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Trinitrobenzenesulfonic Acid: A Possible Chemical Probe to Investigate Lipid-Protein Interactions in Biological Membranes

DAVID V. GODIN AND THERESE WAN NG

Department of Pharmacology, University of British Columbia, Vancouver 8, British Columbia, Canada

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SUMMARY

The modification of erythrocyte membrane protein amino groups by trinitrobenzenesulfonic acid (TNBS) was stimulated by a variety of divalent cations. One group of cations (Mn^{++}, Mg^{++}, Ca^{++}, and Sr^{++}) appeared to act by interacting with, and altering the structure of, membrane phospholipids, and this was proposed to result in an alteration of membrane protein structure as reflected in enhanced reactivity of protein amino groups toward TNBS. A second group of cations (Zn^{++}, Cd^{++}, and Ni^{++}) interacted directly with membrane proteins, probably via proximal pairs of thiol groups, to produce enhancement of TNBS incorporation. A number of drugs, including chlorpromazine and local anesthetics, also interacted with membrane phospholipids, mimicking the stimulatory effects of divalent cations on TNBS incorporation. These studies seem to reflect some fundamental aspect of membrane lipid-protein interaction, and offer an approach for the characterization of drug action at the membrane level in terms of perturbations in membrane structural components.

INTRODUCTION

The nature of the interactions between the lipid and protein components of biological membranes is the most fundamental question to be approached before the relationship between membrane structure and function may be rationalized in molecular terms.

The current theories of membrane structure differ primarily in terms of different emphasis on the role of protein-protein, lipid-lipid, and lipid-protein interactions (1, 2). Such structural considerations are germane to an understanding of membrane function, and the action at the membrane level of such modifiers of biological function as divalent cations, local anesthetics, phenothiazine derivatives, and neurotransmitters must ultimately be described in terms of perturbations of these membrane structural elements.

During the course of our studies concerning the modification of erythrocyte membrane proteins by specific chemical reagents, it was found that the rate of incorporation of trinitrobenzenesulfonic acid, a reagent with considerable specificity for primary amino groups (3), was dependent on the presence of divalent cations in the medium. Although the profound effects of divalent cations on both structural and functional characteristics of natural and artificial membranes are well known (4–6), the mechanisms by which these effects are mediated have thus far remained obscure.

It was therefore decided to utilize TNBS[1]

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[1] The abbreviation used is TNBS, trinitrobenzenesulfonic acid.

as a probe to investigate the interaction of a number of divalent cations and a variety of pharmacologically active agents with the erythrocyte membrane, as measured by the rate and extent of incorporation of the reagent into membrane proteins.

The results obtained suggest an important role of lipid-protein interactions in determining alterations in membrane structure induced by modifiers of membrane function, and these studies might offer a convenient approach to the characterization of such perturbations in membrane systems in general.

METHODS

Membrane preparations. Erythrocyte membranes were prepared from outdated (3–4-week-old) blood stored in acid-citrate-dextrose by a modification of the method of Schrier (7) as described previously (8). Preparations were stored at −20° prior to use and contained between 3.5 and 4.5 mg of protein per milliliter, as estimated by the method of Lowry *et al.* (9), using bovine serum albumin (Armour) as a standard.

Bovine brain microsomes were isolated by standard methods (10) and were stored in 0.25 M sucrose–1 mM EDTA at −20°. Prior to use, the microsomes were washed twice with distilled water by centrifugation to remove the sucrose and EDTA. The washed membrane suspensions used in these studies contained approximately 2 mg of protein per milliliter.

Rabbit skeletal muscle plasma membranes were prepared by the procedure of Kono and Colowick (11) with minor modifications, and were stored at 4° prior to use. The protein concentration of these preparations was between 2 and 3 mg/ml.

Phospholipase C treatment of erythrocyte membranes. In a typical experiment, 2.0 ml of membrane suspension were combined with 0.4 ml of phospholipase C (1 mg/ml) and 0.8 ml of $CaCl_2$ (20 mM) in a final volume of 4.0 ml. This mixture was incubated at 37 ± 0.5° for 15 min and centrifuged for 10 min at 30,000 × *g*, and the pellet was washed by suspension in 40 ml of distilled water and centrifugation. The washed pellet was brought up to a final volume of 2.0 ml.

Butanol and pentanol extraction of erythrocyte membranes. The original procedure of Maddy (12) was modified as follows. Membranes were washed three times with distilled water prior to extraction. The extraction and all subsequent procedures were carried out at temperatures between 0° and 4°. Equal volumes of washed membrane suspension and butanol or pentanol were combined and rapidly mixed, and the samples were immediately centrifuged at 30,000 × *g* for 5 min. After centrifugation the lower, aqueous phase was carefully separated from the interfacial material and from the upper, alcohol phase, and was dialyzed for 24 hr against distilled water lightly buffered with Tris to pH 7.0.

Chemical characterization of membranes and membrane extracts. Phospholipid was estimated by Bartlett's variation of the Fiske-SubbaRow phosphate analysis (13). Cholesterol was analyzed by the method of Zak *et al.* (14). The sialic acid content of the membranes was measured as described by Warren (15).

