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Is calmodulin inhibition involved in shape transformations induced by amphiphiles in erythrocytes?

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Potent antihaemolytic and shape transforming amphiphilic compounds were studied for their ability to inhibit calmodulin-activated phosphodiesterase activity. Some echinocytogenic and stomatocytogenic amphiphiles were potent calmodulin inhibitors. The most potent echinocytogenic and stomatocytogenic amphiphiles, however, had no or only weak inhibitory effect. Our results show that there is no causal relationship between the ability of amphiphiles to induce antihaemolysis or shape transformations in erythrocytes and their ability to inhibit calmodulin-activated phosphodiesterase activity, and it is suggested that calmodulin is not involved in shape transformations induced by amphiphiles.

Introduction

The normal discoid shape of human erythrocytes can be transformed by a variety of amphiphilic compounds to cupped (stomatocytes) or spiculated (echinocytes) forms [1–3]. A common property of shape transforming amphiphilic compounds is that they also have an antihaemolytic effect (protection against hypotonic haemolysis) [2,3]. Several authors have shown that there is an association between the ability to induce antihaemolysis and to inhibit calmodulin-activated functions in a variety of drugs [4,5], and it has been proposed that there may be a causal relationship between antihaemolysis and calmodulin inhibition [5]. The possible relationship between antihaemolysis, shape transforming ability and inhibition of calmodulin was further stressed by Nelson et al. [6]. They examined about 40 drugs for their ability to induce stomatocytes and to inhibit calmodulin-activated functions and found a strong correlation between these two properties. They proposed that calmodulin, through an interaction with membrane-cytoskeletal proteins, participates in the regulation of erythrocyte shape and that inhibition of calmodulin results in a transformation of the discoid shape to stomatocytes. A direct binding of calmodulin to spectrin or a modulation of spectrin phosphorylation via a calmodulin-dependent spectrin kinase were by the authors considered to be possible mechanisms whereby calmodulin could affect cell shape. It has been shown that spectrin is phosphorylated by a calmodulin-dependent kinase [7] and that calmodulin binds to human spectrin [8,9]. Recently it was shown that calmodulin, through an interaction with cytoskeletal proteins, affects membrane stiffness in erythrocytes [10]. It thus seems likely that calmodulin may affect the state of the erythrocyte cytoskeletal network but whether or not calmodulin participates in the regulation of erythrocyte shape is still an open question.

The present study was undertaken in attempt

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to test the proposals that the ability to induce antihaemolysis [5] and stomatocytogenic shape alterations [6] is related to an ability to inhibit calmodulin-activated functions. For this purpose we selected some potent antihaemolytic and shape transforming amphiphilic compounds and studied their ability to affect calmodulin-dependent activation of cyclic nucleotide phosphodiesterase.

Materials and Methods

Chemicals

Alkyltrimethylammonium bromides (C_nH_{2n+1}-$^+N(CH_3)_3$ and chlorpromazine hydrochloride were purchased from Sigma Chemical Co. Zwittergent® detergents (C_nH_{2n+1}-$^+N(CH_3)_2$-$(CH_2)_3$-SO_3^-) were obtained from Calbiochem-Behring, sodium alkyl sulphates (C_nH_{2n+1}-O-SO_3^-) from E. Merck AG and octaethyleneglycol mono *n*-alkyl ethers (C_nH_{2n+1}-$O(CH_2CH_2O)_8H$; R-EO8) from Nikko Chemicals Co.

[^{3}H]Adenosine 3′:5′-cyclic phosphate (31.2 Ci/mmol) was purchased from New England Nuclear, 3′:5′-cyclic nucleotide phosphodiesterase (activator deficient, P9529), 5′-nucleotidase (N4005), calmodulin (bovine brain, P2277), and aluminium oxide (neutral grade I) from Sigma Chemical Co.

