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Interaction of amphiphiles with integral membrane proteins. I. Structural destabilization of the anion transport protein of the erythrocyte membrane by fatty acids, fatty alcohols, and fatty amines

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The effect of model amphiphiles on the structural stability of the anion exchange protein (band 3) of the human erythrocyte membrane was studied by differential scanning calorimetry. The concentration of membranes, as well as the concentration, head group, alkyl chain length, degree of unsaturation, and double bond configuration of a variety of alkane derivatives were all varied in a systematic way. The depression of the denaturation temperature of band 3 per unit membrane concentration of the amphiphile was then determined in order to quantitate the potency of each drug. Saturated fatty acids of chain length C_8 to C_{24} displayed a monotonic decrease in potency up to C_{20}, followed by a dramatic diminution in potency at C_{22} and C_{24}. Unsaturation caused only minor increases in the abilities of fatty acids to perturb the anion exchanger, and surprisingly, there was neither a trend for the number of double bonds nor a significant *cis-trans* distinction. Arachidonic acid, as an exception, was much more effective than any other amphiphile in destabilizing band 3. Fatty acids were about three times more potent than fatty amines and fatty alcohols; however, the enhanced partitioning of the latter into the membrane compensated at certain membrane/buffer ratios for its reduced intrinsic potency. A quantitative model interpretation of the data is presented in an accompanying paper.

Introduction

Small amphiphilic compounds of virtually any charge and configuration can perturb membrane-mediated processes. Tertiary amines, alcohols, hydrophobic acids, polar aromatic compounds and halogenated hydrocarbons have all been shown to block neurotransmission [1–3]. Amphiphilic compounds of a similar variety have also been found to perturb cell motility [4], Ca^{2+}-membrane interactions [5,6], receptor activities [7,8], cytoskeletal interactions [7,9], gross cell morphology [9–12], mitochondrial function [13–15], and numerous ion transport processes [16–26]. Indeed, it has been proposed that the predominant requirement of a membrane perturbant is simply that the perturbing molecule partition strongly into the membrane [27].

While a large number of explanations has been offered for the effect of amphiphilic compounds on membrane function, most of these explanations can be loosely grouped into two major categories: those that attribute a drug's potency to its ability

Abbreviations: DSC, differential scanning calorimetry; SDS, sodium dodecyl sulfate; DTAB, dodecyltrimethylammonium bromide.

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to perturb the lipid phase of the membrane, and those that ascribe the action of the drug to its capacity to bind and modify proteins in the membrane. The lipid-based hypotheses have generally related a drug's potency to its ability to melt, expand, thicken, fluidize or disperse functionally important lipid phases near membrane proteins [25,27–34]. A major strength of these hypotheses is that they easily account for the near absence of structural specificity or uniformity among perturbants of a specific process. However, a major weakness of the hypotheses is that the vast majority of membrane activities are mediated by proteins, and therefore, the proposed lipid perturbation most somehow be communicated through the lipid to the sensitive protein.

The protein-based hypotheses [16,35–38] propose that once the amphiphile partitions into a membrane, it can bind and perturb a protein nonspecifically, much like the wide variety of soluble protein denaturants alter soluble enzyme activities. An obvious strength of these hypotheses lies in their simplicity; i.e., no mediating lipid phase is required to explain the modified membrane function. Furthermore, the perturbation can be described by the simple formalities used to quantitate other protein–ligand interactions, especially those employed to treat protein destabilization by denaturants [39–41]. Unfortunately, both hypotheses have been difficult to verify, since most perturbations of membrane properties cannot be unequivocally assigned to either a protein or a lipid disturbance.

In an effort to take a simpler, more direct approach to these questions, we have engaged in a systematic investigation of the effects of an ordered series of alkane derivatives of varied charge, chain length, and degree/configuration of unsaturation on the structural stability of the membrane-spanning domain of band 3. The amphiphiles in this study were chosen to represent a wide range of hydrophobicities, geometries, and functional group constituents in order to be able to evaluate any degree of structural specificity in the observed protein perturbation. The 55-kDa integral domain of band 3 was chosen because it is an intact ion transport protein which is believed to be comprised of up to 12 membrane-spanning helices [42], a structure thought to be representative of other integral membrane proteins. Furthermore, a completely functional membrane-spanning domain can be readily isolated in the absence of its soluble 43-kDa cytoplasmic domain in abundant amounts [43,44]. Also, the structural stability of the membrane-spanning domain of band 3 can be evaluated accurately and quantitatively by sensitive differential scanning calorimetry (DCS) [44,45]. Sensitive DSC is especially Sensitive DSC is especially attractive for the study of band 3, since the properties of the proteins's calorimetric endotherm have been shown in previous work to quantitatively predict the flux of ions through the protein channel [45,46].