Kinetics of TNBS reaction. The reaction of TNBS with membrane proteins was measured by following the increase in absorbance of the reaction mixture at 335 nm as a function of time. Experiments were usually carried out at 37 ± 0.5° in a total volume of 3.0 ml, containing 1.0 ml of 20 mM Tris buffer (pH 8.0), 0.1 ml of 10 mM TNBS solution (the pH of which had previously been adjusted to 8.0 using NaOH), the divalent cation or drug to be tested in aqueous solution, and water to give a final volume of 2.8 ml. The reaction was initiated by addition of 0.2 ml of membrane suspension (containing 0.5–1.0 mg of protein). The suspension was rapidly mixed and incubated with shaking in an incubator at a temperature of 37 ± 0.5°. In some experiments, when membranes were incubated with the test drug prior to the TNBS assay, the incubation mixture contained membranes, Tris buffer, and water in a total volume of 2.9 ml, and following the incubation period (usually 5 min at 37 ± 0.5°) the TNBS reaction was initiated by adding 0.1 ml of 10 mM TNBS solution. Reactions were terminated by the addition of 2.0 ml of a 1:1 mixture of 1 M HCl and 10% sodium dodecyl sulfate, and the absorbance

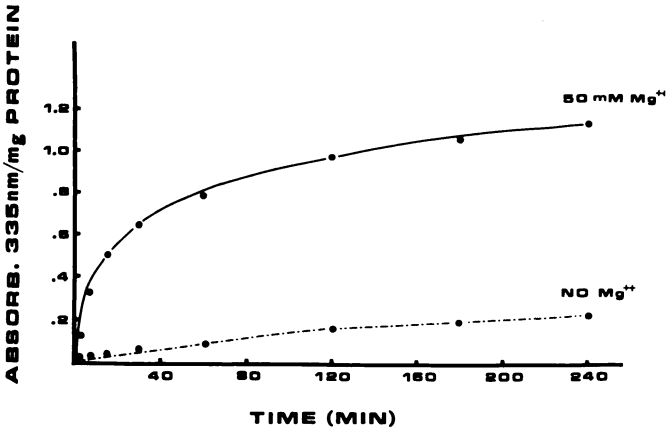

FIG. 1. *Time course of TNBS incorporation into erythrocyte membranes in the presence and absence of Mg^{++}*
The 3.0-ml reaction mixture contained 0.2 ml of membrane suspension, Tris buffer (pH 8.0, 6.67 mM), TNBS (0.33 mM), and divalent cation where indicated. Reactions were terminated by the addition of a mixture of HCl and sodium dodecyl sulfate (see METHODS).

(relative to an appropriate blank without membranes) at 335 nm was measured. In most cases, reaction times of 30–60 sec were found to yield sufficiently large and reproducible absorbance values which increased linearly with time, so that reaction rates were measured during the first minute of the incubation. The reaction rate was found to be a linear function of the protein concentration in the working range of 0.5–1.0 mg of protein per 3.0 ml of reaction mixture. Thus reaction rates were expressed as absorbance at 335 nm per minute per milligram of protein.

Materials. The following chemicals were obtained from Sigma: picrylsulfonic acid (TNBS), 2-thiobarbituric acid, sodium dodecyl sulfate, succinylcholine chloride, Trizma base, and phospholipase C (*Clostridium welchii*). The hydrochlorides of chlorpromazine, promethazine, and procaine were generously provided by Poulenc, Ltd. Aminonaphtholsulfonic acid, acetylcholine chloride and ammonium molybdate were obtained from British Drug Houses. Decamethonium bromide and tetracaine hydrochloride were purchased from K & K Laboratories. The sources of other chemicals were: propranolol, Ayerst; bovine serum albumin, Armour; methylamine, Baker; sodium arsenite, Fisher; hexamethonium chloride, Matheson, Coleman and Bell; and butacaine sulfate, Abbott Laboratories.

RESULTS

Time course of TNBS reaction. The time course of incorporation of TNBS into erythrocyte membrane proteins and the stimulatory effects of Mg^{++} on the incorporation are shown in Fig. 1. The increase in the ionic strength of the reaction medium caused by cation addition was insufficient to explain the enhanced incorporation, as is apparent from the results shown in Fig. 2, where the effects of Na^{+} and Mg^{++} at identical ionic strengths were compared and may be seen to be significantly different.

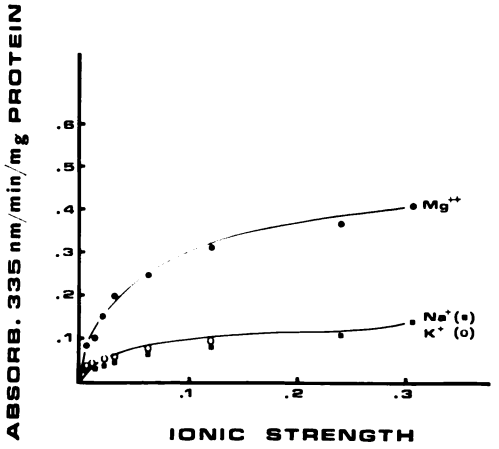

FIG. 2. *Effect of ionic strength increase on TNBS incorporation into erythrocyte membranes*
TNBS incorporation was measured as described in Fig. 1, using reaction times of 30 sec.

