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THE BINDING OF POLYAMINES TO PHOSPHOLIPID BILAYERS

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Abstract—Previous studies have shown that amine groups are ototoxic. The interaction between different polyamines and phospholipid vesicles was studied using vesicle aggregation and fluorescence techniques (DPH and ANS as the fluorescence probes). The results showed that the interaction between polyamines (spermine, spermidine and 1,3-diaminopropane) and acidic phospholipids (PS, PE, PI and PIP_2) is an ionic one. The polyamine with the highest positive charges and the phospholipid with the highest content of negative groups showed the strongest ionic interaction. There was no indication of any hydrophobic interaction within the phospholipid bilayer. The strong interaction between amine groups and PIP_2 support the proposal that the latter is crucially involved in aminoglycoside toxicity in the inner ear and kidney.

The aminoglycoside antibiotics are organic bases with multiple amino groups. They are well known for their toxic effects on the inner ear and the kidney [1, 2]. Previous studies have suggested that the initial effect of aminoglycosides is on cell membranes, probably following their binding to polyphosphoinositides which are present in high quantity in inner-ear tissues and kidney [3–6]. It has been proposed that the interaction is between the amine groups of the aminoglycosides and the monoesterified phosphates of the polyphosphoinositides [6] but the complexity of the aminoglycoside structure makes the study of this interaction difficult.

Recently we have demonstrated that polyamines are also toxic to the inner ear [7]. Polyamines are simpler molecules comprising only an alkyl backbone of varying length with multiple amine groups (imino –NH– and amino $-NH_2$ groups) and this makes them a useful tool for investigating the effects of the amine groups themselves. It is known that polyamines, being polycations under physiological conditions, can bind to anionic sites in cells such as DNA, RNA and membranes [8, 9]. Among the effects of polyamines on membranes that have been noted are stabilization of erythrocytes and protoplasts against lysis and inhibition of some enzymes and transport processes [10–13]. Some studies have been done on the interaction of polyamines with phospholipids [14–16] but there has been no comparative study of the effects of the differing number of amine groups on this interaction, especially with reference to polyphosphoinositides. We have therefore studied the ability of the polyamines, diaminopropane, spermidine and spermine, to aggregate vesicles of several phospholipids including the polyphosphoinositides and used fluorescence probes to study the mechanism of their interaction. 1-Anilino-8-naphthalene sulphonate (ANS)‡ is an anionic fluorescent agent that is useful for studying the hydrophilic surface regions of lipid bilayers [17]. On the other hand, 1,6-diphenyl-1,3,5-hexatriene (DPH) is a hydrophobic fluorescent probe widely used to study the physical state of the hydrocarbon chains in the interior of the bilayer [18].

MATERIALS AND METHODS

Materials. Phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol-4,5-bisphosphate (PIP_2), all from bovine brain, together with phosphatidylinositol (PI) from bovine liver, dimyristoylphosphatidylcholine (PC), polyamines and ANS were all from Sigma Chemical Co., Poole, Dorset, U.K. Polyamines were purchased as the free bases and their aqueous solutions were adjusted to pH 7.2 with hydrochloric acid before dilution to the required strength. DPH was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Methods. Phospholipid vesicles, mainly unilamellar and of less than 50 nm in diameter were prepared and fluorescence measurements made on them as previously described [19]. To prevent oxidation of the lipids, butylated hydroxytoluene (1 μg) was added to 10 mg of phospholipid before it was dispersed in water.

To determine the index of polarization (P) fluorescence polarization studies using DPH were carried out as described by Wharton *et al.* [19]. Two sets of experiments were performed. In the first, the concentrations used (phospholipids, 0.5 mg/ml; polyamines, 0.01 mM) were chosen so that no aggregation occurred and the samples remained optically

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‡ Abbreviations: ANS, 1-anilino-8-naphthalene sulphonate; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP_2, phosphatidylinositol-4,5-bisphosphate.

