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ASSOCIATION OF CALMODULIN INHIBITION, ERYTHROCYTE MEMBRANE STABILIZATION AND PHARMACOLOGICAL EFFECTS OF DRUGS

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The present study was designed to determine whether there is an association of drug-induced inhibition of calmodulin functions, drug-induced membrane stabilization (protection against osmotic lysis), and pharmacological effects of drugs. First, data on drugs which have been studied for both calmodulin inhibition and membrane antihemolysis were collected from the literature and an association of the two properties was established. Second, ten additional drugs were selected for study of all three properties. Four drugs, with known antihemolytic effects, were studied for calmodulin inhibition. One drug, which was a known calmodulin inhibitor, was studied for antihemolysis. Our results show that membrane-stabilizing drugs are usually calmodulin inhibitors, and vice versa; that drugs in certain therapeutic classes inhibit calmodulin-activated functions and protect against osmotic lysis; and finally, that there is a significant correlation ($P < 0.01$) in terms of potency between these two actions of drugs. Data from the literature which bear on these mechanisms of drug actions suggest that the interactions between drugs and calmodulin, and drugs and the membrane, appear to be hydrophobic in nature. At this point, we do not know whether there is some causal relationship between calmodulin inhibition and the antihemolytic effect of drugs, or whether the two are simply a result of hydrophobic properties of drugs. Similarly, the roles of calmodulin inhibition and/or membrane antihemolysis in producing therapeutic efficacy are unknown.

Introduction

In this paper, we present evidence that drugs in certain pharmacological classes share two interesting properties. These properties are: (1) inhibition of the function of a calcium-activated protein called calmodulin; and (2) protection of erythrocytes against osmotic lysis. The classes of drugs which share these properties include tranquilizers, other neuroleptics, local anesthetics, cardiac anti-arrhythmic agents, and membrane anti-sickling agents.

Calmodulin is a low-molecular-weight, high affinity calcium-binding protein which mediates many of the intracellular effects of calcium in eukaryotic cells [1–3]. Calmodulin has been shown to activate or modulate intracellularly a large series of proteins in a calcium-dependent manner, including the ($Ca^{2+} + Mg^{2+}$)-ATPase [3], cyclic nucleotide phosphodiesterase [1,2] and adenylate cyclase [4,5]. As indicated above, many drugs from the therapeutic classes mentioned inhibit calmodulin-activated functions [6–12].

The second associated characteristic of these drugs is their effect on membrane antihemolysis. We have observed that a large number of drugs which produce calmodulin inhibition also have a membrane-stabilizing effect on human erythro-

cytes. This characteristic, which was studied in the 1960's primarily by Seeman and his colleagues [13–15] was suggested to involve an expansion of the membrane, since the measured increase in membrane area was found to be considerably in excess to that calculated for simple physical displacement by drug molecules. More recently it was shown that an expansion of membrane surface area is accompanied by a decrease in membrane thickness, and that the increase in membrane volume is approximately equal to the volume of drug molecules occupying it [16].

In this paper we attempt to pull together the literature related to these topics, and add additional data of our own. Then, in the discussion, we cover possible explanations for these associations.

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Materials and Methods

Calcium adenosine triphosphatase (Ca^{2+}-ATPase) assay of 310 mosM imidazole (I-310) erythrocyte membranes