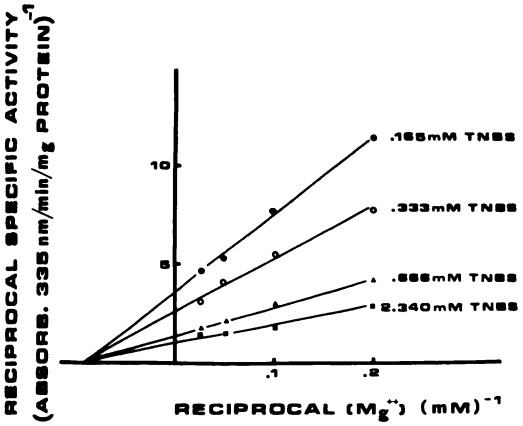

FIG. 3. *Effect of increasing TNBS concentrations on apparent K_{diss} of Mg^{++} for stimulation of TNBS incorporation into erythrocyte membranes*

Experimental details were the same as described in Fig. 2, except that the final concentrations of TNBS and $MgCl_2$ in the reaction medium were varied as indicated in the figure.

One possible explanation for the stimulatory effect of Mg^{++} would be complex formation between the divalent cation and the anionic TNBS molecule, which might in some way enhance the reactivity of the TNBS and/or increase its accessibility to sites of attack. If this were so, the concentration of Mg^{++} exhibiting half-maximal stimulation (the apparent K_{diss} value) would be expected to be dependent on the concentration of TNBS. However, the fact that the K_{diss} of Mg^{++} was unchanged over a 14-fold range of TNBS concentrations (Fig. 3) indicated that the stimulatory effects of Mg^{++} did not depend on complex formation with TNBS.

A direct interaction of Mg^{++} with protein amino groups, the most probable sites of attack of TNBS (3), did not seem likely, since in a simple model system the interaction of TNBS with the amino group of methylamine was unaffected by ionic strength increase in general, and by Mg^{++} in particular.

The remaining alternative was an effect of divalent cation at the level of membrane (or protein) structure.

Analysis of concentration dependence of cation stimulation. The concentration dependence of cation-stimulated TNBS incorporation for a variety of cations was determined. Concentration dependence data were analyzed by means of Eadie plots (16) and were expressed in terms of maximal velocity (V_{max}) and apparent dissociation constant (K_{diss}) values (Table 1A). Typical plots for Mn^{++} (which exhibited two distinct slopes and hence two K_{diss} values) and Co^{++} are illustrated in Fig. 4.

TABLE 1

Cation stimulation of TNBS incorporation

Assays were performed, as described under METHODS, in a total volume of 3.0 ml containing between 0.6 and 0.8 mg of membrane protein. The final concentration of Tris buffer (pH 8.0) was 6.67 mM, and that of TNBS was 0.33 mM. The reaction was carried out at 37 ± 0.5°. The range of cation concentrations tested was between 1.0 and 40 mM. Apparent K_{diss} and V_{max} values were evaluated by means of Eadie plots (for typical plots, see Fig. 4). V_{max} values are expressed as maximal absorbance at 335 nm per minute per milligram of membrane protein.

Cation	A. Control membranes		B. Phospholipase C-treated membranes	
	Apparent K_{diss}	V_{max}	Apparent K_{diss}	V_{max}
	mM		*mM*	
Mn^{++}	2, 7	0.710	2	0.150
Sr^{++}	10	0.510	2	0.140
Mg^{++}	2, 10	0.525	1	0.150
Ca^{++}	3, 9	0.530	2	0.140
Co^{++}	1	0.350	1	0.160
Ni^{++}	0.3	0.170	1	0.100
Cd^{++}	0.6	0.380	1	0.235
Zn^{++}	0.5	0.196	1	0.130

The existence of two groups of cations exhibiting high or low V_{max} values suggested that these cations might be interacting with two different types of membrane sites. The possibility that phosphoryl groups of membrane phospholipids might represent sites of interaction of divalent cations was explored by examining the effects of phospholipase C on the cation stimulatory effects. Phospholipase C treatment, which resulted in removal of 70% of the membrane lipid phosphorus (Table 2), substantially decreased the stimulatory effects of Mn^{++}, Sr^{++}, Mg^{++}, and Ca^{++}, those cations characterized by high V_{max} values in untreated membranes,

