecithin) but we have not pursued this possibility.

Discussion

The most striking finding of this work was the ability of some amphipathic compounds to reseal erythrocyte ghosts, as seen in augmented resealing at low temperature.

Cationic amphiphiles were effective in resealing ghosts, while inhalational anesthetics were not. This is only partially consistent with the hypothesis that membrane lipid fluidity influences the rate of resealing. The local anesthetics, dibucaine and tetracaine, increase the fluidity of artificial lipid bilayers [13, 14] and erythrocyte membrane [15]. For example, the microviscosity of erythrocyte ghosts, as determined with perylene fluorescence depolarization, is markedly increased by 0.6 mM tetracaine [15]. The inhalational anesthetics, which did not induce resealing, are also less effective in fluidizing membranes. For example, changes in erythrocyte ghost microviscosity required 36 mM $CHCl_3$, 40 mM halothane or 250 mM diethyl ether [16].

Conrad and Singer [17] have recently proposed that cationic amphiphiles such as chlorpromazine also do not dissolve in the lipids of biological membranes, and that earlier binding data are artefactual. It is clear, however, that these compounds affect lipid fluidity [13–16], although the mechanism may not be intercalation into the lipid bilayer [17].

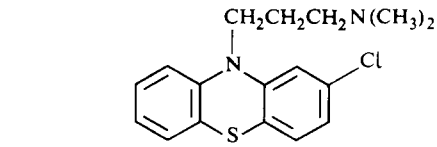
Not all the data are consistent with the hypothesis that membrane repair by spontaneous resealing is entirely a lipid-mediated process. For example, cholesterol depletion,

Table 1. Abilities of various compounds to reseal ghosts*

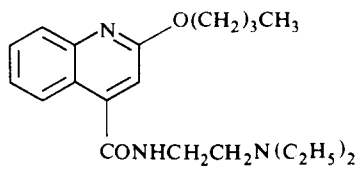
Compound	R_{50}	AH_{50}†
Chlorpromazine	0.7	0.008
Dibucaine	1.6	0.10
Tetracaine	4.5	0.50
Pentobarbital	>100	0.30
Ethanol	>2000	1600
Butanol	95	42
Benzyl alcohol	>100	22
Chloroform	No effect at 36 mM	11
Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane)	No effect at 15 mM	100
Diethyl ether	No effect at 250 mM	260

* All concentrations were millimolar. R_{50} is the concentration at 10° that induces 50% of maximum resealing. The 100% value indicates resealing at 37° without additions. AH_{50} is the concentration that inhibits hemolysis by 50%.

† These data are taken from Ref. 8, with the permission of the authors.



Chlorpromazine
pK = 9.3



Dibucaine
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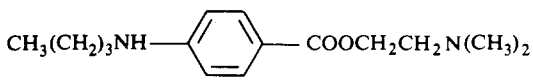
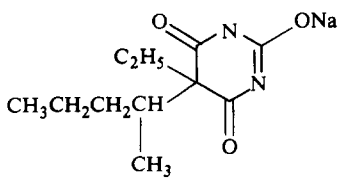


Tetracaine
pK = 8.9



Pentobarbital
pK = 8.1

Fig. 3. Structures of chlorpromazine, dibucaine, tetracaine and pentobarbital and their pK values. The pK values are from D. D. Perrin, *Dissociation Constants of Organic Bases in Aqueous Solution*, Butterworths, London (1965), except for pentobarbital, which is from A. Goldstein, L. Aronow and S. M. Kaufman, *Principles of Drug Action*, John Wiley, New York (1974).

which lowers lipid microviscosity [10], and pentobarbital, which appears to fluidize artificial bilayers [4], do not promote resealing. It is perhaps significant that cationic compounds promote resealing, whereas altered levels of anionic or neutral compounds have no effect. It is possible that fluidization of a region of negatively charged phospholipids is essential for membrane resealing. In any case, the use of cationic drugs will permit trapping of heat-labile solutes within resealed ghosts and may aid in investigations of the mechanism of spontaneous membrane repair.

In summary, we have observed a surprising effect of anesthetics on biological membranes. Cationic anesthetics were able to reseal lysed erythrocyte membranes at very low temperatures. Some resealing was observed even at 0°, and a survey of a number of representative compounds was carried out at 10°. This phenomenon may be related to lipid fluidity, but not all the data can be reconciled with present ideas of fluidity.

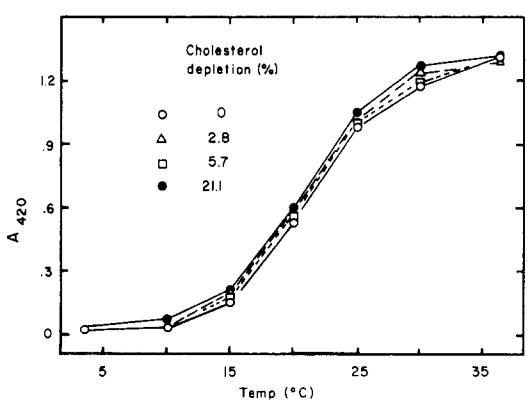


Fig. 4. Effect of cholesterol depletion on resealing. The amount of cholesterol removed by incubation with serum [5] is given as a percentage of normal cholesterol level.