Phosphodiesterase assay

The determination of cyclic phosphodiesterase activity was carried out essentially as previously described [5,11]. The determination is based on the conversion of ^{3}H-labelled 3′:5′-cAMP to 5′-AMP by phosphodiesterase. The reaction product, 5′-AMP, is converted to [^{3}H]adenosine by the action of 5′-nucleotidase. [^{3}H]Adenosine is then separated from the reaction mixture by an aluminium column and counted in a scintillation counter. The assay medium (total sample volume 100 μl) consisted of 50 mM Tris-HCl, 5 mM $MgSO_4$, 30 μM $CaCl_2$, 0.6 mM dithiothreitol (pH 7.5). Calmodulin in excess (1 μg calmodulin/sample) and amphiphiles separately dissolved in assay medium was added and the samples were preincubated for 10 min at 37 °C. The concentrations of the amphiphiles inducing maximum protection against hypotonic haemolysis (CAH_{max}), at a cell density of $(1.6–1.7) \cdot 10^8$ cells/ml, have been determined in previous studies [12,13] and in the present study the amphiphile concentrations used corresponded to 0.1 ×, 0.5 ×, 1 × and 2 × CAH_{max}. Following preincubation phosphodiesterase (2.8 mU/sample) was added to the samples and the reaction was started by addition of ^{3}H-labelled 3′:5′-cAMP (0.5 μCi/sample). The samples were incubated in a shaking thermostat bath at 37 °C for 20 min and the reaction was stopped by placing the tubes in boiling water for 75 s. The tubes were then chilled on ice and 5′-nucleotidase (10 mU/sample) was added. The samples were then reincubated for 30 min at 37 °C and the reaction was stopped by addition of 400 μl of 0.1 M ammonium acetate (pH 4.0). Following this the samples were applied to columns containing 1.8 g aluminium oxide equilibrated with 0.1 M ammonium acetate (pH 4.0) and eluted with 2 ml of the same buffer. 10 ml scintillation fluid was added to the eluants and the samples were counted in a liquid scintillation counter.

Unactivated phosphodiesterase activity (basal activity) was measured in samples containing all ingredients except calmodulin. Nonspecific production of [^{3}H]adenosine was determined in samples containing all ingredients except phosphodiesterase, calmodulin and amphiphiles and the counts in these samples were subtracted from the experimental values. The difference in [^{3}H]adenosine production in the presence and absence of calmodulin was taken to represent calmodulin-activation of phosphodiesterase.

The statistical significance of differences in enzymatic activity in the absence and presence of amphiphiles were tested by using Student's *t*-test. Probability values less than 0.05 were taken as statistically significant.

Results

The effects of amphiphiles on calmodulin-dependent activation of phosphodiesterase and on basal phosphodiesterase activity are shown in Fig. 1. The alkyltrimethylammonium derivatives (Fig. 1, a–c) and the alkyl sulphates (Fig. 1, d and e) were potent inhibitors of calmodulin action. The concentrations inducing 50% inhibition of calmodulin-activated phosphodiesterase activity (I_{50}) are listed in Table I, and as can be seen the