All blood was drawn by venipuncture of healthy laboratory personnel and collected into heparinized vacutainers, and membranes were prepared by a slightly modified method of Farrance and Vincenzi [17,18]. This method was selected because it promotes the binding of calmodulin to the Ca^{2+}-ATPase and results in a high level of Ca-ATPase activity in the absence of added calmodulin. Red blood cells from about 20 ml whole blood were washed three times in 0.154 M NaCl, then lysed in 0.151 M (310 mosM) imidazole buffer containing 30 μM EDTA (pH 7.4) for 20 min at 0–5°C. The lysate was spun at $49000 \times g$ for 20 min, the supernatant was decanted and the membranes washed three times, twice with 40 ml 0.01 M imidazole buffer (pH 7.4), once with 40 ml 40 mM histidine/40 mM imidazole buffer (pH 7.1), and each wash spun at $49000 \times g$ for 20 min. The resulting membrane pellet was diluted with an equal volume of 40 mM histidine/40 mM imidazole buffer (pH 7.1) and the protein concentration determined by the method of Lowry et al. [19]. Red blood cell I-310 membrane Ca^{2+}-ATPase was assayed by suspending sufficient membrane preparation to produce 1 mg/ml membrane protein in a solution containing final concentrations of 50 mM NaCl, 120 mM KCl 3 mM $MgCl_2$, 50 μM $CaCl_2$, 3mM ouabain, an appropriate amount of drug dissolved in 40 mM histidine/40 mM imidazole buffer (pH 7.1) and the last ingredient added, 3 mM adenosine triphosphate (ATP) (pH 7.2) all brought up to a final volume of 3 ml. For no-drug controls, sufficient 40 mM histidine/40 mM imidazole buffer was added to replace the volume of buffer used for the drug solution to bring the final volume up to 3 ml. In the presence of ouabain and 50 μM $CaCl_2$, 90% or more of the ATPase activity is due to Ca^{2+}-ATPase. The remaining 10% or less, which is due to Mg^{2+}-ATPase, is not stimulated by calmodulin and does not significantly alter the results [3]. The tubes were incubated in a water bath at 37°C for 1 h, during which time two 0.5 ml aliquots were taken, the first immediately following the addition of the last ingredient, ATP, the second after 60 min incubation. Since Ca^{2+}-ATPase activity is linear with time of incubation [3], additional aliquots were not collected. The aliquots were immediately mixed with 2.0 ml 6.7% trichloroacetic acid and immediately stored at −70°C until assayed for ATP. ATP was assayed using a modified method of Sigma Technical Bulletin No. 366-UV. In the ATP assay, as used, 0.5 M triethanolamine was substituted for water as one of the solvents for nicotinamide adenine dinucleotide (NADH). Triethanolamine stabilized NADH against oxidation prior to the onset of the reaction. For the assay of Ca^{2+}-ATPase activity, the consumption of ATP was employed rather than the production of inorganic phosphorus, because of our finding that drugs precipitated during the phosphorus assay, possibly due to formation of complexes with reagents used. Concerning possible decomposition of ATP during incubation at 37°C for 1 h, preliminary experiments under comparable conditions in the absence of Ca^{2+}-ATPase have shown that the amount of ATP decomposed was less than 5% and did not significantly alter the results.

Phosphodiesterase assay

Cyclic nucleotide phosphodiesterase activity was determined following a slightly modified method

of Filburn and Karn [20] which is based on the conversion of ^3H labeled cAMP to 5'AMP by phosphodiesterase, then the conversion of the reaction product, 5'AMP, to adenosine and inorganic phosphate by 5'-ribonucleotide phosphohydrolase (5'-nucleotidase). The adenosine, which has the ^3H label, is separated from the reaction mixture by alumina columns. The labeled adenosine is then counted in a scintillation counter. Phosphodiesterase as a crude preparation was prepared from bovine brain by the method of Klee and Krinks [21]. A crude preparation was used because further purification results in the degradation and instability of the enzyme. Calmodulin was purified from bovine brain by the method of Dedman et al. [22] to apparent homogeneity as shown by the formation of a single band on polyacrylamide gel electrophoresis. The standard phosphodiesterase assay mixture in 100 μl contained 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgSO_4$, 30 μM $CaCl_2$, 0.6 mM dithiothreitol, 10 ng phosphodiesterase preparation, 1 μg calmodulin, an appropriate amount of drug dissolved in 40 mM histidine/40 mM imidazole buffer (pH 7.1) and 20 μM cAMP (0.2 mCi [^3H]cAMP per assay). A larger amount of calmodulin than necessary to produce maximal stimulation of phosphodiesterase was used to make sure calmodulin was present in excess. The following controls, with final volumes of 100 μl, were run with each assay. Nonspecific production of adenosine from [^3H]cAMP was determined by employing control tubes containing all ingredients except phosphodiesterase, calmodulin and drug. Baseline phosphodiesterase activity (noncalmodulin-activated) was measured by including tubes containing all ingredients except calmodulin and drugs. Maximally stimulated phosphodiesterase activity was determined by measurements on tubes containing all ingredients except the drugs. The reaction was started with the addition of [^3H]cAMP to each tube. The tubes were incubated in a water bath at 37°C for 10 min, afterwards in a dry bath at 95°C for 1 min and then placed on ice. 0.01 unit of 5'-nucleotidase (from snake venom) was added and the tubes incubated in a water bath at 37°C for 30 min. The reaction was terminated by the addition of 400 μl of 0.018 M ammonium acetate buffer (pH 4.0). The contents of each tube were eluted over a column containing 2 ml grade I alumina (ICN High Grade Alumina) and collected in a scintillation vial. Each column was washed down with 1.5 ml 0.018 M ammonium acetate buffer (pH 4.0) and the wash eluant from each column was collected into the same scintillation vial containing the sample from that column. 13.5 ml aqueous counting scintillant was added to each vial and the samples counted in a Beckman Liquid Scintillation Counter (L800100).