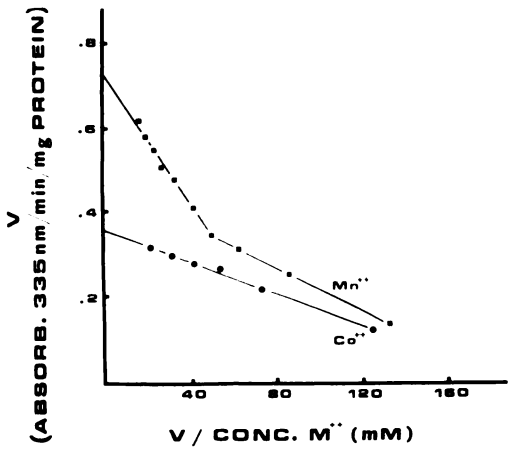

FIG. 4. *Typical Eadie plots for stimulation of TNBS incorporation by Mn^{++} and Co^{++}*

Each point represents the average of triplicate determinations using three different membrane preparations. The values obtained in each case were highly reproducible from preparation to preparation and were not significantly different from each other. Experimental details were the same as described for Fig. 2.

but this treatment had considerably less effect on the behavior of Ni^{++}, Cd^{++}, and Zn^{++}, with Co^{++} occupying an intermediate position (Table 1B). In fact, phospholipase C treatment appeared to abolish the differences between the two groups of cations.

The foregoing results suggested that maximal stimulation by Mg^{++}, Ca^{++}, Sr^{++}, and Mn^{++} required integrity of membrane phospholipid and protein interactions, whereas Zn^{++}, Cd^{++}, and Ni^{++} would interact directly with protein residues to produce the observed alteration in protein reactivity toward TNBS. In order to test this hypothesis further, water-soluble membrane fractions differing in phospholipid content were prepared by butanol or pentanol extraction (17). The chemical compositions of these two fractions are given in Table 2, and it is apparent that the butanol-fractionated material contains only 13% of the original membrane phosphorus, whereas the material derived from the pentanol extraction retains about 60% of the original membrane phosphorus. Since, on the basis of the phospholipase C data, it seemed that the stimulatory effects of Mg^{++} required the integrity of membrane phospholipids—in contrast to the situation with Zn^{++}—it would be predicted that the stimulatory effects of Mg^{++} on TNBS incorporation would be substantially greater in the pentanol-extracted membranes as compared with the phospholipid-depleted, butanol-extracted membranes, but that the effects of Zn^{++}, being rather independent of phospholipid, should be essentially the same in both pentanol-extracted and butanol-extracted membranes. The results illustrated in Fig. 5 are consistent with these predictions.

Role of protein dithiol groups. It is known that certain heavy metal divalent cations, such as Cd^{++}, are able to interact with proximal pairs of protein sulfhydryl groups (18). These effects may be abolished by prior treatment with arsenite, a reagent with a high affinity for dithiols (19). We therefore examined the effects of arsenite treatment on the stimulatory effects of cations on TNBS incorporation. The results of this experiment are shown in Table 3. The stimu-

TABLE 2

Chemical characterization of membrane preparations

Sample	Phospholipid	Cholesterol	Sialic acid	Phospholipid : cholesterol	Sialic acid : phospholipid	Sialic acid : cholesterol
	nmoles/mg protein					
Erythrocytes (untreated)	818	625	87	1.31	0.11	0.14
Phospholipase C-treated	245	662	27	0.37	0.11	0.41
Butanol-extracted	104	336	167	0.31	1.60	0.49
Pentanol-extracted	475	625	164	0.76	0.34	0.26
Muscle	272	229	11	1.19	0.04	0.05
Brain	694	502	53	1.38	0.76	0.11

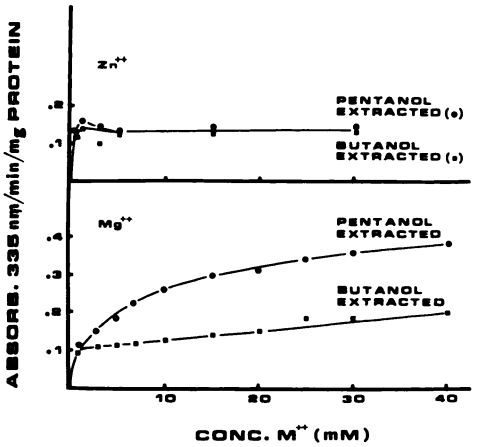

FIG. 5. *Stimulation of TNBS incorporation by Mg^{++} and Zn^{++} into pentanol- and butanol-extracted erythrocyte membranes*

Membranes were washed three times with distilled water and extracted with cold pentanol or butanol (see METHODS). The water-soluble membrane extracts were dialyzed at 4° for 24 hr against distilled water lightly buffered to pH 7.0 with Tris. The protein contents of both extracts were 1.5 mg/ml, and 0.4-ml aliquots (0.60 mg of membrane protein) were assayed as usual for TNBS incorporation.

TABLE 3

Effect of arsenite treatment on stimulatory effects of cations on TNBS incorporation

Membranes (0.87 mg of membrane protein) were incubated in a total volume of 2.6 ml containing 1.0 ml of 20 mM Tris buffer, pH 8.0, and 0.3 ml of 100 mM $NaAsO_2$ for 30 min at 37°. Following this, 0.3 ml of divalent cation (100 mM) and 0.1 ml of TNBS (10 mM, pH 8.0) were added, so that the final concentrations of Tris buffer and TNBS were the same as those in Table 1. The mixture was incubated for 30 sec at 37 ± 0.5°. The reaction was terminated by adding 2.0 ml of HCl (1 M) and sodium dodecyl sulfate (10%), 1:1, and the absorbance at 335 nm was measured.