Acknowledgements—This work was supported by the National Institutes of Health and the Michigan Heart Association. I thank Mr. Gregory Taylor for excellent technical assistance.

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Biochemical Pharmacology, Vol. 31, No. 9, pp. 1787–1790, 1982.
Printed in Great Britain.

0006-2952/82/091787-04 $03.00/0

Displacement of Ca^{2+} bound to synaptosomal membrane sialoglycoconjugates by serotonin and serotonergic drugs and the effect on endogenous sialidase (neuraminidase) activity

(*Received* 13 *July* 1981; *accepted* 4 *November* 1981)

Gangliosides are a class of complex glycosphingolipids which contain one or more residues of the negatively charged sugar, sialic acid (*N*-acetylneuraminic acid) (for review, see Ref. 1). Gangliosides are abundant in nervous tissue and are thought to enrich neuronal synaptic membranes [2, 3]. Recent evidence suggests that membrane gangliosides modify various neuronal enzyme activities [4, 5], drug interactions [6, 7] and receptor properties for certain neurotoxins [8] and peptide hormones [9, 10]. The possibility of a membrane ganglioside being the receptor for serotonin was first suggested by Woolley and Gommi [11, 12], and in recent years this concept has again received much attention [13–15].

Synaptic membranes also contain an intrinsic sialidase (*N*-acetyl neuraminosyl glycohydrolase, EC 3.2.1.18), which resides in close association with its preferred substrate, membrane gangliosides [16]. By enzymatic removal of sialic acid from gangliosides in synaptic membranes, oligosialosyl gangliosides are reduced to the monosialosyl ganglioside G_{M1} (for a complete description of ganglioside nomenclature, see Ref. 17). One of the many consequences of such an intrinsic enzyme–substrate system functioning to modify the synaptic membrane glycocalyx may be an alteration of net surface negative charge density [18]. Calcium ions have been shown to interfere with this system by inhibiting endogenous sialidase activity [19]. Others have demonstrated that gangliosides bind Ca^{2+} with high affinity [20–22], and it has been suggested that Ca^{2+} binding to sialic acid residues of gangliosides in synaptic membranes is a critical step in synaptic transmission [22, 24]. The present experiments were designed to examine more closely the interactions of serotonin and serotonergic drugs with Ca^{2+} bound to synaptosomal sialoglycoconjugates and the effect of these interactions on the endogenous membrane sialidase activity.

Synaptosomes were prepared from bovine brain frontal cortical gray matter by techniques previously described [25]. After hypo-osmotic lysis of the synaptosomes at pH 8.4 [26], the plasma membranes were collected by centrifugation at 100,000 *g* for 60 min. Synaptosomal plasma membranes (2 mg protein) were suspended in 2 ml of 1 mM HEPES* buffer, pH 7.5, containing 10 mM [^{45}Ca]Cl_2 (New England Nuclear Corp., Boston, MA, 0.8 mCi/mmole). After incubation at room temperature for 10 min, membrane samples were collected by centrifugation and were washed twice with buffer. Samples were then suspended in 2 ml of the same buffer containing one of the following compounds at various concentrations: serotonin, quipazine [2-(1-piperazinyl)quinoline maleate], fluoxetine [Lilly 110140, 3-(*p*-trifluoromethylphenoxy)-*N*-methyl-3-phenylpropylamine], acetylcholine, ruthenium red and EGTA. Membranes were collected by centrifugation after 10 min at room temperature, and radioactivity in the supernatant fraction was determined by liquid scintillation spectrometry in Aquasol (New England Nuclear Corp.). After suspension in 20 mM acetate buffer, pH 3.9, endogenous sialidase activity (i.e. activity of the intrinsic membrane enzyme toward native membrane sialoglycoconjugates) was determined as described elsewhere [27].

The displacement of bound [$^{45}Ca^{2+}$] from synaptosomal membranes by various agents, and the effect of this displacement on the endogenous sialidase activity are shown in Fig. 1. Exposure of $^{45}Ca^{2+}$-labeled synaptosomal membranes to 10 mM $^{40}Ca^{2+}$ (non-radioactive) resulted in a 40% inhibition of the endogenous sialidase activity, whereas less than 5% of the radioactive [$^{45}Ca^{2+}$] was displaced. This observation suggests that bound [$^{45}Ca^{2+}$] does not readily exchange with subsequently added Ca^{2+}. The degree of inhibition of sialidase activity is similar to that reported previously [19].

Acetylcholine (ACh) at 2 and 10 mM displaced approximately 50 and 63% of bound [$^{45}Ca^{2+}$] respectively; sialidase activity was increased 10 and 20% above the level seen in the presence of 10 mM Ca^{2+}. Serotonin (5-HT) displaced 90% of the bound [$^{45}Ca^{2+}$] at each concentration studied. Endogenous sialidase activity increased 15 and 22% above the Ca^{2+} level at 2 and 10 mM serotonin. The finding that serotonin was more effective than acetylcholine in displacing Ca^{2+} bound to synaptosomal membranes parallels that made by others when examining displacement of bound Ca^{2+} from purified gangliosides by the same agents [28].

* Abbreviations: HEPES 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid; and EGTA, ethyleneglycolbis(amino-ethylene)tetra-acetate.