Material and Methods

Materials. Octanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, eicosanoic acid, docosanoic acid, tetracosanoic acid, *trans*-9-octadecenoic acid, *cis*-9-octadecenoic acid (Na-salt), *cis*-9,12-octadecadienoic acid, *cis*-9,12,15-octadecatrienoic acid, 1-octadecanamine, 1-octadecanol, and *cis*-5,8,11,14-eicosatetraenoic acid were purchased from Aldrich Chemical Co. Decanoic acid was obtained from Fastman Chemical Co., octanol from Mallinckrodt, octadecanoic acid, trypsin, and phenylmethylsulfonyl fluoride from Sigma Chemical Co., and sodium dodecyl sulfate (SDS) from Fisher Scientific Co. Octanoic acid, octylamine, and octadecylamine were converted into salts by dissolving them in methanol and titrating with NaOH or HCl, followed by evaporation of the solvent and vacuum drying. Ali solvents were spectrophotometric grade. Fresh human blood was obtained from the Central Indiana Regional Blood Bank with citrate dextrose solution added, and stored at 3°C until used.

Inside-out vesicle preparation. The trypsin-digested membrane vesicles were prepared as described [45], except that the peripheral proteins were removed with 1 M KCl, 20 mM phosphate (pH 8.00) for 60 min at 37°C. The resulting vesicles contained the 53-kDa membrane-spanning, C-terminal fragment of band 3 as the predominant polypeptide. This fragment retains the transport function of the parent protein [43] but lacks the water soluble 43000-Da cytoplasmic domain which serves mainly as a peripheral protein

anchoring site [46]. The membrane vesicles were finally washed three times in phosphate-buffered saline (125 mM NaCl, 20 mM Na_3HPO_4, titrated to pH 7.40 with HCl, 3 mM NaN_3, and resuspended in the same buffer to give a stock suspension with 30–40 mg dry membrane/ml (determined gravimetrically). When scanned in the calorimeter, these vesicles yield a single major transition (see Fig. 1) which has previously been shown to derive from the denaturation of the membrane-spanning domain of band 3 [44]. The transition of the minor components of these vesicles (e.g. glycophorin) are either too small to detect or at significantly different temperature. The batch which was to be treated with unsaturated fatty acids was degassed by three cycles of evacuation and regassing with N_2 and the resulting deoxygenated sample was stored until use under nitrogen in order to avoid lipid peroxidation.

Sample preparation. All alkane derivatives with a chain length of more than 10 carbons (except SDS) were dissolved at 2–10 mM concentration in chloroform/methanol (2:1) and aliquots were removed and dried first under a stream of N_2, and then by evacuation for 2 h, Unsaturated fatty acids were invariably handled under N_2. To the dry amphiphile films phosphate-buffered saline was added and sonicated in a bath sonicator at 60–70 °C just long enough to give a clear solution (except for suspensions with pure octadecnol and octadecylamine), and after rapid cooling to 30–35 °C, the inside-out vesicle suspension was injected during vortexing. SDS and decanoic acid were dissolved in phosphate-buffered saline (pH 7.40 readjusted), to give 5 mM stock solutions. Sodium-octanoate and octanamine · HCl were dissolved in water/saline mixtures to give a final amphiphile concentration of 60 mM and a final osmolyte concentration of 310 mosM. The aliquots from these aqueous stock solutions were then mixed with buffer and inside-out vesicle suspensions to give the desired samples. The stock solution of octanol was prepared by dissolving the octanol in a 50-fold molar amount of ethanol. Aliquots from this stock solution were then introduced directly into the inside-out vesicle samples. All amphiphile-treated inside-out vesicles samples were incubated for 30 min at 37 °C to ensure complete equilibration of the drug. The various sample preparation procedures were necessary because of the vast differences in the aqueous solubilities of the amphiphiles. However, the objective in each case was to achieve complete equilibration of the amphiphile with the inside-out vesicle suspension in a solution of the same final osmolarity.

DSC. The calorimetric was performed on a Microcal-1 differential scanning calorimeter (Amherst, MA) at a heating rate of 1 C°/min. The sample volume was 1.00 ml and the reference buffer was phosphate-buffered saline in all experiments.

