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MODULATION OF PHOSPHOLIPID TRANSFER PROTEIN ACTIVITY

INHIBITION BY LOCAL ANESTHETICS

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The transfer of phospholipid molecules between biological and synthetic membranes is facilitated by the presence of soluble catalytic proteins, such as those isolated from bovine brain which interacts with phosphatidylinositol and phosphatidylcholine and from bovine liver which is specific for phosphatidylcholine. A series of tertiary amine local anesthetics decreases the rates of protein-catalyzed phospholipid transfer. The potency of inhibition is dibucaine > tetracaine > lidocaine > procaine, an order which is compared with and identical to those for a wide variety of anesthetic-dependent membrane phenomena. Half-maximal inhibition of phosphatidylinositol transfer by dibucaine occurs at a concentration of 0.18 mM, significantly lower than the concentration of 1.9 mM required for half-maximal inhibition of phosphatidylcholine transfer activity of the brain protein. Comparable inhibition of liver protein phosphatidylcholine transfer activity is observed at 1.6 mM dibucaine. For activity measurements performed at different pH, dibucaine is more potent at the lower pH values which favor the equilibrium toward the charged molecular species. With membranes containing increasing molar proportions of phosphatidate, dibucaine is increasingly more potent. No effect of Ca^{2+} on the control transfer activity or the inhibitory action of dibucaine is noted. These results are discussed in terms of the formation of specific phosphatidylinositol or phosphatidylcholine complexes with the amphiphilic anesthetics in the membrane bilayer.

Introduction

Phospholipid transfer between membranes is catalyzed by a family of soluble, low-molecular-weight proteins. These phospholipid transfer proteins are eukaryotic in origin, generally specific for certain classes of phospholipid molecules, and capable of interacting with biological and artificial membranes alike. Rates of protein-catalyzed intermembrane transfer have been shown to be sensitive to the polar and nonpolar lipid composition of the membranes and to the ionic properties of the medium, suggesting that both hydrophilic and hydrophobic forces play significant roles in the overall process [1–4].

It was observed several years ago in this laboratory that stearylamine had a profound effect on the activity of bovine brain phosphatidylinositol transfer protein [3]. As increasing proportions of the alkylamine were incorporated into egg phosphatidylcholine vesicles, the membranes became less competent as acceptors of phospholipid from rat liver microsomes. Indeed, at the highest concentration (40 mol%) of stearylamine less than 10% of the original activity remained.

In the present communication, we have extended this investigation of amphiphilic amines.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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The kinetics of phospholipid transfer between microsomes and vesicles and between two populations of vesicles are determined for phosphatidylinositol transfer protein in the presence of a series of local anesthetics: dibucaine, tetracaine, lidocaine and procaine. From this series, dibucaine was selected for further studies dealing with membrane lipid composition, pH and divalent cations. The precise mechanism by which these compounds are able to block nerve transmission remains to be established. Morever, many other biological and physical properties of membranes are modulated by local anesthetics, properties which include changes in Na^+ and K^+ permeability, displacement of bound Ca^{2+}, alteration of membrane fluidity, influence on phospholipid organization, and modification of phospholipase A_2 activity [5–8].

Materials and Methods

Lipids

Phosphatidylcholine, sodium phosphatidate, and phosphatidylinositol were isolated from natural sources, as previously described [3]. Egg yolk phosphatidylcholine, labelled with [9,10 – ^{3}H]oleic acid at the *sn*-2 position, and rat liver microsomal phosphatidylinositol, labelled with *myo*-[2 – ^{3}H]inositol, were synthesized biochemically [9,10]. The above radioisotopes and cholesteryl [1 – ^{14}C]oleate were obtained from Amersham Corporation, Arlington Heights, IL, or New England Nuclear, Boston, MA. *N*-Palmitoyllactosylceramide was a product of Miles Laboratories, Elkhart, IN. All lipids were pure on thin-layer chromatographic analysis and were stored in chloroform/methanol (2:1, v/v) under N_2 at −20°C.

Proteins

Phosphatidylinositol transfer protein was isolated from fresh bovine cerebral cortex and purified to chromatographic homogeneity [9]. Phosphatidylcholine transfer protein was obtained from fresh bovine liver [11]. Both proteins were stored in 10 mM Hepes/50 mM NaCl/1 mM Na_2EDTA (pH 7.4) containing glycerol (50%, v/v) at −20°C. The galactose-specific agglutinin was purified from raw castor beans (*Ricinus communis*) through the anion-exchange chromatography stage [12]. Fatty acid-free bovine plasma albumin was purchased from Miles Laboratories.