Cation (10 mM)	Reaction velocity	
	No arsenite	Arsenite-treated
	A_{335}/min/mg protein	
Cd^{++}	0.282	0
Zn^{++}	0.084	0
Co^{++}	0.179	0.061
Ni^{++}	0.076	0
Sr^{++}	0.134	0.693
Ca^{++}	0.197	0.840
Mg^{++}	0.174	0.880

latory effects of Cd^{++}, Zn^{++}, and Ni^{++} were completely abolished by arsenite treatment. In contrast, the effects of Sr^{++}, Ca^{++}, and Mg^{++} were strikingly increased under the same conditions. Co^{++} appeared to occupy an intermediate position, since its activity was decreased but not abolished by arsenite.

Reversibility of cation effects. The reversibility of divalent cation binding giving rise to enhanced TNBS incorporation into erythrocyte membranes was examined. This problem was approached by incubating membranes with increasing concentrations of cations under the same conditions used to measure TNBS incorporation, and at the end of this incubation membranes were washed by centrifugation and were assayed for TNBS incorporation in the absence of any further addition of cation (Fig. 6). These experiments showed that those cations whose binding sites were retained on treatment with phospholipase C and were abolished by arsenite (namely, Cd^{++}, Zn^{++}, and Ni^{++}) were bound much more firmly than Mg^{++} and Mn^{++}. The results with Co^{++} tended to parallel the effects of arsenite treatment, in that Co^{++} exhibited mixed properties, intermediate between the two extremes represented by the other divalent cations.

Other membrane systems. It was of interest to determine whether or not the cation-stimulated incorporation of TNBS and the cation activation patterns observed in the erythrocyte membrane reflected some basic aspect of membrane structure in general. We therefore examined the behavior of brain microsomal membranes and a plasma membrane preparation derived from skeletal muscle.

The results of Mg^{++}-stimulated TNBS incorporation studies are shown in Table 4, and it is apparent that there is a striking similarity in the behavior of the three membrane systems, with the erythrocyte and brain preparations exhibiting almost identical specific activities. The effects of Mg^{++} and Zn^{++} on TNBS incorporation were compared in both the brain and muscle preparations, and the results obtained (Fig.

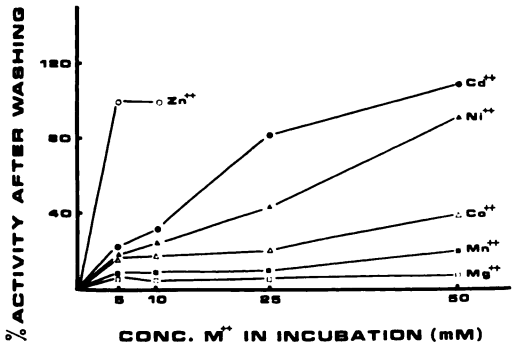

FIG. 6. *Relative strengths of binding of divalent cations to erythrocyte membranes as measured by retention of stimulatory effects on TNBS incorporation following washing*

Membranes were initially incubated in a total volume of 3.0 ml containing Tris buffer, pH 8.0 (6.67 mM), and divalent cation at the final concentration indicated, for 5 min at 37 ± 0.5°. Then the mixture was diluted to 10 ml and centrifuged at 30,000 × *g* and the pellet was resuspended and assayed for TNBS incorporation as usual without any further addition of divalent cations.

TABLE 4

Effect of $MgCl_2$ on incorporation of TNBS into erythrocyte, brain microsome, and skeletal muscle membranes

Mg^{++}	Reaction velocity		
	Erythrocytes	Brain microsomes	Skeletal muscle
mM	*A_{335}/min/mg protein*		
1	0.060	0.069	0.054
5	0.170	0.144	0.089
10	0.191	0.188	0.118
25	0.326	0.272	0.178
50	0.356	0.357	0.210

7) qualitatively paralleled those in the erythrocyte membrane studies.

A comparison of the chemical compositions of the muscle and brain membrane preparations relative to that of erythrocyte membranes (Table 2) revealed that although the phospholipid, cholesterol, and sialic acid contents (all expressed per milligram of membrane protein) vary considerably, the ratio of phospholipid to cholesterol in all three membranes is remarkably constant, being of the order of 1.2–1.4. This may correspond to some particular physical arrangement of phospholipids and cholesterol which is in some way related to the analogous effects of divalent cations on TNBS incorporation in these three different membrane systems.

Effects of pharmacological agents on TNBS incorporation. Since a number of the divalent cations studied appeared to exert stimulatory effects on TNBS incorporation via perturbations induced at the level of membrane phospholipids, the question arose as to whether the interaction of some pharmacologically active molecules with membranes might not also be characterized by similar alterations in phospholipid-protein interactions, and as such be amenable to analysis in terms of TNBS incorporation studies.