Data analysis. In order to describe the effect of an amphiphile on a membrane protein two parameters must be defined. First, it is necessary to know the concentration of the amphiphile in the membrane, i.e. in the vicinity of the target protein. This membrane concentration (N_m/V_m) is related to the buffer concentration (N_b/V_b) by the partition coefficient K_p in the following way:

$$\frac{N_m}{V_m} = K_p \frac{N_b}{V_b} = \frac{K_p N_t}{V_t + V_m(K_p - 1)} \quad (1)$$

where N and V refer to the moles of drug and to volume, and the subscripts m, b, and t refer to membrane, buffer, and total system, respectively. The membrane volume was determined directly from the dry weight of the membranes by assuming a density of 1 kg/l. Second, because the effect of different drugs at the same membrane concentration may differ in magnitude, each drug has to be characterized by a potency value (P) which relates the perturbing activity of a drug to its membrane concentration. We quantitated the perturbing potency of each drug calorimetrically by measuring its depression of the denaturation temperature (ΔT) of the membrane-spanning domain of band 3 and have found this to be linearly related to its membrane concentration (N_m/V_m):

$$\Delta T = P \frac{N_m}{V_m}; \; P = \text{constant} \quad (2)$$

Combination of Eqns. 1 and 2 yields an expression which allows one to predict the destabilization of band 3 (ΔT) from the two model parame-

ters, P and K_p, and from the experimental conditions given by N_t, V_t, and V_m.

$$\Delta T = \frac{PK_pN_t}{V_t + V_m(K_p - 1)} \quad (3)$$

This equation can be simplified under two limiting conditions which depend on the fraction of the total drug which resides in the membrane, as described below.

If the inside-out vesicle concentration (V_m/V_t) or the membrane solubility (K_p) of the drug is very small, then $V_m(K_p - 1) \ll V_t$ and Eqn. 3 becomes:

$$\Delta T_0 = PK_p\frac{N_t}{V_t} \quad (4)$$

The subscript '0' refers to this situation where the fraction of drug in the membrane is negligible, and therefore, its buffer concentration is not diminished by the presence of the membranes. It should be emphasized that only under these limiting conditions will the drug concentration necessary to achieve a constant effect ΔT be independent of the concentration of membranes in the sample. Eqn. 4 can then be restated to define an apparent potency value (P_{app}) under these low partitioning conditions:

$$\Delta T_0\frac{V_t}{N_t} = PK_p = P_{app} \quad (5)$$

In the opposite limiting case, practically all of the amphiphile is assumed to partition into the membrane ($N_m = N_t$); therefore, the calorimetric effect is simply derived from Eqn. 2 as:

$$\Delta T = P\frac{N_t}{V_m} \quad (6)$$

Obviously in this situation the degree of sample dilution has a strong influence upon the overall amphiphile concentration which is necessary to achieve a constant effect ΔT.

Results

Destabilization of the membrane-spanning domain of band 3 by saturated long chain fatty acids

As outlined under Material and Methods, the effect of an amphiphile on the stability of an integral membrane protein can be described by one of three equations, depending on the fraction of the drug which resides in the membrane.

Fatty acids having more than 12 carbons in their alkyl chains were found to partition totally into the bilayer, and therefore, their effects on band 3 stability were found to follow Eqn. 6. Fig. 1 shows the denaturation transition of the anion channel in the presence and absence of octadecanoic acid. In the absence of modifier, the integral domain of band 3 denatured at 69.8°C. While this value varied by nearly 1 C° from individual to individual, the denaturation temperature was observed to be constant for any single preparation of membranes. Also, the shift in denaturation temperature due to the presence of an amphiphile was invariant within a single batch of membranes. Therefore, all ΔT values were obtained by comparison of the band 3 transition temperatures in control and drug-treated membranes from the same batch of inside-out vesicles.

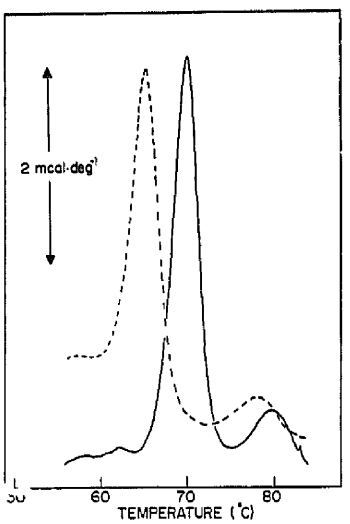

Fig. 1. DSC scan of peripheral protein-depleted, trypsin-digested inside-out erythrocyte membrane vesicles containing the membrane-spanning domain of band 3 as the predominant (> 70%) polypeptide. The vesicles were equilibrated in phosphate-buffered saline (pH 7.40) containing 3 mM NaN_3 either in the absence (———) or presence (- - - - -) of octadecanoic acid. The vesicle concentration was 27.0 mg/ml and the fatty acid concentration was 104 mmol/kg of membrane.