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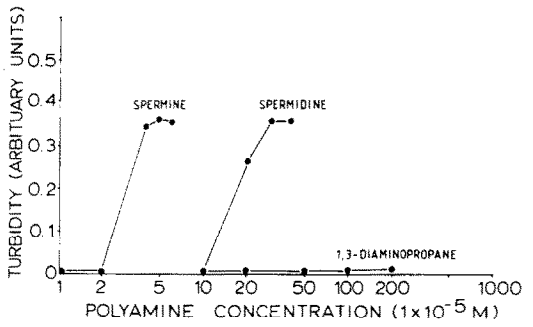

Fig. 1. Effects of polyamines on the aggregation of vesicles of phosphatidylcholine and phosphatidylinositol-4,5-bisphosphate (4:1, w/w). Polyamines were added in increasing concentration to the vesicles (0.25 mg/ml) phospholipid) and the extinction at 400 nm was measured 2 min after each addition.

clear. In the second, in order to maximise any effects, a much higher ratio of polyamine to phospholipid was used (phospholipids, 0.2 mg/ml; polyamines, 0.1 mM). At these concentrations, the spermine and spermidine samples became slightly opalescent so that light scattering could have interfered with the measurements. However, as the two sets of experiments gave essentially the same results, only the second is reported.

The ANS experiments were performed as described by Ma *et al.* [20]. Solutions of polyamines (10 mM) and the ANS (50 μM) were incubated together in 0.01 M Tris buffer, pH 7, for 30 min in the dark. The phospholipid vesicles (0.5 mg/ml) were then added in increments to the required final concentration and the fluorescence spectrum recorded after each addition. The maximum dilution of the sample caused by addition of the vesicles was about 2.5%.

Aggregation of the vesicles (0.5 mg/ml phospholipid except for PC/PIP_2 which was 0.25 mg/ml) was determined from the change in extinction at 400 nm 2 min after addition of the polyamines [15]. To prevent too rapid aggregation of the negatively charged vesicles, mixtures of PS, PI and PIP_2 with PC (1:4 w/w) were used rather than the pure compounds. Vesicles containing lower proportions of acidic phospholipid to PC (1:99 and 1:19 w/w) were also tested but there was no aggregation of any of the vesicles by any polyamine at concentrations up to 2 mM. In addition, a mixture of PC, PE, PS, PI and cholesterol (4:10:5:1:8, by weight) approximating to the contents of the major components of the inner monolayer of the erythrocyte membrane [21] was tested.

RESULTS

Aggregation of phospholipid vesicles by polyamines

Examples of the results obtained in the aggregation experiments are shown in Fig. 1 and the results are summarised in Table 1 where the polyamine concentrations needed to produce half the maximum aggregation (i.e. 50% of the maximum increase in turbidity) are listed. It can be seen that, as expected, no aggregation of the uncharged PC vesicles was obtained at any of the polyamine concentrations tested. In addition, diaminopropane, with two amine groups, caused no measurable aggregation of any of the vesicles even when present in millimolar concentration. Spermine, with four amine groups, is most efficient at aggregating vesicles containing negatively charged phospholipids (PS, PI and PIP_2) since all such vesicles show half maximum aggregation at 50 μM spermine or less. Spermidine, with three amine groups, falls between the two in its aggregating activity. The results obtained with PC and PS containing vesicles agree with those obtained by others [14–16]. Schuber *et al.* also mention that the diamino compound putrescine failed to aggregate negatively-charged vesicles as we found for diaminopropane [15].

The phospholipid most readily aggregated is that with the highest content of negative groups (i.e. PIP_2) and the most effective agent is the one with the highest positive charge (i.e. spermine). However, the spermidine and spermine results show that the addition of a fourth amino group to the molecule increases the effectiveness by a factor of at least five. The similarity of the results obtained with PS and PI are consistent with the finding that their association constants for spermine are close in value [16]. Addition of cholesterol (40 mole %) to the PC/acidic phospholipid mixtures lowered the concentration of spermine needed for 50% aggregation of the dif-

Table 1. Polyamine-induced aggregation of phospholipid vesicles*

Phospholipid	Concentration needed for 50% aggregation (mM)† Diaminopropane	Spermidine	Spermine
PC	>7	>1	>1
PC/PS	>1	>2	0.05 (0.053; 0.046)
PC/PI	>2	>2	0.05 (0.051; 0.047)
PC/PIP_2	>5	0.23 (0.19; 0.27)	0.03 (0.025; 0.03)
PC/PE/PS/PI/cholesterol	>5	0.16 (0.15; 0.17)	0.03 (0.03; 0.034)

* Increasing amounts of polyamines were added to the phospholipid vesicles and the extinction measured as described in Materials and Methods.