Chlorpromazine was found to cause a marked stimulation of TNBS incorporation into erythrocyte membranes at concentrations below 1 mM, and this effect was greatly diminished by phospholipase C treatment (Fig. 8).

Study of a series of local anesthetics revealed a considerable variation in effectiveness in enhancing TNBS incorporation. The results are shown in Fig. 9, and it is apparent

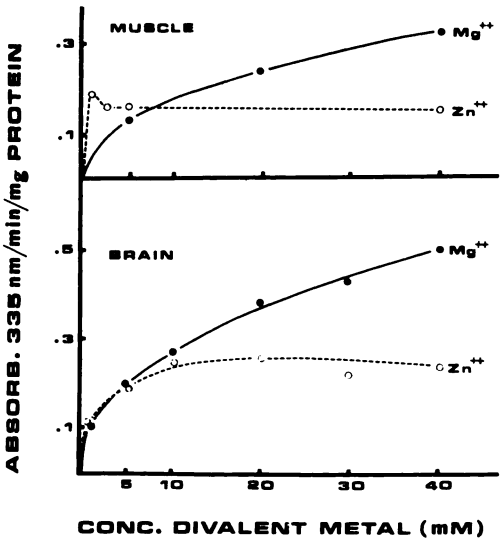

FIG. 7. *Effects of Mg^{++} and Zn^{++} on incorporation of TNBS into bovine brain microsomal membranes and rabbit skeletal muscle plasma membranes*

Experimental details were the same as described in Fig. 2.

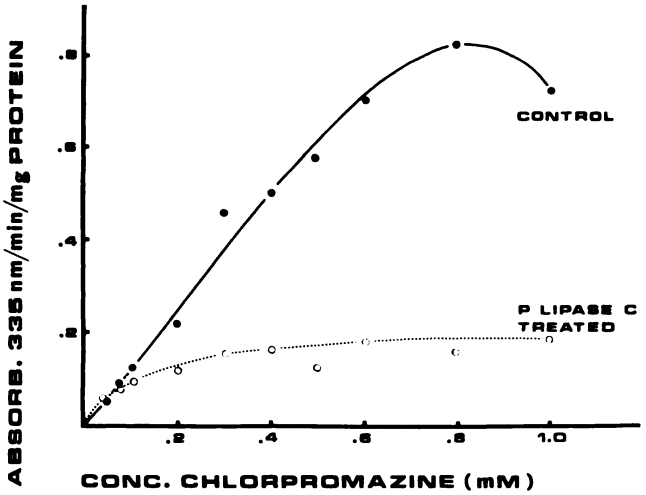

FIG. 8. *Stimulatory effect of chlorpromazine HCl on incorporation of TNBS into untreated and phospholipase C-treated erythrocyte membranes*

The control membranes were treated in the same way as the phospholipase C-treated membranes regarding the incubation in the presence of calcium and the washing procedure (see METHODS), except that the phospholipase C was omitted.

that xylocaine was the least effective member, procaine occupied an intermediate position, and tetracaine and butacaine were the most effective in stimulating the reaction.

The effects of two antiarrhythmic agents, diphenylhydantoin and propranolol, on the TNBS reaction were also determined, and the results showed that although both were able to enhance TNBS incorporation, propranolol was by far the more potent of the two (Fig. 10).

A variety of other compounds were examined with regard to their effects on TNBS incorporation. A homologous series of straight-chain aliphatic alcohols (C_1–C_5) and also benzyl alcohol were all ineffective in stimulating TNBS incorporation in the concentration range of 10–500 mM. Other compounds which proved to be inactive were acetylcholine, succinylcholine, and hexamethonium (at concentrations between 0.5 and 20 mM), as well as decamethonium (in the range 0.5–15 mM).

In order to gain some information regarding the possible role of phospholipids in the stimulatory effects exerted by the various pharmacological agents studied on TNBS incorporation, the ratio of TNBS incorporated into butanol-extracted membranes relative to pentanol-extracted membranes was determined for each drug. A high ratio would be expected for compounds interacting primarily with protein sites, whereas compounds interacting primarily with phospholipid should exhibit a ratio considerably less than 1. The results are summarized in Fig. 11, and Mg^{++} and Zn^{++} have been included for the sake of comparison. As has already been established, the stimulatory effects of Zn^{++} arise from interaction with protein sites, and this is reflected in a ratio very close to unity. All the other compounds were characterized by ratios considerably less than 1, suggesting varying degrees of phospholipid involvement in the interaction of these compounds with the membrane. The actions of chlorpromazine, tetracaine, and butacaine seemed particularly dependent upon integrity of membrane phospholipid-protein interaction on this basis.