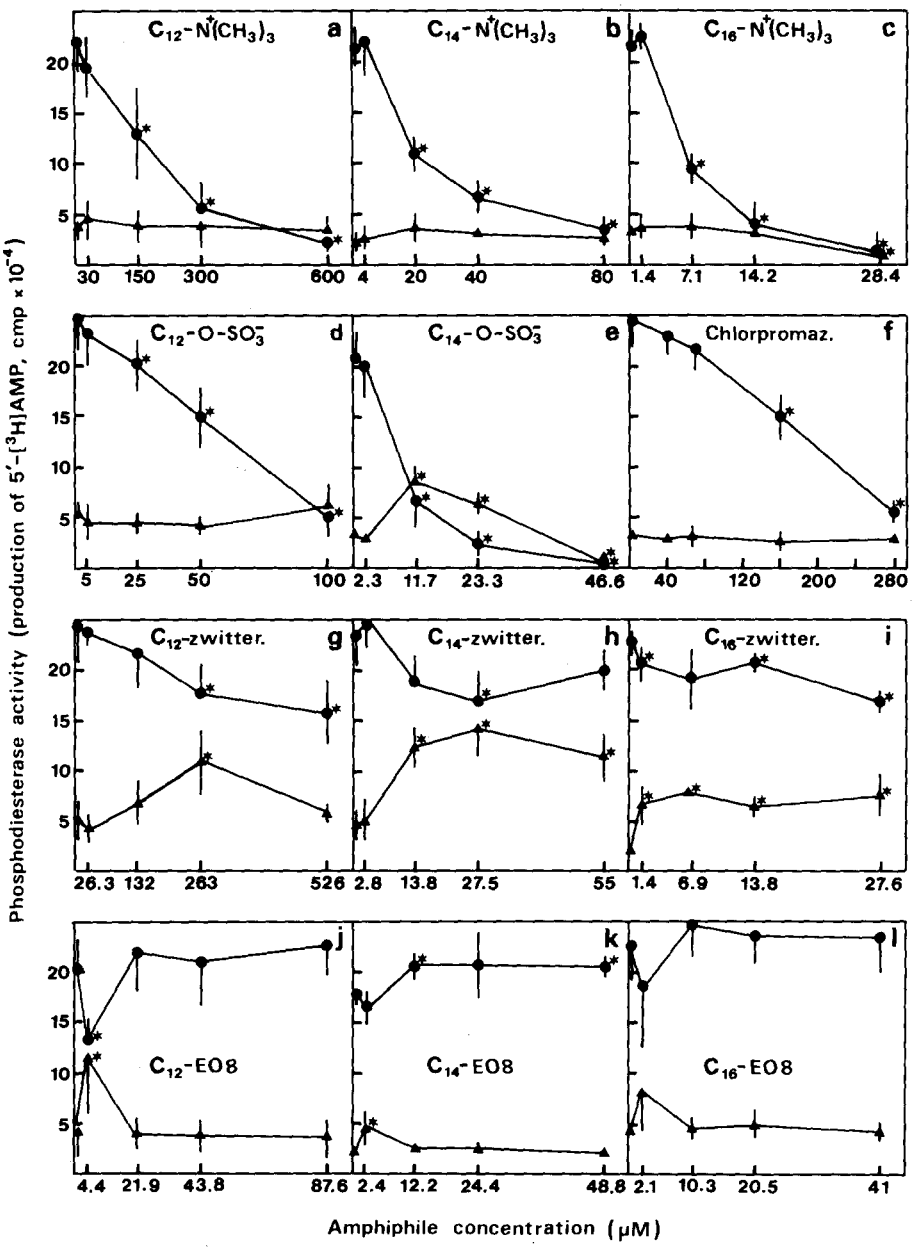

Fig. 1. Effect of amphiphiles on cyclic nucleotide phosphodiesterase activity in the presence (●) and absence (▲) of calmodulin. The assays contained 1 μg calmodulin and 2.8 mU phosphodiesterase. Incubation was carried out for 20 min at 37 °C. The concentration of the amphiphiles (except chlorpromazine) corresponds to 0.1×, 0.5×, 1× and 2×CAH_{max} (CAH_{max} is the concentration resulting in maximal protection against hypotonic haemolysis). Each symbol represents the mean of three separate determinations. Vertical bars indicate S.D. When not indicated, S.D. is in the range of the symbols. * indicates a statistically significant ($P < 0.05$) difference from corresponding control (assay without amphiphile).

inhibitory potency of the amphiphiles increased with an increasing alkyl chain length. The alkyl sulphates were more efficient inhibitors than the corresponding alkyltrimethylammonium derivatives. The shape transforming ability of chlorpromazine and its inhibitory effect on calmodulin-activated functions has been thoroughly studied and for this reason we included it

as a reference compound in our study. The I_{50} obtained for chlorpromazine (177 μM, Table I) is about four times higher than that reported by Levin and Weiss [15]. This discrepancy is apparently due to the fact that a higher concentration of calmodulin was used in our study. Chlorpromazine was a less potent inhibitor than the alkyl sulphates and the C_{14} and C_{16} homologues of the alkyltrimethylammonium derivatives and contrary to these amphiphiles chlorpromazine did not inhibit calmodulin-activated phosphodiesterase activity at a concentration where it induces maximum protection against hypotonic haemolysis (Table I, Fig. 1f).