Osmotic lysis as determined by release of hemoglobin

Osmotic hemolysis of red blood cells in the presence of drugs was measured following the method of Seeman and Weinstein [13]. An erythrocyte suspension was prepared from freshly drawn human blood in a heparinized vacutainer by centrifuging 0.925 ml whole blood (+0.075 ml heparin initially present in the tube) at $1500 \times g$ for 5 min. The plasma and buffy coat were removed, and the erythrocytes suspended in 145 mM NaCl in 10 mM sodium phosphate buffer (pH 7.0) or 145 mM NaCl in 10 mM Hepes buffer (pH 7.0) for a final volume of 12.5 ml. The choice of red cell suspension buffer depended on which buffer was used as the solvent for a drug. Drugs were dissolved in a solution composed either of 60.6 mM NaCl in 10 mM sodium phosphate buffer or in some cases, if solubility of a drug was a problem, 60.6 mM NaCl in 10 mM Hepes buffer (pH 7.0). It was shown in preliminary work that the choice of buffer did not affect the results. 0.1 ml erythrocyte suspension and 1.5 ml drug solution were combined, mixed and allowed to sit at room temperature (23°C) for 5 min and then centrifuged for 45 s at $1500 \times g$. The hemoglobin content of the supernatant was measured by recording the absorbance at 543 nm in a spectrophotometer.

Drugs and reagents

Drugs used in this study were obtained from the following sources: procaine and quinidine from the University of Michigan Hospital Pharmacy; dibucaine from Ciba Pharmaceutical Co.; thioridazine from Sandoz Pharmaceuticals; cetiedil from Innothera, Arceuil, France; propranolol, procainamide, and zinc from Sigma Chemical Co.; UM 272 (1-dimethyl-isopropylamino-3-[1-naph-

thyloxy]-propan-2-ol) and UM 424 (1-dimethylisopropyl-amino-3-[2-phenylphenoxy]-propan-2-ol) from Dr. Lucchesi, Department of Pharmacology, University of Michigan. [^{3}H]cAMP was obtained from New England Nuclear. All other substances used were reagent grade.

Results

Coincidence of calmodulin inhibition and membrane antihemolysis based on the literature

As described in the Introduction, the focus of this paper is on two properties, erythrocyte membrane antihemolysis and calmodulin inhibition, which a group of pharmacologically diverse drugs share. Although these properties of drugs have been studied independently by various investigators, no systematic attempt appears to have been made to see if they are related. We have compiled data from the literature on drugs which have already been shown to produce either membrane antihemolysis or inhibition of calmodulin-activated functions. In Table I, we have listed the eight drugs on which both types of study have been done, along with appropriate references, and seven of the eight drugs show both properties. To our knowledge, the literature shows only one exception to the association of these properties: D-lysergic acid diethylamide causes membrane antihemolysis, but does not inhibit calmodulin-activation of phosphodiesterase. Four of the drugs have been studied for calmodulin binding and all four have been found to be positive (Table I).