DISCUSSION

Although the interaction of small molecules and ions with biological membranes has been studied using a variety of approaches—including binding studies (20, 21), nuclear magnetic (22) and electron paramagnetic (23) resonance, fluorescent probe analysis (24, 25), and erythrocyte membrane stabilization (26)—it has generally not been

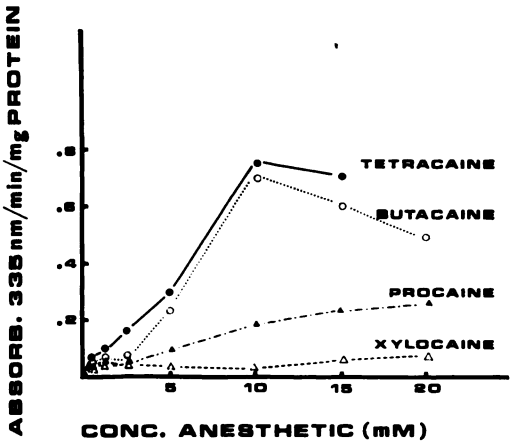

FIG. 9. *Effects of local anesthetics on incorporation of TNBS into erythrocyte membranes*

The local anesthetics were first incubated in a total volume of 2.9 ml, containing membranes (0.87 mg of membrane protein) and Tris buffer, pH 8.0 (6.67 mM), for 5 min at 37 ± 0.5°. At zero time TNBS was added (final concentration, 0.33 mM), and the incorporation was measured as described previously.

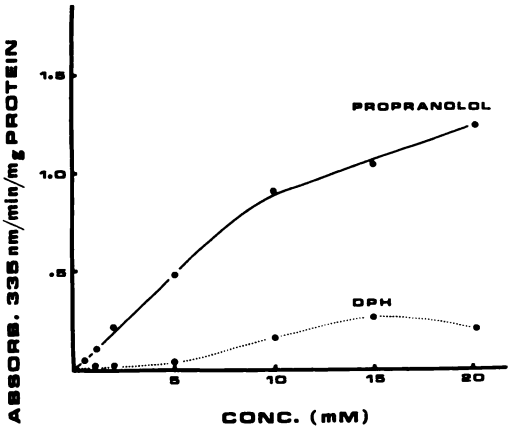

FIG. 10. *Effects of propranolol and diphenylhydantoin (DPH) on incorporation of TNBS into erythrocyte membranes*

Preliminary incubation conditions and the measurement of TNBS incorporation were the same as described in Fig. 9.

possible to obtain information simultaneously regarding the chemical nature of binding sites and the consequences of binding in terms of perturbations of membrane protein and lipid components. The present study represents an approach to this problem.

The rate and extent of trinitrophenylation of erythrocyte membrane amino groups by trinitrobenzenesulfonic acid (3, 27) was used to monitor alterations in membrane structure induced by the binding of divalent cations. Mg^{++} was found to enhance the incorporation of TNBS (Fig. 1), and this effect could not be attributed to ionic strength (Fig. 2) or to the direct interaction of divalent cation with amino groups or with the TNBS molecule (Fig. 3). The effects of divalent cations therefore seemed to be exerted at the level of membrane structural components.

The effects of a variety of divalent cations on enhancement of TNBS incorporation were examined, and the cations fell into two general categories. One group (Mn^{++}, Sr^{++}, Ca^{++}, and Mg^{++}) required integrity of membrane phospholipids for maximal stimulation of TNBS incorporation (Table 1; Figs. 5 and 11). The binding sites for these cations abol-

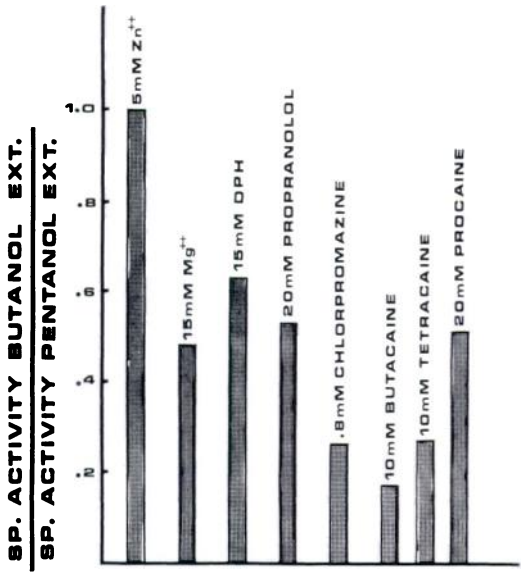

FIG. 11. *Relative stimulatory effects of various agents on TNBS incorporation into butanol- and pentanol-extracted erythrocyte membranes*

Experimental details for the butanol and pentanol extraction of erythrocyte membranes and for the measurement of TNBS incorporation were the same as in Fig. 5. All substances tested were equilibrated with the membrane extracts in Tris buffer for 5 min at 37 ± 0.5° prior to the addition of TNBS. DPH, diphenylhydantoin.

ished by phospholipase C (*C. welchii*) treatment should be largely the phosphoryl groups of lecithin (28). Sialic acid carboxyl groups (29) did not appear to contribute functionally important binding sites for these cations, since although the phospholipase C-treated membranes were depleted of sialic acid (Table 2), the pentanol- and butanol-extracted membranes, which corresponded to the untreated and phospholipase C-treated membranes, respectively, in terms of phospholipid content and susceptibility to Mg^{++} stimulation of TNBS incorporation (Fig. 5), had identical sialic acid contents (Table 2). It should also be pointed out that the maximal incorporation of TNBS into erythrocyte membranes seemed relatively independent of the phospholipid content of the membrane, so that the amino groups of phosphatidylserine and phosphatidylethanolamine probably did not contribute significantly to the incorporation of TNBS.