Phospholipid transfer activity measurements

Phosphatidylinositol transfer from rat liver microsome donor membranes to single bilayer vesicle acceptor membranes (microsome-vesicle assay) was measured as outlined by Helmkamp et al. [9]. Phosphatidylcholine and phosphatidylinositol transfers between two single bilayer vesicle populations, one of which contained 8–10 mol% lactosylceramide (vesicle-vesicle assay), were carried out according to Kasper and Helmkamp [13]. Microsomes and vesicles were prepared as previously outlined [9,11]. The buffer throughout was 50 mM Hepes/50 mM NaCl/1 mM Na_2EDTA (pH 7.4), unless otherwise specified. Acceptor vesicles were routinely added last to the assay mixture. Incubations were performed at 37°C for 30 min in triplicate; corrections were made for spontaneous transfer in the absence of transfer proteins. Results are expressed as nmol phospholipid transferred per hour (mean ± S.D.). Acceptor vesicles always contained a trace quantity (less than 0.5 mol%) of cholesteryl [1 – ^{14}C]oleate in order to monitor recovery of these particles at the conclusion of an assay.

Other materials

Sepharose 4B was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Dibucaine hydrochloride, tetracaine hydrochloride, lidocaine, and procaine hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO.

Results

Inhibition of phosphatidylinositol transfer

A series of amphiphilic amines which function as local anesthetics was investigated for effects on protein-catalyzed phospholipid transfer. In the transfer of phosphatidylinositol from rat liver microsomes to single bilayer vesicles, all of the local anesthetics tested were inhibitory (Fig. 1). It was apparent, however, that the concentration in the bulk phase necessary to modulate transfer activity differed greatly according to the specific amine. Dibucaine was the most potent inhibitor, followed by tetracaine, lidocaine and procaine in that order.

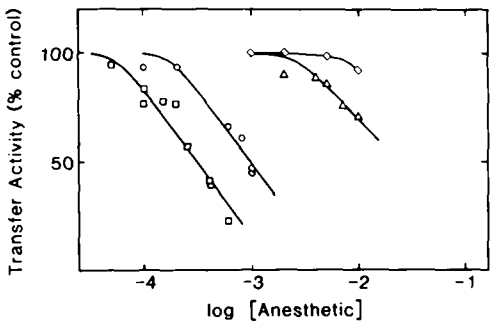

Figure 1. Local anesthetic inhibition of phosphatidylinositol transfer from microsomes to vesicles. Assays consisted of rat liver microsomes (1.0 mg protein, 34 nmol phosphatidyl[^{3}H]inositol, 775 dpm·nmol^{-1}), single bilayer vesicles (1.0 μmol egg phosphatidylcholine containing 5 mol% egg phosphatidate), and the indicated concentration of amine, expressed as M, in a total volume of 2.5 ml. These were incubated at 37°C for 30 min in the presence and absence of 0.62 μg bovine brain phosphatidylinositol transfer protein. The transfer activity in the presence of protein alone was 5.98 nmol per 30 min; the activity in the absence of protein was 0.68 nmol per 30 min. The points represent means of triplicate determinations, with blanks subtracted. The following amines were tested: dibucaine (□), tetracaine (○), lidocaine (△) and procaine (◇).

The concentrations which elicited a 50% inhibition of transfer activity were 0.30 mM dibucaine, 0.96 mM tetracaine, 24.5 mM lidocaine and more than 75 mM procaine. In these experiments, the control level of transfer activity was 17.6 ± 0.7% of the microsomal pool per 30 min, or 5.98 nmol · h^{-1}. Incubations carried out in the absence of transfer protein and amine gave transfers of 0.68 nmol · h^{-1}; in the presence of the higher concentrations of the various amines, these blanc values increased to 1.70 nmol · h^{-1}. The marked sensitivity of phosphatidylinositol transfer protein to local anesthetics prompted further studies toward elucidating the inhibitory mechanism, using dibucaine as the model compound.