Coincidence of calmodulin inhibition and membrane antihemolysis based on our studies

Based on the premise that membrane antihemolysis, calmodulin inhibition, and certain types of therapeutic activity of drugs are related, and in general, predictive of one another, we have selected additional drugs for study according to the following criteria: (1) four drugs which are known to stabilize membranes against osmotic lysis for determination of their calmodulin inhibitory proper-

TABLE I

COMPARISON OF PUBLISHED DATA ON DRUG-INDUCED INHIBITION OF CALMODULIN FUNCTIONS AND MEMBRANE ANTIHEMOLYSIS

Class of drug	Drug	Calmodulin inhibition [a]			Membrane antihemolysis [b]		Calmodulin binding	
		Enzyme	I_{50} (μM)	Ref.	C_{50} (μM)	Ref.	Result	Ref.
Phenothiazine	Chlorpromazine	AC	200	6	10	15	+	11
		CaATPase	22,54	7,8				
		PDE	6,42	9,10				
	Fluphenazine	CaATPase	10	7	4	15		
	Trifluoperazine	CaAPTase	9,50	7,23	1.2	15	+	11
		PDE	10	10				
	Thioridazine	PDE	18	10	0.5	15		
	Promethazine	PDE	340	10	16	15		
Butyrophenone	Haloperidol	PDE	41,60	20,11	22	15	+	11
Tricyclic antidepressant	Imipramine	PDE	125	11	10	15	+	11
Halucinogen	D-lysergic acid diethylamide	PDE	no effect	10	30	15		

[a] Calmodulin-activated functions for which data are available are adenylate cyclase (AC), erythrocyte Ca^{2+}-ATPase (CaATPase) and cyclic nucleotide phosphodiesterase (PDE). I_{50} refers to the concentration of the drug which inhibits 50% of the calmodulin-activated enzyme activity. More than one I_{50} value per enzyme is listed for some drugs and reflects variations in experimental conditions employed by different investigators.

[b] C_{50} refers to the concentration of drug which causes 50% antihemolysis of human erythrocytes.

ties; (2) one drug which is a known calmodulin inhibitor, for determination of its membrane antihemolytic property, and; (3) five drugs which belong to specific therapeutic classes, for determination of either or both calmodulin inhibition and membrane antihemolysis. The two properties of calmodulin examined were calmodulin-activation of Ca^{2+}-ATPase and cyclic nucleotide phosphodiesterase. All drugs studied are reported here; the following data have not been preselected.

Drugs selected for study of calmodulin inhibition because they were known membrane antihemolytic agents. It can be seen in Table IIA that the four drugs, dibucaine, procaine, propranolol, and procainamide, which are known from Seeman's work to have an antihemolytic effect on membranes and to produce membrane expansion, also inhibit calmodulin-activated Ca^{2+}-ATPase and phosphodiesterase activities. (See also Fig. 1 for results on dibucaine). The concentrations of drugs at which 50% Ca^{2+}-ATPase activity was inhibited (I_{50}) are given in Table IIA. Two substances, barbital sodium and triethanolamine, were run as controls and even at high concentration ranges (1–100 mM) had little effect on Ca^{2+}-ATPase activity.

The I_{50} values for inhibition of calmodulin-activated phosphodiesterase activity by three of these drugs are also given in Table IIA (propranolol was not determined). In the absence of added calmodulin, these three drugs inhibited phosphodiesterase only slightly (about 5%).

Drug selected for study of membrane antihemolysis because it was known to inhibit calmodulin functions. Earlier work in this laboratory has shown that zinc is an inhibitor of calmodulin-activated Ca^{2+}-ATPase and phosphodiesterase activities [22,23]. The present study (Table IIB and Fig. 2), shows that zinc also protects membranes against osmotic lysis. The C_{50} value for zinc is 190 μM.

The control substances for membrane antihemolysis, $MgSO_4$, $CaCl_2$ and NaCl had no effect on the erythrocyte membrane in the range of concentrations at which zinc and other drugs (dis-

TABLE II

Drugs selected according to three criteria were observed for their effects on either or both calmodulin inhibition and membrane antihemolysis. These criteria are listed as A,B and C below. The fourth category of drugs, category D, was selected for study of Ca^{2+}-ATPase activity because of existing data on inhibition of PDE activity and membrane antihemolysis by these drugs. All of the data listed are from our study, except where indicated by parentheses. The values in parentheses are from literature sources as referenced. ND, not determined.