The Zwittergent® detergents (Fig. 1, g–i) and the C_{14} homologue of the alkyl sulphates (Fig. 1e) caused an activation of the basal phosphodiesterase activity. The concentration dependence of the activation showed by the derivatives follows essentially the same pattern as that observed with some fatty acids [16], i.e. a concentration-dependent activation up to optimal concentration followed by a decrease in the activating effect. The stimulation of basal phosphodiesterase activity induced by the Zwittergent® detergents may, however, have interfered with the evaluation of the calmodulin-inhibiting property of these derivatives. If an activation of basal phosphodiesterase activity occurs without a concomitant increase in the calmodulin-activated activity, the calculated calmodulin-activated activity (total minus basal activity) will show an inhibition although there is no inhibition of calmodulin action. Such a situation may occur if the activation of phosphodiesterase by calmodulin and the amphiphiles is due to similar interactions with the same site on the enzyme. The slight inhibitory effect obtained for the Zwittergent® detergents may thus be false. A similar phenomenon occurred with the octaethyleneglycol alkyl ethers (Fig. 1, j–l). At a concentration corresponding to $0.1 \times CAH_{max}$ these amphiphiles induced an activation of basal phosphodiesterase activity and a concomitant inhibition of the calculated calmodulin-activated activity. At concentrations above $0.1 \times CAH_{max}$ a slight increase in the production of 5′-[^{3}H]AMP occurred with the octaethyleneglycol alkyl ethers, but since this increase did not show a concentration dependence it is apparently not due to an activation of calmodulin.

Summarizing our findings concerning the effect of the amphiphiles on calmodulin-activated phosphodiesterase activity at antihaemolytic concentrations, it is evident that the nature of the polar head of the amphiphiles has crucial role in

TABLE I

EFFECTS OF AMPHIPHILES ON CALMODULIN-ACTIVATION OF PHOSPHODIESTERASE AND ON ERYTHROCYTE SHAPE

Values are mean ± S.D. of three separate experiments. The shape of erythrocytes is classified according to Bessis [14].

Amphiphile	CAH_{max} (μM) (Ref. 3)	% inhibition (−) or stimulation (+) of calmodulin at CAH_{max}	I_{50} (μM)	Dominating shapes at CAH_{max} (Ref. 3)
C_{12}-$N^+(CH_3)_3$	300	−75.4 ± 9.6	185 ± 58	mixed; stomatocyte I to echinocyte III
C_{14}-$N^+(CH_3)_3$	40	−68.7 ± 5.0	21.7 ± 3.9	mixed; stomatocyte II to echinocyte III
C_{16}-$N^+(CH_3)_3$	14.2	−79.9 ± 12.8	6.9 ± 0.8	mixed; discocyte to sphero-echinocyte I
C_{12}-O-SO_3^-	50	−40.8 ± 5.6	61.3 ± 6.8	sphero-echinocyte I and II
C_{14}-O-SO_3^-	23.3	−88.6 ± 4.2	8.7 ± 0.9	sphero-echinocyte I and II
Chlorpromazine	20	0	177 ± 18	stomatocyte I and II
C_{12}-Zwittergent®	263	−27.4 ± 10.4 [a]		sphero-echinocyte II
C_{14}-Zwittergent®	27.5	−27.2 ± 6.2 [a]		sphero-echinocyte II
C_{16}-Zwittergent®	13.8	−9.0 ± 1.9 [a]		sphero-echinocyte II
C_{12}-EO8	43.8	+6.7 ± 0.8 [a]		stomatocyte I and II, sphero-stomatocyte I
C_{14}-EO8	24.4	+14.1 ± 13.0 [a]		stomatocyte II and sphero-stomatocyte I
C_{16}-EO8	20.5	+5.9 ± 2.7 [a]		stomatocyte I and II, sphero-stomatocyte I

[a] These derivatives are probably not real inhibitors or activators of calmodulin action (see Fig. 1).

the interaction with calmodulin. Positively and negatively charged amphiphiles were potent calmodulin inhibitors, zwitterionic possibly weak inhibitors whereas nonionic amphiphiles had no inhibitory effect.