Effect of vesicle composition

The incorporation of a small amount of an anionic phospholipid, such as phosphatidate, serves to facilitate single bilayer vesicle formation. At the same time, the bilayers acquire a net negative surface charge and might be expected to interact in a specific manner with the cationic anesthetic amines. Therefore, acceptor vesicles were constructed to contain 2, 5 or 10 mol% egg phosphatidate, the remainder of the lipid being egg phosphatidylcholine. As acceptors of microsomal phosphatidylcholine, no difference was observed for the 2, 5 and 10 mol% phosphatidate vesicles in the absence of dibucaine, a finding in agreement with earlier observations [3]. However, the 2 mol% phosphatidate vesicles were less sensitive to the inhibitory action of dibucaine than the 5 and 10 mol% species, which were essentially identical (Fig. 2). These data suggest that the maximal effect of dibucaine is at least partially dependent on membrane phospholipid composition, to the extent that ionic interactions between phospholipid and amine are significant.

Kinetic analysis of dibucaine inhibition

In order to describe further the mode of inhibition of phospholipid transfer by anesthetic amines, a kinetic analysis was carried out at a constant vesicle concentration and a variable microsome concentration. With increasing levels of dibucaine in the assay system, linear double-reciprocal plots could be constructed (Fig. 3). These lines intersected on the abscissa, consistent with a non-competitive inhibitory mechanism. The calculated inhibitor constant, K_i, was 0.10 mM, in good agreement with the half-maximal value obtained from the dose-response curve (Fig. 1).

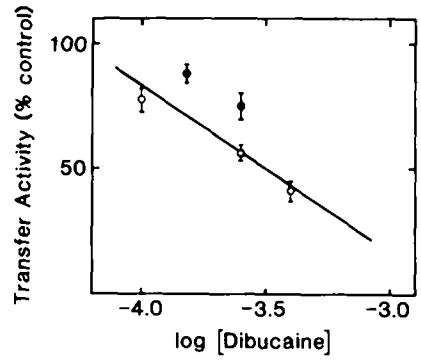

Fig. 2. Variation in phosphatidate content of acceptor vesicles. Phosphatidylinositol transfer from microsomes to vesicles was carried out as described in Fig. 1 in the absence (control) or presence of dibucaine. The points represent means ± S.D. for 4–6 determinations. Acceptor vesicles contained the following molar proportion of phosphatidate: 2% (●), 5% (from Fig. 2, least-squares fit; correlation coefficient, 0.94), and 10% (○). The blank-corrected control transfer activities were 4.60 nmol per 30 min at 2 mol% phosphatidate, 5.71 at 5 mol% and 6.90 at 10 mol%. Dibucaine concentration is expressed as M.

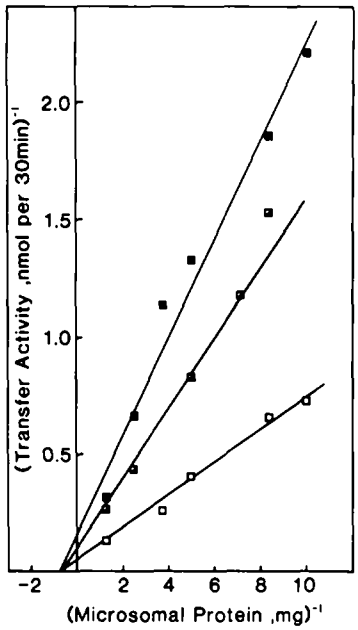

Fig. 3. Kinetic analysis of dibucaine inhibition of phosphatidylinositol transfer. Microsome-vesicle assays were performed as outlined in Fig. 1, using different amount of donor microsomes. The results are depicted in a double-reciprocal plot. The data were fitted by least-squares linear regressions, the correlation coefficients being in the range 0.95 to 0.98. Dibucaine was present at the following concentrations: none (□), 0.1 mM (◪) and 0.3 mM (■).

Interaction between dibucaine and membranes

Dibucaine is capable of associating with both microsome and vesicle membranes in the assay system. Each membrane was treated independently with 1 mM dibucaine for 30 min at 37°C and then separated from excess amine. Microsomes were pelleted at 200000 × *g* for 45 min at 4°C and resuspended in dibucaine-free buffer; vesicles were chromatographed on a column of Sepharose 4B. Control membranes were carried through the entire procedure. The anesthetic-treated and control vesicles displayed identical elution profiles. When the dibucaine-treated and control membranes were compared in microsome-vesicle assays, no differences were observed. These results suggest that the dibucaine-membrane interaction is a reversible one; moreover, dibucaine forms a long-term, stable complex with neither microsome nor vesicle.