Mode of selection of drug		Drug	Calmodulin inhibition results (I_{50}, μM)		Membrane antihemolysis results (C_{50}, μM)
			CaATPase	PDE	
A.	Known membrane antihemolytic agent	Procaine	1400	5000	(400000)[14]
		Dibucaine	2500	520	(100)[14]
		Procainamide	40000	30000	(5000)[15]
		Propranolol	5000	N.D.	(250)[15]
B.	Known calmodulin inhibitor	Zinc	(40)[25]	(75)[26]	190
C.	Therapeutic class				
	(1) Membrane antisickler	Cetiedil	800	800	45
	(2) Antiarrhythmic agent	Quinidine	1000	1000	450
		UM 272	N.D.	N.D.	240
		UM 424	N.D.	N.D.	350
		Bretylium tosylate	N.D.	N.D.	no effect
D.	Known membrane antihemolytic agent and inhibitor of one calmodulin-activated function	Imipramine	1000	(125)[11]	(0.5)[15]
		Thioridazine	72	(18)[10]	(10)[15]

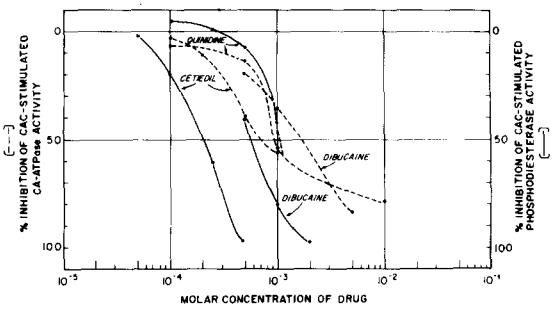

Fig. 1. Inhibition by cetiedil, quinidine, and dibucaine of calmodulin activated Ca^{2+}-ATPase activity of 310 mosM imidazole erythrocyte membranes (— — —) and calmodulin-activated phosphodiesterase activity (———).

cussed below) were effective (Fig. 2).

Drugs selected for study of either or both calmodulin inhibition and membrane antihemolysis because of therapeutic class. Five additional agents from two therapeutic classes (Table IIC) were studied to see if the therapeutic class of a drug is predictive of its actions on calmodulin functions and membrane stabilization. The two therapeutic classes are: (1) membrane antisickling agents represented by the drug cetiedil, and (2) cardiac antiarrhythmic agents represented by the drugs quinidine, bretylium tosylate, and two University of Michigan experimental drugs, UM 272 and UM 424.

Two of the five drugs were observed for inhibition of calmodulin-activated functions. It can be seen in Fig. 1 and Table IIC that cetiedil and quinidine inhibit calmodulin-activated Ca^{2+}-ATPase and phosphodiesterase activities. The I_{50} values are given in Table IIC.

All five drugs were tested for membrane antihemolysis and four were found to be positive (Table IIC and Fig. 2). The C_{50} values for these four drugs are given in Table IIC. The fifth drug, bretylium tosylate, had no effect on membrane antihemolysis. The controls employed in determination of membrane antihemolysis have already been discussed above.

Test of the quantitative relationship between calmodulin inhibition and membrane antihemolysis

Earlier, we have stated that the two properties of drugs, that is, membrane protection against osmotic lysis and calmodulin inhibition, may be related and, in general, predictive of one another, and data to support this view have been presented in Tables I and II. Further support for this relationship is shown by the correlations presented in