Perhaps the simplest interpretation of these cation effects would be in terms of charge neutralization, wherein the divalent cation would interact with phospholipid negative charges, allowing greater accessibility of anionic TNBS molecules to adjacent protein sites. If this were so, it might be predicted that phospholipase C treatment should enhance TNBS incorporation, whereas in fact a drastic decrease in TNBS incorporation was observed. Also, no apparent correlation was found between potential membrane anionic binding sites and the stimulatory effects of Mg^{++}, which, despite considerable differences in sialic acid and phospholipid contents (Table 2), were remarkably similar in the three different membrane systems studied (Table 4 and Fig. 7). A striking correlation was found, however, between the phospholipid to cholesterol ratio in different membrane preparations and the stimulatory effects of Mg^{++}, and this suggested that the observed cation effects might be mediated via configurational changes in membrane phospholipids, the physical state of which may be governed by the content and distribution of cholesterol within the membrane matrix (30). It is therefore proposed that the effects of Mg^{++} and the other cations of this group are best rationalized in terms of cation-induced alterations in membrane phospholipids, which in turn give rise to changes in the structure (and reactivity) of membrane proteins.

The other group of cations, comprising Zn^{++}, Cd^{++}, and Ni^{++}, the effects of which were largely unaffected by phospholipase C treatment (Table 1 and Fig. 5) but were abolished by sodium arsenite (Table 3), appeared to act by direct interaction with protein sites, probably via proximal pairs of protein thiol groups (18, 19). The mixed effects of Co^{++} (Tables 1 and 3; Fig. 6) suggested that this cation shared properties in common with both groups of cations. This was significant, since Co^{++} can substitute effectively for Zn^{++} at the active site of certain zinc-containing enzymes (31), but Co^{++} can also interact with the same cation transport system as Mg^{++} in *Escherichia coli* (32).

Support for the validity of this functional approach to the nature of membrane cation-binding sites came from the findings of Jacobus and Brierley (33), who by direct binding studies were able to show that in heart mitochondria Mn^{++}, Ca^{++}, and Mg^{++} interact preferentially with phospholipids, whereas Zn^{++} interacted primarily with protein sites. Furthermore, our findings that the cation activation patterns observed in erythrocyte membranes seem also representative of the behavior of two other membrane systems examined (Table 4 and Fig. 7) suggest that the results obtained here may be relevant to membrane lipid-protein interactions in general.

It was therefore particularly significant that chlorpromazine (Fig. 8) and several local anesthetics (Fig. 9) were effective in stimulating TNBS incorporation into erythrocyte membranes. The importance of membrane phospholipid integrity in the mediation of these effects (Figs. 8 and 11) supports proposals that chlorpromazine and local anesthetics are capable of interacting with membrane phospholipids (34, 35) and indicates that binding of these molecules induces perturbations in membrane lipid and protein components. The greater affinity of chlorpromazine (half-maximal effect at 0.3 mM) compared with calcium (half-maximal effect at 9 mM) for membrane phospholipids is consistent with the observation that chlorpro-

mazine, at concentrations between 0.1 and 1.0 mM, is able to displace Ca^{++} bound to erythrocyte membranes (36). The order of effectiveness of local anesthetics in stimulating TNBS incorporation (tetracaine > butacaine > procaine) is also the order of potency of these compounds in coagulating cephalin sols and as local anesthetics *in vivo* (34).

In attempting to correlate the effects of the pharmacological agents on TNBS incorporation with their pharmacological properties, it was pertinent to consider whether the molecular features determining activity in the TNBS model system corresponded to those responsible for the local anesthetic activity of these compounds (36, 37) or for their membrane-stabilizing properties (26). The fact that aliphatic alcohols, which are known to act as membrane stabilizers, did not affect TNBS incorporation over a wide range of concentrations would suggest that there may be a correlation between the local anesthetic properties of these agents and their ability to stimulate TNBS incorporation. Furthermore, the local anesthetic activity of these compounds may in turn be related to their ability to induce perturbations in membrane structure by interacting with phospholipids, and the differences in the properties of alcohols and local anesthetics as manifested here may be relevant to the proposed differences in the mechanism of action of general compared with local anesthetics (38).

Finally, mention must be made of the functional significance of the proposed link between membrane phospholipid structural alterations and protein structural alterations as described here. Although direct experimental evidence has generally been lacking, the existence of such a functional link, which, for example, could enable cation movements across membranes to be coupled to enzymatic processes within the membrane, has been implicit in most theories of excitation and active cation transport (39, 40). The present studies have provided evidence for such a functional link between membrane lipid and protein components and offer a molecular approach which may eventually enable the interrelationships between membrane structural components to be analyzed in functional terms.

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