Effect of pH on dibucaine inhibition

The transfer of phosphatidylinositol between two populations of single bilayer vesicles was investigated at pH 6.8, 7.4 and 8.2. In the control assay system, transfer activities were measured to be 5.0 ± 0.2 nmol · h^{-1} at pH 6.8, 4.3 ± 0.4 nmol · h^{-1} at pH 7.4 and 3.8 ± 0.3 nmol · h^{-1} at pH 8.2. In the presence of dibucaine transfer activity was again inhibited, with half-maximal concentrations of 0.18 mM at pH 6.8 and 7.4 and 0.22 mM at pH 8.2. The value at pH 7.4 for the vesicle-vesicle system is slightly less than that noted for the microsome-vesicle system and could be indicative of a somewhat greater partition coefficient of dibucaine for the artificial phospholipid bilayers. The trend toward higher half-maximal concentrations under more alkaline conditions may be interpreted to mean that the protonated form of the anesthetic is the more potent inhibitor. With a pK_a of 8.5, it was impractical to evaluate directly the inhibitory activity of the uncharged dibucaine species. It should be noted that the contribution of amphiphile charge to various amphiphile-membrane interactions remains an unresolved issue [14–16].

Effect of Ca^{2+} on dibucaine inhibition

Phosphatidylinositol transfer was measured between single bilayer vesicles in the usual buffer system, but lacking EDTA as a divalent chelation agent. The addition of Ca^{2+} in the range 50 μM–2 mM had no effect on the rate of phospholipid transfer or on the extent of acceptor vesicle recovery. Furthermore, the inhibition of transfer by 0.4 mM dibucaine was essentially identical in the absence on presence of 2 mM Ca^{2+}. Such observations were made with acceptor vesicles containing 10 mol% phosphatidylinositol or 20 mol% phosphatidylserine, both of which should exhibit high affinitites for metal ions and amphiphilic amines [7]. Direct binding of Ca^{2+} to phosphatidylcholine would be insignificant under these conditions [15]. These results demonstrate that Ca^{2+} and probably other divalent ions play no role in the protein-catalyzed transfer of phospholipid or in the inhibition of this process by compounds known to displace cations from membrane surfaces. Another important conclusion to be drawn from these results is the unlikely participation of non-bilayer

membrane phases, such as the hexagonal phase, in the mechanism of local anesthetic inhibition [18,19]. While polymorphic phases are induced by dibucaine or Ca^{2+}, only the former can effectively modulate phospholipid transfer protein activity.

Inhibition of phosphatidylcholine transfer

Using the vesicle-vesicle assay system the intermembrane flux of phosphatidylcholine was measured in the presence of bovine liver phosphatidylcholine transfer protein and brain phosphatidylinositol transfer protein, the latter protein being active toward phosphatidylcholine as well as phosphatidylinositol. As can be seen in Fig. 4, phosphatidylcholine transfer was sensitive to dibucaine, but with dose-response relationships which were unique to the specific transfer protein. The half-maximal concentrations for the inhibition of phosphatidylcholine transfer by dibucaine were 1.6 mM with phosphatidylcholine transfer protein and 1.9 mM with phosphatidylinositol transfer protein. The shapes of the dose-response curves differed markedly.