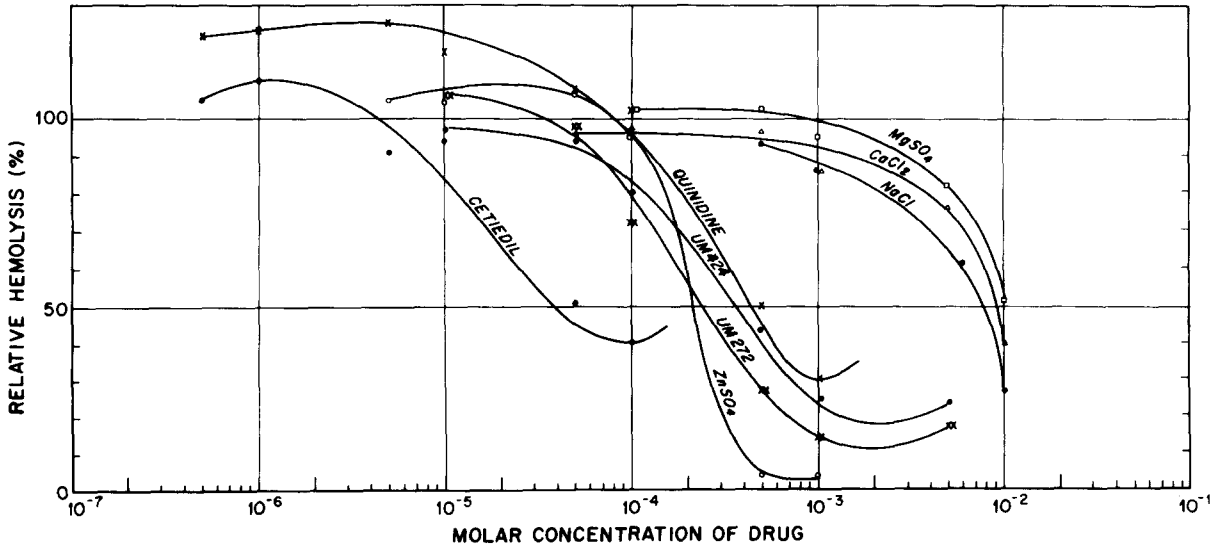

Fig. 2. Protection of erythrocytes against osmotic lysis by zinc, cetiedil, quinidine, UM 272, UM 424. The control substances are $MgSO_4$, $CaCl_2$ and NaCl. The reduction in osmotic lysis of erythrocyte membranes caused by drugs is expressed as a proportion of the amount of lysis which occurred in the presence of buffered saline solution alone.

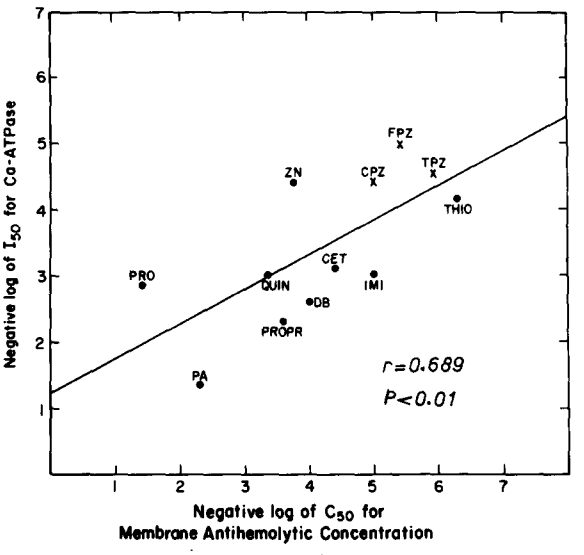

Fig. 3. Relationship between Ca^{2+}-ATPase inhibition and membrane antihemolysis. The inhibitory concentrations of drugs are negative logarithmic transformations of the I_{50} and C_{50} values expressed in molar concentrations of drugs presented in Table I and II. ●, our values; ×, literature values; PRO, procaine, PA, procainamide; QUIN, quinidine; PROPR, propranolol; DB, dibucaine; ZN, zinc; CET, cetiedil; IMI, imipramine; CPZ, chlorpromazine; FPZ, fluphenazine; TFP, trifluoperazine; THIO, thioridazine.