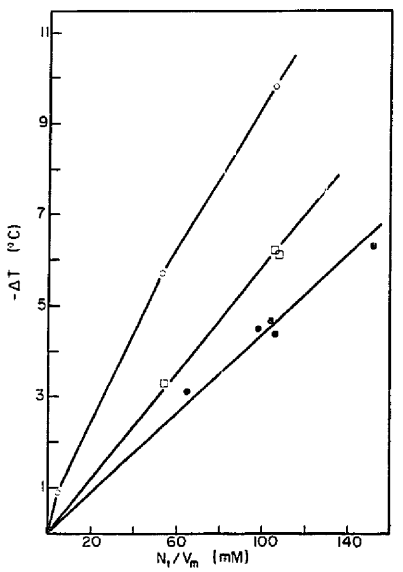

Fig. 2. Depression ($-\Delta T$) of the denaturation temperature of band 3 by increasing of membrane concentrations (N_t/V_m) of long chain fatty acids. The inside-out vesicle concentration was 27.0 mg/ml in all experiments. ●, octadecanoic acid; □, dodecanoic acid; ○, arachidonic acid. The lines for octadecanoic acid and dodecanoic acid were calculated by a least-squares fit to Eqn. 6. The membrane concentration (N_t/V_m) was calculated by assuming a density of 1 kg/l of membrane.

In the membrane preparation used in Fig. 1, the membrane-spanning domain of band 3 denatured at 65.1°C in the presence of 0.104 mol octadecanoic acid per liter membranes, yielding a ΔT of −4.7°C.

A study of the effect of the membrane concentration of a fatty acid on the shift in the transition temperature of band 3 showed the relationship to be nearly linear, as seen for the cases of arachidonic, octadecanoic and dodecanoic acids in Fig. 2. This linearity was extremely useful, since it permitted the use of the proportionality constant, termed potency (see Eqn. 2), to quantitate the perturbing strength of each amphiphile in the membrane. This proportionality constant will be used throughout this report to compare the abilities of different amphiphiles at the same intramembrane concentration to destabilize the anion channel.

Table I shows that chain length has a significant influence on the intrinsic perturbing potency of a fatty acid. Thus, tetracosanoic acid (24:0) has a potency of only −13 C°·M^{-1} while dodecanoic acid displays a potency of −58 C°·M^{-1}. This general trend of reduced potency with increased chain length is maintained throughout the entire series from C_8 to C_{24}, with the most dramatic change occurring between the C_{20} and C_{22} species. Although all fatty acids from C_{12} to C_{24} partition essentially completely into the inside-out vesicles, the shorter chain amphiphiles clearly distinguish themselves as better destabilizers of band 3.

TABLE I

SHIFT IN THE DENATURATION TEMPERATURE OF BAND 3 BY FATTY ACIDS AND SDS

Fatty acid	N_m/V_m [a] (mM)	ΔT (C°)	P [b] (C°·M^{-1})
24:0	104	−1.3	−13
22:0	104	−1.6	−16
20:0	104	−4.3	−41
18:0	104	−4.7	−44 [c]
16:0	106	−5.1	−48
14:0	106	−5.5	−52
12:0	108	−6.1	−58 [d]
10:0	–	–	−65 [e]
8:0	–	–	−72 [f]
trans-18:1	106	−6.2	−58
cis-18:1	106	−7.0	−66
cis-18:2	106	−6.4	−60
cis-18:3	106	−6.8	−64
cis-20:4	106	−9.8	−93 [g]
SDS	135	−6.3	−47

[a] The membrane concentration of the fatty acid in the sample, calculated with the assumption that $N_t = N_m$, i.e. that all of the fatty acid is in the membrane. V_m was calculated from the dry weight of membranes by assuming a density of 1 kg/l.

[b] The potency of each fatty acid calculated from the data point given in this table via Eqn. 6 unless stated otherwise.

[c] Calculated from all data points shown in Fig. 2 by a least-squares analysis with Eqn. 6.

[d] Calculated from the data shown in Fig. 4 by a least-squares analysis with Eqn. 3.

[e] Calculated from the data shown in Fig. 3 by a least-squares analysis with Eqn. 3.

[f] Calculated from the data shown in Fig. 5 under additional assumptions on the buffer-membrane partition coefficient as described in the text.

[g] Calculated from the interpolated data point at $N_t/V_t = 100$ mM in Fig. 2 via Eqn. 6.

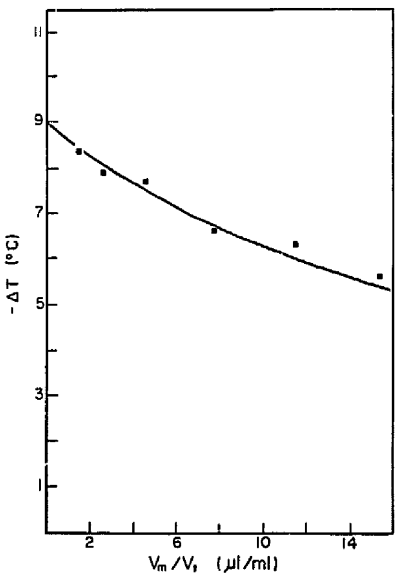

Fig. 3. Destabilization of band 3 (ΔT) by decanoic acid (3.08 mM sample concentration) at increasing inside-out vesicle concentrations. The solid line represents the nonlinear least-squares best fit of the data to Eqn. 3.