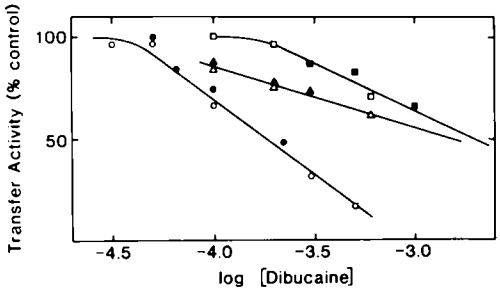

Fig. 4. Effect of dibucaine on transfer of phospholipids between single bilayer vesicles. Assays were performed in a total volume of 0.5 ml and consisted of donor vesicles (200 nmol egg phosphatidylcholine with 10 mol% phosphatidylinositol, using either [^{3}H]phosphatidylcholine, 248 dpm · nmol^{-1}, or [^{3}H]phosphatidylinositol, 1030 dpm · nmol^{-1}), acceptor vesicles (200 nmol egg phosphatidylcholine containing 5 mol% egg phosphatidate), 1 mg fatty acid free bovine plasma albumin, and the indicated concentration of dibucaine (M). The open data points represent experiments in which 8 mol% lactosylceramide was added to the donor vesicles; the closed data points represent 8 mol% lactosylceramide in the acceptor vesicles. The following phospholipids, proteins and control activities were measured: phosphatidylinositol in presence of 0.12 μg bovine brain phosphatidylinositol transfer protein, 2.16 nmol per 30 min (○, ●); phosphatidylcholine in presence of 1.25 μg bovine brain phosphatidylinositol transfer protein, 21.4 nmol per 30 min (□, ■); phosphatidylcholine in presence of 0.59 μg bovine liver phosphatidylcholine transfer protein 44.3 nmol per 30 min (△, ▲).

Of greater interest, however, was the effect of dibucaine on the activities of phosphatidylinositol transfer protein (Fig. 4). Protein-catalyzed transfer of phosphatidylinositol was significantly more sensitive to the presence of the local anesthetic in comparison to the transfer of phosphatidylcholine, the half-maximal concentrations differing by an order of magnitude. To our knowledge, this is the first demonstration of a selective modulation of the multiple catalytic properties of this bovine protein.

Discussion

The tertiary amine anesthetics are typical amphiphilic molecules which at physiological pH bind to phospholipid membranes through hydrophobic and electrostatic interactions. The hydrophobic interactions involve the aliphatic fatty acyl moieties of the phospholipids and the aromatic ring systems of the anesthetics; these interactions have been shown to be sensitive to fatty acid composition, temperature, and the phase behavior of the membrane. The electrostatic interactions between the charged polar head groups of the phospholipids and the (protonated) amine functions of the anesthetics are a function of pH and ionic strength [21–23]. Not surprisingly, membranes composed solely of acidic phospholipids or with a high proportion of these lipids are significantly more affected by local anesthetics than neutral phosphatidylcholine membranes [17]. Current evidence supports a model of interaction between anesthetic and membrane phospholipid molecules based upon the hydrophobic aromatic nucleus of the anesthetic penetrating well into the internal fatty acyl chain region of the bilayer and the charged, alkyl-substituted amine moiety associating with the external head-group region [5,22,23].

Local anesthetics were shown in the present investigation to decrease the rate of protein-catalyzed phospholipid transfer from microsomes to single bilayer vesicles and between single bilayer vesicles. The activities of phosphatidylcholine transfer protein, isolated from bovine liver, and phosphatidylinositol transfer protein, isolated from bovine brain, are inhibited by these tertiary amines.

The potency of inhibition, determined by the concentration required for a 50% loss of transfer activity, decreased in the order; dibucaine > tetracaine > lidocaine > procaine. This same order of potency has been observed for a wide variety of pharmacological and biochemical phenomena, including blockage of nerve transmission [24], penetration into phospholipid monolayers and bilayers [5,14,25], reduction of the zeta potential [26], displacement of membrane-bound Ca^{2+} [27], increased permeability to sodium ion [6], and inhibition of phospholipase A_2 activity [7,8,28]. A comparison of these effects is summarized in Table I. The orders of potency (Table I) exhibit a remarkable correlation with the partition coefficients (total lipid phase concentration/total aqueous phase concentration) for these compounds in egg phosphatidylcholine multilamellar liposomes: dibucaine, 235; tetracaine, 22; procaine, 2 [14,17].

Also of particular relevance to the present work is the recent observation that the partition coefficient for chlorpromazine, another membrane-active amphiphile, was 1490 with large (500 nm diameter) vesicles and 1350 with small (25 nm diameter) vesicles [16]. We, therefore, feel confident in extrapolating many of the above-mentioned results obtained with a variety of lipid membranes to the small single bilayer vesicles employed in our phospholipid transfer measurements.