In Table I the shape alterations induced by the amphiphiles in human erythrocytes at CAH_{max} ($(1.6–1.7) \cdot 10^8$ cells/ml) are shown together with the effects of the amphiphiles on calmodulin-activated phosphodiesterase activity. As can be seen in Table I the ability to induce stomatocytes or other shape alterations is not related to the ability to inhibit calmodulin-activated phosphodiesterase activity. The most potent stomatocytogenic amphiphiles (octaethyleneglycol alkyl ethers) and the most potent echinocytogenic amphiphiles (Zwittergent® detergents) had no or only weak inhibitory effect. The potent calmodulin inhibitors were echinocytogenic (alkyl sulphates) as well as stomatocytogenic (chlorpromazine) or weakly stomatocytogenic (alkyltrimethylammonium derivatives; at concentrations slightly above CAH_{max} these amphiphiles induce mainly stomatocytes [3]). Since all the amphiphiles studied are potent antihaemolytic compounds it is also clear that there is no correlation between the ability to induce antihaemolysis and to inhibit calmodulin-activation of phosphodiesterase.

Discussion

Our study clearly shows that there is no causal relationship between the ability of amphiphiles to induce antihaemolysis or shape alterations in erythrocytes and their ability to inhibit calmodulin-activated phosphodiesterase activity. A calmodulin inhibiting property does not seem to be an intrinsic quality of stomatocytogenic compounds, as proposed by Nelson et al. [6], and it seems fairly unlikely that calmodulin is involved in shape alterations induced by amphiphiles.

Shape alterations and protection against hypotonic haemolysis can be induced by a variety of structurally unrelated amphiphilic compounds and these effects seem to be due to nonspecific interactions of the amphiphiles with the erythrocyte membrane. The potency of amphiphiles to induce these effects is related to the hydrophobicity of the amphiphiles. An excellent correlation between the concentration required for half-maximum protection against hypotonic haemolysis and the oil/water partition coefficient has been shown for a great number of compounds [17]. At concentrations where amphiphiles protect erythrocytes against hypotonic haemolysis they also induce shape alterations [2,3]. However, the inhibitory potency of calmodulin inhibitors is apparently also related to the hydrophobicity of the inhibitors. A correlation between inhibitory potency and the oil/water partition coefficient has been demonstrated for a variety of calmodulin inhibitors [18] and for those amphiphiles which in our study proved to be potent inhibitors of calmodulin-activated phosphodiesterase activity (alkyl sulphates and alkyltrimethylammonium derivatives), the inhibitory potency was positively related to the length of the alkyl chain. Consequently, if a group of amphiphilic calmodulin inhibitors are selected and studied for their antihaemolytic potency, a good correlation between the concentration required for half-maximum protection and their inhibitory potency will be obtained, especially in the case of a congeneric set of molecules or structurally related compounds. If the compounds are structurally related, as was the case with those studied by Nelson et al. [6], it is very likely that they induce the same type of shape alteration and a good correlation between the ability to induce a certain shape and the ability to inhibit calmodulin-activated functions will also be obtained. Thus, in trying to relate functions depending on hydrophobic properties of ligands to effects such as protection against hypotonic haemolysis and shape transformations, caution should be taken.

Although we did not attempt to study inhibition of calmodulin per se, some findings deserving attention emerged from our study. In an attempt to specify the structural requirements for a compound to be a potent inhibitor of calmodulin-activated functions Weiss et al. [18] concluded that the essential features are a large hydrophobic region, consisting of two aromatic rings, and a side-chain amino group bearing a positive charge at neutral pH. The finding that negatively or positively charged simple amphiphilic alkylderivatives may be more potent inhibitors of calmodu-

lin-activated phosphodiesterase activity than chlorpromazine is therefore surprising. These simple alkylderivatives could possibly be valuable tools in attempts to elucidate the character of the domains on calmodulin important for its interaction with phosphodiesterase and other proteins. Neither the zwitterionic nor the nonionic amphiphiles had a clear inhibitory effect. Thus it appears that besides a hydrophobic character, a positive or negative net charge is essential for inhibitory effect of amphiphilic alkyl derivatives.

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