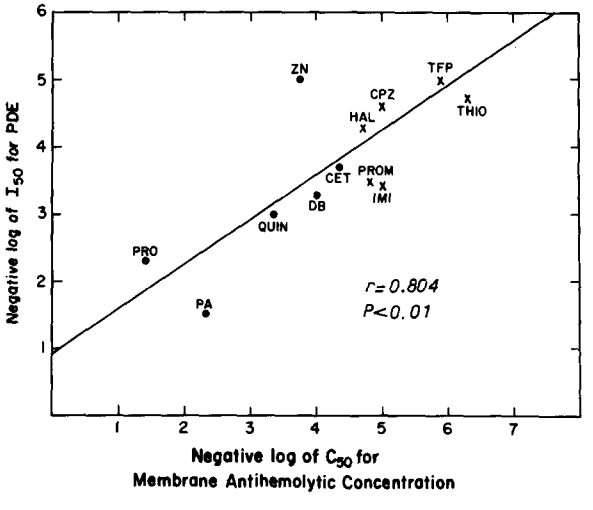

Fig. 4. Relationship between phosphodiesterase (PDE) inhibition and membrane antihemolysis. The inhibitory concentrations of drugs are negative logarithmic transformations of the I_{50} and C_{50} values expressed in molar concentrations of drugs presented in Tables I and II. ●, our values; ×, literature values. All abbreviations used for drugs are listed in the legend to Fig. 3 except two. These are: HAL, haloperidol; PROM, promethazine.

Figs. 3 and 4. Here we have compiled the data from Tables I and II and correlated each calmodulin-activated function with membrane antihemolysis. Where more than one I_{50} value per enzyme was listed for a drug in Table I, we have averaged them and used the average value. The correlation between the C_{50} values for the membrane antihemolytic effect and the I_{50} values for Ca^{2+}-ATPase inhibition for the combined sample of drugs is 0.689 (significant at the 0.01 level; Fig. 3). The correlation between the C_{50} values for membrane antihemolysis and the I_{50} values for phosphodiesterase inhibition is 0.804 (significant at the 0.01 level; Fig. 4). Finally, the correlation between the I_{50} values of the two calmodulin-activated functions, Ca^{2+}-ATPase and phosphodiesterase activities, is 0.938 (significant at the 0.01 level).

Discussion

Two independent sets of data are presented here which show that a variety of drugs from different therapeutic classes have parallel actions with respect to erythrocyte membrane stabilization and inhibition of calmodulin-activated functions. The first set of data comes from the literature. Of the eight drugs which have been studied for both actions, seven are shown to be positive (Table I). These include representatives from the phenothiazine, butyrophenone, and tricyclic antidepressant drug classes.

The second set of data presented in this paper is based on our studies. Drugs were selected according to one of three criteria – membrane antihemolysis, calmodulin inhibition or certain therapeutic activities – and a remarkable degree of concordance of the three characteristics was seen (Table II). Four known antihemolytic drugs were shown to be calmodulin inhibitors (four cases of concordance of the properties). One known calmodulin-inhibiting drug was shown to cause membrane antihemolysis (one case of concordance). Two drugs which were selected becasuse they belong to specific therapeutic classes were shown to both inhibit calmodulin-activated functions and cause membrane antihemolysis (four cases of concordance). Two of three drugs, which were also selected because of certain therapeutic actions,

showed membrane antihemolysis (two of three cases of concordance). With the latter three drugs, calmodulin inhibition was not studied. Thus in 11 of 12 opportunities, the association of therapeutic class, calmodulin inhibition and membrane antihemolysis were found to be present.

Our results indicate that it is possible to predict calmodulin inhibition if a drug has membrane antihemolytic properties, and vice versa. A close association between these properties is further supported by highly significant correlations between the C_{50} values for the membrane antihemolytic effect and the I_{50} values for Ca^{2+}-ATPase and phosphodiesterase activities for the combined data.

What is the reason for these associations? The first of two hypotheses we will consider is one of 'causality' [27,28]. This hypotheses proposes, first, that calmodulin inhibition causes a reduction in osmotic lysis of erythrocyte membranes, giving calmodulin a role in somehow modulating membrane stabilization. According to this hypothesis, any drug that is a calmodulin inhibitor would also be a membrane antihemolytic agent, although membrane stabilization could occur throught routes other than calmodulin inhibition. A second part of the causality hypothesis holds that calmodulin inhibition results in certain kinds of pharmacological effects, such as membrane antisickling effects, cardiac antiarrhythmic effects, certain central nervous system effects, and local anesthesia. Such a concept appears plausible because of the knowledge that calcium has important regulatory roles in many cell types. For example, calcium is known to be intimately involved with platelet, spermatozoal, and neutrophil activation [31]. On the other hand, calmodulin inhibitors, such as the phenothiazines, inhibit all of these activations [31].