The inhibition of phosphatidylinositol transfer protein activity by dibucaine yielded a dramatic difference between phosphatidylinositol and phosphatidylcholine transfer, the former being more sensitive to the presence of the local anesthetic. Since the chemical compositions of the donor vesicles in these experiments were identical except for the molecular placement of the radioisotopic label, the selective action of dibucaine could represent a difference in the interaction between the amine and the two phospholipids. Indeed, a stronger interaction should exist between dibucaine and phosphatidylinositol, based on electrostatic forces alone. One consequence of a dibucaine-membrane interaction could be the formation of distinct anionic and zwitterionic phospholipid phases in the bilayer. Like the donor membranes, acceptor vesicles also contain an anionic lipid, phosphatidate. At the low levels of these phospholipids (10 mol% phosphatidylinositol, 1–20 mol% phosphatidate) used in these studies, such lateral phase separations would be unlikely. Papahadjopoulos and coworkers [17] suggested that a minimum level of 33 mol% phosphatidylserine in phosphatidylcholine membranes in required for a close-packed arrangement of the

TABLE I

COMPARISON OF POTENCIES OF LOCAL ANESTHETICS ON BIOLOGICAL AND PHOSPHOLIPID MEMBRANES

Activity	Membrane system	Local anesthetic concentration (mM)				Ref.
		Procaine	Lidocaine	Tetracaine	Dibucaine	
Inhibition of phosphatidylinositol transfer (50%)	vesicle, microsome	>75	24.5	0.96	0.30	
Inhibition of phospholipase A_2 activity (50%)	monolayer	3.8	2.8	0.52	0.15	28
Reduction of phase transition cooperative unit (50%)	liposomes	8.7	0.80	0.75	0.30	22
Displacement of bound Ca^{2+} (half maximal)	human erythrocytes	38	14.1	1.7	0.4	27
Reduction of Na^+ self-diffusion rate (50%)	vesicles	7.0	1.9	0.16	0.04	6
Reduction in zeta potential (5 mV)	liposomes	5.0	–	0.1	0.02	26
Blocking of nerve conduction (minimum)	frog sciatic nerve	4.6	2.6	0.01	0.005	24

anionic molecules. They further reported that neither 0.4 mM dibucaine nor 0.6 mM Ca^{2+} significantly altered the thermotropic properties of mixed phosphatidylserine-phosphatidylcholine (20:80, mol%) vesicles. Such an ion-pair complex in the bilayer could impede the translocation of phosphatidylinositol from the lipid bilayer to the transfer protein. The formation of a diffusable protein-phospholipid complex is a critical step in the transfer process and has been documented by kinetic and physical evidence [1,2,29]. The zwitterionic character of the phosphatidylcholine molecule should result in decreased attraction to a cationic anesthetic molecule. Higher bulk concentrations and, in turn, membrane concentrations would be needed to effect the same degree of inhibition. Correspondingly, a 10-fold difference in the half-maximal concentration of dibucaine for the inhibition of phosphatidylcholine and phosphatidylinositol transfer was noted. On the other hand, nearly identical concentrations of dibucaine inhibited phosphatidylinositol transfer from two dissimilar donor membranes, microsomes and vesicles.

Local anesthetics exhibit effects on both the synthesis and degradation of phospholipids. In general, these effects are directed toward hydrolytic enzymes, such as phosphatidate phosphohydrolase and phospholipase A_2 [7,8,28,30], which are inhibited by these compounds in the decreasing order of potency: dibucaine > tetracaine > lidocaine > procaine. Other steps in phospholipid metabolism sensitive to inhibition by local anesthetics include group transfer reactions for cytidylyl, fatty acyl and inositol moieties [31–33]. The mechanism through which local anesthetics operate on these metabolic activities is not well understood. The present results suggest yet another potential site of action of amphiphilic amines in biological systems, namely the protein-catalyzed intracellular transfer of phospholipids among membranes. A principal common feature in all sensitive reactions is a phospholipid membrane or substrate molecule. Interestingly, the protein catalyst may be an integral membrane enzyme, such as phosphatidate phosphohydrolase, or a more loosely membrane-associated protein, such as phospholipase A_2 or phospholipid transfer protein. Ionic and hydrophobic interactions between the cationic amphiphile and a phospholipid molecule could explain many of the observations. We feel that the inhibition of phospholipid transfer described here is best accounted for by a similar association between amine amphiphile and phospholipid. Less likely, but still not ruled out, is a direct interaction of these amphiphilic amines with the phospholipid transfer proteins.

Acknowledgements

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