Destabilization of band 3 by saturated fatty acids of intermediate chain lengths

If the potency of decanoic acid is calculated assuming complete partitioning into the membrane, an anomalously low value is obtained (-30 $C^\circ \cdot M^{-1}$). We suspected that this was not due to a real drop in intrinsic potency but rather to the fact that the fatty acid partitions incompletely into the membrane. Therefore, we carefully measured the partition coefficients of all fatty acids up to C_{12}. Experimentally, this was accomplished by varying the amount of membranes in the sample, while keeping the total fatty acid concentration constant (Fig. 3). The partition coefficient was then calculated by fitting the data to Eqn. 3 using the method of nonlinear least squares. The resulting partition coefficient and potency value for decanoic acid were found to be 45 and -65 $C^\circ \cdot M^{-1}$, respectively. This potency value clearly fits much better with the dependence of potency on chain length seen for the other fatty acids in Table I.

Dodecanoic acid was also examined as described above and the resulting curve differed slightly from the expected curve for a fatty acid which partitions totally into the membrane. In order to more accurately measure the small fraction of dodecanoic acid which remains in the buffer, the plot shown in Fig. 4 was generated. In this study a constant amount of membranes plus dodecanoic acid was diluted with increasing volumes of buffer, and the resulting destabilization (ΔT) of the anion channel was measured. Nonlinear least-squares analysis of this data according to Eqn. 3 yielded a partition coefficient of 620 and a potency value of -58 $C^\circ \cdot M^{-1}$. Since an identical potency of -58 $C^\circ \cdot M^{-1}$ was determined under the assumption of complete partitioning, it is clear that the approximation was valid within the resolution of our calorimetric technique for the high inside-out vesicle concentrations used in the experiments shown in Table I and Fig. 2.

Destabilization of band 3 by short chain fatty acids

Octanoic acid behaved exactly as expected for a highly buffer-soluble amphiphile. When examined in the same way as decanoic acid, it was found that the destabilization of band 3 was independent of the amount of membrane in the buffer (Fig. 5). Therefore, the depression in the denaturation temperature of the anion channel, which as usual was

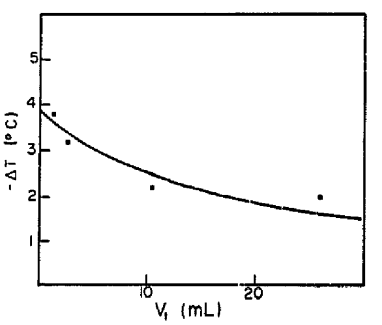

Fig. 4. Destabilization of band 3 (ΔT) by dodecanoic acid at increasing sample dilutions. The amount of fatty acid ($N_t = 2.0$ μmol) and inside-out vesicles ($V_m = 30$ μl) was held constant while the total sample volume V_t was varied. The solid line represents the nonlinear least-squares best fit of the data to Eqn. 3.

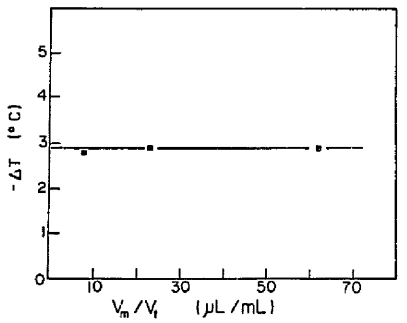

Fig. 5. Destabilization of band 3 (ΔT) by octanoic acid (10 mM total concentration) at increasing inside-out vesicle concentrations. The solid line represents the least-squares best fit of the data to Eqn. 3.

found to be a linear function of the membrane concentration of the drug, was also found to be directly proportional to the total concentration of the amphiphile in the suspension as given in Eqn. 5. Unfortunately, it was not possible to evaluate K_p and P independently from the data. Instead, only the product of the two parameters, the apparent potency (P_{app}) could be determined. This value was $-290\ C^\circ \cdot M^{-1}$. For hexanoic acid the partition coefficient was so low that shifts in the denaturation temperature of band 3 were observed only at unreasonably high buffer concentrations of the amphiphile.