However, a number of factors mitigating against the causal hypothesis can be brought to bear. With respect to the expansion of the membrane greater than can be accounted for by the incorporation of the drug [13–15], which suggests the need for a modulation of membrane expansion, recent work has offered an explanation. Jain et al. [35] studying local anesthetics and phenothiazines, have shown by scanning calorimetry that membrane expansion is associated with a disordering of the phospholipid bilayer. Snow et al. [36], working with local anesthetics, and Krishnan and Brandts [37] studying alcohols and phenothiazines, have reported alterations of the phospholipid transition profile and in some cases the transmembrane protein, called band 3. In other words, it appears that membrane antihemolytic drugs may disorder the phospholipid bilayer around the region of band 3 protein, perhaps accounting for membrane expansion and for an increase in membrane surface area in relation to a decrease in membrane thickness [16]. These findings argue against the need to invoke an effect of a regulator, such as calmodulin, to account for this membrane effect.

Regarding the relationship of the pharmacologic effects of drugs to calmodulin inhibition, recent work indicates that calmodulin inhibition is not involved in neuroleptic effects. Norman et al. [9] have studied the effect of therapeutically active and inactive isomers of neuroleptic drugs on the inhibition of calmodulin-activated phosphodiesterase activity, and found that both stereoisomers of these drugs are equally inhibitory to calmodulin. Thus, calmodulin inhibition does not appear to be related to therapeutic efficacy with the neuroleptics. Levin and Weiss [12] have found some stereospecificity in the binding of drug isomers to calmodulin; however, the difference in binding between isomers was small, and the significance of this difference is uncertain. Similar studies have not been carried out with other classes of therapeutic agents, and it remains possible that calmodulin inhibition is involved in the mechanism of action of many of these drugs.

The second hypothesis to explain the associations may be called the 'hydrophobic hypothesis'. This hypothesis holds that calmodulin binding and inhibition, membrane antihemolysis and certain kinds of therapeutic effects are all related to relatively nonspecific hydrophobic properties of drugs, and that this hydrophobicity is the common denominator. LaPorte et al. [38] have shown that the interaction between calmodulin, which reveals a hydrophobic domain when it binds calcium, and target proteins or drugs, is hydrophobic is character. Based on these results, LaPorte et al. have suggested that the hydrophobic domain is the site of interface between calmodulin and numerous interacting substances including drugs and target proteins. It is possible that a correlation might

exist between the degree of hydrophobicity of a drug and its potency as an inhibitor of calmodulin function on the one hand, and its potency as a membrane stabilizer on the other. Seeman [15] has demonstrated that relative hydrophobicity correlates with membrane antihemolytic potency. Norman et al. [9] have shown that hydrophobicity of neuroleptic drugs as measured by octanol/water partition coefficients correlates well with I_{50} for calmodulin-activated phosphodiesterase activity. A related study was earlier reported by Roufogalis [39] who showed hydrophobicity/membrane action relationships for various local anesthetics, antihistamines, tricyclic antidepressants, and phenothiazine tranquilizers. He showed a positive correlation between hydrophobicity and inhibition of erythrocyte membrane $(Na^+ + K^+)$-ATPase. Thus, a hydrophobicity hypothesis could explain the inhibition of calmodulin, could explain membrane antihemolysis, and could presumably explain the high correlation in drug potencies producing these effects.

A third possible explanation is suggested by recent reports that drug actions may be associated with the effects of drugs on the distribution of calmodulin within the membrane and cytosolic portions of cells in certain tissues. Thus a 'distributional hypothesis' would propose that drugs indirectly modulate the calmodulin-activated enzymes within these compartments by virtue of these effects [40,41].

The causality hypothesis is the more exciting of the two major proposals. However, even if relatively nonspecific hydrophobicity is the explanation for these associations, the associations will remain interesting and potentially useful from the standpoint that the presence of one effect is predictive of the other two. That is, one might screen for other properties if one finds one of the three properties. This is exactly what we our doing in our efforts to find better membrane antisickling agents. That is, we are screening known membrane antihemolytic agents and calmodulin inhibitors for their antisickling potency.

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