† Defined as the concentration at which turbidity reaches half of the maximum value obtained after two minutes incubation with the polyamines [15].

Values are means of determinations made on two separate vesicle preparations, with the individual values in parenthesis.

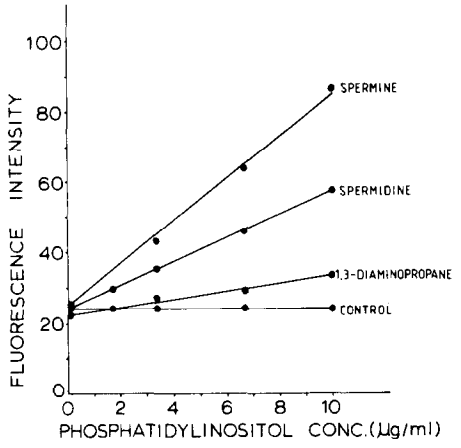

Fig. 2. Effect of phosphatidylinositol on the efficiency of fluorescence of ANS in the presence of polyamines. Increasing amounts of the vesicles were added to a solution of ANS (50 μm) and the appropriate polyamine (10 mM) as described in Materials and Methods. No polyamine was added to the control sample.

ferent vesicles by between 30 and 50% (result not shown). Thus it appears that either the spacing out of the phospholipids by cholesterol facilitates interaction of the polyamines with the negatively-charged head groups or in the ternary systems there is an increased proportion of acidic phospholipid present in the outer leaflet of the vesicle bilayer.

Fluorescence studies. For these experiments, vesicles of pure PS, PI and PIP_2 were used rather than the mixture with PC used for the aggregation studies. The results obtained with the polar fluorescent probe, ANS, are illustrated in Fig. 2 and summarized in Table 2. ANS in aqueous solutions shows a low level of fluorescence and this was not affected by the presence of polyamines. When an uncharged phospholipid such as PC is added to ANS, it binds to the vesicles and there is an increased emission of fluorescence [17, 20]. Because both are negatively-charged, ANS does not bind to vesicles containing PS, PI, or PIP_2 and there is no increase in fluorescence when they are added to the ANS solution (Table 2). When polyamines are added to the system, there is an increase in fluorescence efficiency that is linear with the amount of phospholipid added over the range used (Fig. 2). The slopes of the lines obtained from such plots are given in Table 3. The ability to promote ANS interactions with acidic phospholipids increases in the order diaminopropane, spermidine, spermine and the interaction with each polyamine increases in the order PS, PI, PIP_2, as for their aggregation (Table 1).

The results obtained with DPH, whose fluorescence polarization value (P) gives a measure of the state of the hydrocarbon region of the bilayer, are shown in Table 3. Because the fatty acid compositions of the different phospholipids and the interaction between their head groups differ, the control values, before addition of the polyamines vary. Very little difference was observed between the P values obtained with the different polyamines despite the very large differences seen in the previous experiments. All gave values similar to those of the control preparations. Thus it appears that polyamines do not produce effects on the interior of the bilayer comparable to their effects at the surface. These findings are consistent with work using ANS and hydrophilic fluorescent probes to study polyamine interactions with mitochondria, which are organelles rich in acidic phospholipids [21, 22] and studies of amphipathic monoamine binding to PC, PS and phosphatidylglycerol vesicles [20].

DISCUSSION

The results of both aggregation and ANS experiments confirm that the interaction between polyamines and acidic phospholipids is an ionic one. The strength of the interaction increases with an increase in the number of negative charges on the phospholipids and of positively charged groups on the polyamines. The differences between the three polyamines (Tables 1 and 2) confirm that their effects are not the result of simple charge neutralization. It is probable that the polyamines adsorb to the phospholipids and interact with several negative groups [14–16]. Chung *et al.* have proposed that spermine lies flat on the surface of vesicles of acidic phospholipids and the fluorescence results are consistent with this [16].