Chain length profile of the intrinsic potency of saturated fatty acids

With the added potency values of the intermediate chain length fatty acids, it became of interest to plot the intrinsic potencies of the entire series of saturated fatty acids as a function of chain length. Fig. 6 shows that the relationship between these two parameters is nearly mono-exponential, with the obvious exception of the discontinuity between C_{20} and C_{22}. Importantly, the nearly linear logarithmic plot of P against chain length allows one to estimate the undetermined potency of octanoic acid. Extrapolation of the line in Fig. 6B to C_8 yields a potency value of $P = -72\ C^\circ \cdot M^{-1}$. Assuming this value to be accurate, K_p can be calculated from the product ($K_p/P = P_{app} = -290\ C^\circ \cdot M^{-1}$) reported above. The value of K_p so derived was $K_p = 4.0$. In order to check whether this way of estimating P and K_p is correct, a plot of log K_p versus chain length was constructed. The estimated partition coefficient of octanoic acid was found to fit perfectly into the expected linear relationship determined using K_p values of longer chain fatty acids as shown in Fig. 6B. Thus, the extrapolation of the P value made in Fig. 6B is probably valid.

Effect of configuration, number, and position of double bonds on the potency of fatty acids

The effect of unsaturated fatty acids of different structures on the destabilization of band 3 can be seen from Table I. When compared with octadecanoic acid, the C_{18} unsaturated fatty acids all appear to have increased potencies; however, the increase is small and the degree of unsaturation seems to exert little effect. The small magnitude of the *cis/trans* effect is also very surprising in view

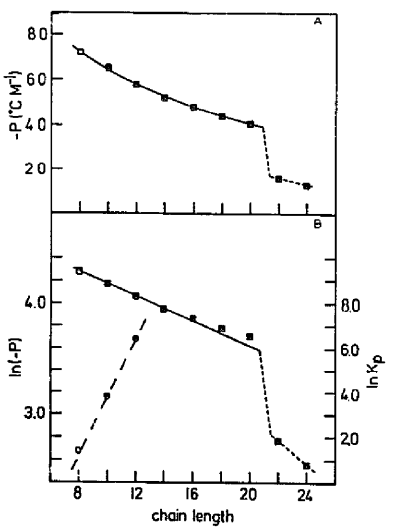

Fig. 6. Chain length dependence of the intrinsic potency (P) of saturated fatty acids in perturbing band 3 stability. (A) All P values were calculated from experimental data, except for octanoic acid. (B) Semilogarithmic plot of the intrinsic potency (P) values (———, ■) and of the partition coefficients (K_p) of fatty acid partitioning into erythrocyte membrane inside-out vesicles at the denaturation temperature of band 3, i.e. between 65 °C and 70 °C (- - - - -, ●). The intrinsic potency (□) for octanoic acid was extrapolated in this plot under the assumption of a mono-exponential chain length dependence of P; the partition coefficient was determined as outline in the test.

of the prediction of others on this matter [7,48]. Only arachidonic acid appears to be significantly more potent than the corresponding saturated fatty acid. Interestingly, arachidonic acid was also the only fatty acid to significantly reduce the enthalpy of the band 3 transition.

Destabilization of band 3 by fatty alcohols

When octadecanol was incubated with erythrocyte membrane inside-out vesicles, an indication of the same phase separation as reported by Grunze et al. [12] was observed. Thus, in addition to the denaturation transition of band 3, other endotherms were observed for presumably octadecanol-rich lipid phases in the membrane. Furthermore, a transition near the melting temperature of pure octadecanol also appeared. In order to guarantee an even dispersion of octadecanol in the membrane lipid phase, we found it necessary to introduce a 'catalyst', octadecanoic acid, to promote complete equilibration of the alcohol in the membrane. Thus, in the presence of a 4-fold smaller or an equal concentration of octadecanoic acid, these extraneous endotherms were abolished. However, in order to evaluate the effect of octadecanol alone, we had to treat the effect of the octadecanol/octadecanoic acid mixture as though the perturbations by the two amphiphiles were additive. Unfortunately, this assumption is clearly

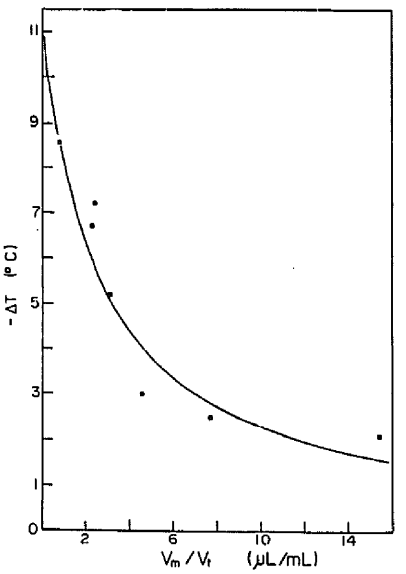

Fig. 7. Destabilization (ΔT) of band 3 by octanol (1.54 mM total concentration) in the presence of ethanol (77 mM total concentration) at increasing inside-out vesicle concentrations. The destabilization by 77 mM ethanol (−0.5 C°) was subtracted from all experimentally determined ΔT values. The solid line represents the nonlinear least-squares best fit of data to Eqn. 3.

TABLE II

EFFECT OF OCTADECANOL AND OCTADECYLAMINE ON THE THERMAL STABILITY OF THE MEMBRANE-SPANNING DOMAIN OF BAND 3 [a]

Perturbing amphiphile	Dispersing agent	ΔT (C°)	Excess P [b] (C°·M^{-1})
None	0.98 μmol stearic acid	−1.2	
3.94 μmol octadecanol	0.98 μmol stearic acid	−2.5	−12
None	3.94 μmol stearic acid	−4.7 [c]	
3.94 μmol octadecanol	3.94 μmol stearic acid	−4.7	0
None	0.79 μmol DTAB	−0.2	
3.94 μmol octadecylamine	0.79 μmol DTAB	−0.8	negl. [d]
1.97 μmol octadecylamine	0.39 μmol DTAB	−0.6	negl. [d]

[a] In all experiments 36.4 mg inside-out vesicles were incubated with the amphiphile in a volume of 4.50 ml, centrifuged at room temperature and resuspended after removal of 3.20 ml of supernatant.

[b] Excess potency was calculated under the assumption of complete partitioning after subtracting the ΔT effect of the dispersing agent alone from the overall effect of perturbing amphiphile plus dispersing agent.

[c] Obtained by interpolation in Fig. 2.

[d] Negligible effect.

inadequate at high acid/alcohol ratios, where octadecanol makes no additional contribution to perturbation by octadecanoic acid (Table II). However, at low acid/alcohol ratios, an additional destabilization of band 3 was observed upon addition of octadecanol. Based on this additional perturbation we estimate the potency of octadecanol to be approx. -12 C° · M^{-1}, i.e. a value which is nearly four times smaller than that of the corresponding fatty acid.

For ease of handling, octanol was added to the inside-out vesicles from an ethanol stock solution such that the final ethanol concentration was always 77 mM. This concentration of ethanol produced a small shift of -0.5 C° in the denaturation temperature of band 3 which was subtracted from all values in the presence of octanol. As can be seen from Fig. 7, the partitioning behaviour of octanol is similar to that of the moderately partitioning fatty acid, decanoic acid. Based on a nonlinear least-squares analysis of the data according to Eqn. 3, K_p and P values of 400 and -18.5 C° · M^{-1}, respectively, were determined.

Destabilization of band 3 by fatty amines

As with octadecanol, it was difficult to dissolve octadecanamine into the membrane without the help of an exogenous 'catalyst'. Because anionic detergents are known to precipitate upon mixing with alkylamines [49], DTAB, a cationic detergent, was used as such a 'catalyst'. The effects of DTAB alone and of DTAB in conjunction with octadecanamine are given in Table II. The data indicate that the long chain fatty amine exerts little influence on the stability of the membrane-spanning domain of band 3. DTAB also appears to exert little effect at the concentrations employed in this study.

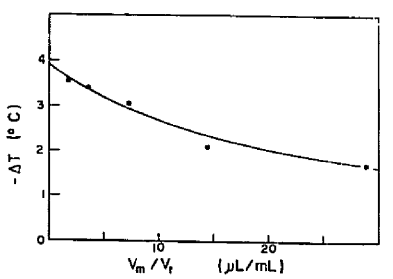

Fig. 8. Destabilization of band 3 ($-\Delta T$) by octanamine (3.73 mM total concentration) at increasing inside-out vesicle concentrations. The solid line represents the nonlinear least-squares best fit of the data to Eqn. 3.

Octanamine was then introduced directly into the membranes from an aqueous stock solution of its hydrochloride. As had been found for octanol and decanoic acid, octanamine was observed to partition only moderately into the membrane. Based on the data in Fig. 8, a partition coefficient and a potency value of 46.5 and -22.5 C° · M^{-1}, respectively, were calculated.

Discussion

The influence of lipid-soluble drugs on the properties of ion channels and other related membrane proteins has received considerable attention in recent years [1–26]. While these studies have predominantly concerned the effects of an amphiphile on the functional characteristics of a membrane transport protein, our investigation has focused on the influence of amphiphiles on the structural properties of a transport protein. We have chosen to monitor the structural destabilization of the erythrocyte anion exchanger with sensitive DSC because this method provides a more direct indication of the degree of structural distortion than an assay of transport kinetics. For model membrane perturbants, we have selected the simplest and most generic forms of an amphiphilic drug, i.e. various monofunctional polar head groups linked to a series of alkyl tails.