When ANS interacts with an uncharged phospholipid such as PC, not only does the fluorescence intensity increase sharply but there is a blue shift of the fluorescence maximum from 510 to 480 nm, as the ring system inserts into the less polar environment of the bilayer [17, 20]. When the acidic phospholipids were added to the aqueous ANS/polyamine solution, the maximum also shifted progressively to 480 nm as the fluorescence efficiency increased. Thus the ANS was not just loosely bound to the surface of the vesicles where it would remain

Table 2. Enhancement of ANS fluorescence by phospholipids in the presence of polyamines*

Phospholipid	Fluorescence enhancement (fluorescence units per μg added phospholipid) No polyamine	Diaminopropane	Spermidine	Spermine
PS	0	0.14	0.58	1.1
PI	0	0.38	1.12	1.9
PIP_2	0	0.70	2.15	4.15

* Phospholipid vesicles were added incrementally to a solution of ANS and the polyamine as described in Materials and Methods.

Table 3. Effect of polyamines on the polarization of diphenylhexatriene fluorescence in phospholipid vesicles*

Phospholipid	Fluorescence polarization value (P) No addition	Diaminopropane	Spermidine	Spermine
PS	0.29	0.25	0.27	0.29
PI	0.19	0.17	0.18	0.19
PIP_2	0.21	0.20	0.24	0.24
PC/PE/PS/PI/cholesterol	0.32	0.33	0.32	0.32

* 2.5 μg DPH were added to 2 mg of phospholipid (1 mg for PIP_2) in 10 ml water and the polarization of its fluorescence was measured as described in Materials and Methods before and after addition of 0.1 mM polyamine (0.05 mM for PIP_2).

in a polar environment but it is able to insert into the bilayer in the same way as it does with PC in the absence of polyamine. There is no indication from the DPH experiments of any hydrophobic interaction within the bilayer. There was no quenching of the fluorescence when polyamines were added which would suggest that the probe had been displaced from its hydrophobic binding sites by the polyamines [20] and the P values did not reflect the interactions shown in Tables 1 and 2. Thus there is no evidence of any penetration of the centre of the bilayer by the inter-amine hydrocarbon segments of the polyamines.

It was shown previously that polyamines with a higher number of amine groups, when injected into the middle ear of the guinea pig, are more cochleotoxic than the polyamines with a lower number of amine groups in the molecule [23]. The aim of this work was to compare the interaction between polyamines and phospholipid vesicles with their previously determined cochleotoxicities. The findings indicate that the two phenomena are similar. On an equimolar basis, spermine is most cochleotoxic, followed by spermidine and then 1,3-diaminopropane. This is the same order as that seen in the aggregation and the ANS experiments with the polyamines and acidic phospholipids. Both are related to the number of amine groups present in the molecule. These results support the theory that aminoglycosides, a group of polyamino compounds, exert their well-known ototoxic effects by interaction between the amino groups and the phospholipid of the cell membrane. Several groups have studied the binding of aminoglycosides to biological membranes [24], to phospholipid monolayers [25], and to phospholipid bilayers [26]. It has been reported that there is a high degree of correlation between the ototoxic potential of an aminoglycoside and its ability to interact with monomolecular films of polyphosphoinositides [25].

The present results together with those previously cited establish that any compound with multiple amine groups will interact strongly via ionic forces with any acidic phospholipid. It can be seen from Table 1 that polyamines interact with vesicles containing a lipid mixture similar to that of the major lipids of the inner leaflet of the erythrocyte membrane [27] and this is generally taken as the pattern for other mammalian cells. To explain the ototoxicity and nephrotoxicity of the polyamino aminoglycosides, it is argued from these results that the tissue specific toxicity cannot result from general binding to negatively charged phospholipids but must be a consequence of a specific interaction. The phospholipid that shows the most marked effects with polyamines is the most acidic, PIP_2. This phospholipid is currently of great interest because of its role in mediating cell responses to a variety of physiological stimuli [28]. As this phospholipid is present in high concentration in the kidney and inner ear, it has been proposed to be crucially involved in the toxicity of aminoglycosides in these tissues [3–6, 16, 25, 29]. Our results with the polyamine drug analogues support this proposal.

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