It is clear from our data that the net destabilizing potency of an amphiphile (P_{app} in the case of short chain amphiphiles) derives from two major contributions: (i) the drug's membrane-buffer partition coefficient (K_p), and (ii) the intrinsic perturbing potency (P) of the drug once present in the membrane. Based on the data in Table III, the net perturbing capacity of short chain alcohols stems mainly from the former contribution, while the apparent potency of short chain fatty acids is strongly determined by the latter.

Our ability to distinguish and selectively examine intrinsic rather than apparent or net potencies allows us to begin to evaluate the structural features of a membrane perturbant which render it most destabilizing to its target protein.

TABLE III

THE ROLE OF THE HEAD GROUP IN DETERMINING THE PARTITION COEFFICIENT (K_p), THE INTRINSIC POTENCY (P) AND THE APPARENT POTENCY (P_{app}) OF OCTANE DERIVATIVES

Amphiphile	K_p	$-P$ ($C^\circ \cdot M^{-1}$)	$-P_{app}$ ($C^\circ \cdot M^{-1}$)
$C_8H_{17}OH$	400.0	18.5	7400
$C_8H_{17}NH_2$	46.5	22.5	1046
$C_8H_{17}COOH$	12.1 [a]	69.0 [a]	835

[a] Determined by interpolation in Fig. 6.

First, at least for the case of band 3, short chain fatty acids are about three times more intrinsically potent than similar chain length fatty alcohols or fatty amines (Table III). However, before immediately attributing this difference to the net charge on the amphiphile, it is important to note that pK_a of both fatty acids [50,51] and hydrophobic amines [52], when membrane-associated, lies in the physiological pH range; i.e. a substantial fraction of each amphiphile may be in its uncharged state. Therefore, it is especially interesting to compare the intrinsic potencies of the permanently charged species, SDS and DTAB. SDS exhibited an intrinsic potency of -47 $C(^\circ \cdot M^{-1}$, a value similar to that of dodecanoic acid (-58 $C^\circ \cdot M^{-1}$), while DTAB displayed a corresponding potency which was hardly measurable. Thus, it can be concluded that the ionized form of the fatty acid contributes significantly to the destabilizing capacity of this amphiphile, while the charged form of the amine is probably inactive. This ineffectiveness of cationic amines has also been previously documented for the tertiary amine, lidocaine, which was only able to perturb band 3 in its neutral state [45]. The measurable potency of octylamine reported in Table III, therefore, must be attributed to the presence of the uncharged species in the membrane. In summary, it is not unlikely that the enhanced potency of the fatty acids over corresponding amines and alcohols is due largely to their added ability to disrupt band 3 structure in their charged or ionized state.

The findings that the intrinsic potencies of C_{18} fatty acids change little upon unsaturation and even less upon *cis/trans* isomerism (Table I) seem to suggest that double bond stereochemistry and abundance are relatively unimportant in determining perturbing potency. These results are of particular interest because others have found a fundamental distinction between the perturbing effects of *cis*-unsaturated fatty acids on the one hand, and saturated or *trans*-unsaturated fatty acids on the other [7,48]. Based on these differences, they have proposed a lipid-oriented hypothesis of fatty acid action, where *cis*-unsaturated fatty acids are thought to perturb those proteins situated in rigid lipid environments, while saturated/*trans*-unsaturated fatty acids alter proteins located in fluid lipid environments. The absence of a strong distinction between these two classes of fatty acids in our study suggests either that the postulated sensitivity of the lipid environment of band 3 to fatty acid unsaturation is lost in the calorimetric experiment or that band 3 represents an example of a protein which does not support the above hypothesis. Our earlier studies demonstrating a quantitative relationship between the shift in denaturation temperature (ΔT) of band 3 and ion flux rates measured at 37°C suggest that the calorimetric data are quite relevant to the functional state of band 3 [45]. Thus it is conceivable that band 3, like the sarcoplasmic reticulum ATPase [25], represents an exception to the hypothesis cited above. The unusual potency of arachidonic acid, which could be interpreted in terms of the above lipid domain hypothesis, might alternatively derive its potency from the unique position of the first double bond near the head group of the fatty acid.

The influence of alkyl chain length on intrinsic potency is surprisingly opposite to that generally seen in similar studies of the membrane-perturbing capacities of homologous amphiphiles. In these studies an increase in the hydrophobicity of a lipophilic drug was commonly found to enhance its capacity as a membrane modifier. In contrast, we have found that addition of methylenes to the alkyl chains of fatty amines, fatty alcohols and fatty acids invariably diminishes their intrinsic potencies as perturbants. Thus, while increased hydrophobicity may cause more drug to enter the membrane where it is active, the added non-polarity simultaneously reduces the potency of the drug once in the membrane. This inverse relationship between intrinsic potency and hydrophobicity may

derive from a preference of the more hydrophobic species for association with lipid rather than protein domains within the membrane. A quantitative treatment of a physical model describing this behaviour is provided in the accompanying paper.

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