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Exogenous polyamines alter membrane fluidity in bean leaves – a basis for potential misinterpretation of their true physiological role

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Abstract. Changes in the rotational motion of paramagnetic and fluorescent lipid-soluble probes were used to assess the effects of putrescine, spermidine and spermine on the fluidity of microsomal membranes from primary leaves of bean (*Phaseolus vulgaris* L.). Surface probes were more strongly immobilized by physiological concentrations of the polyamines than probes that partitioned deep into the bilayer interior. Spermidine and spermine were more effective than putrescine at reducing membrane fluidity, and at equimolar concentrations, the polyamines and calcium had similar effects on the mobility of the membrane probes. Spermine had essentially equivalent effects on the fluidity of native membranes, heat-denatured membranes and liposomes prepared from the total lipid extract of the membranes, indicating that polyamines associate with membrane lipid. These results raise the possibility that some of the physiological effects previously attributed to exogenously added polyamines could reflect membrane rigidification rather than a true physiological response.

Key words: Membrane fluidity – *Phaseolus* (polyamine effects) – Polyamine – Senescence.

Introduction

Polyamines appear to be localized in the vacuole or cytosol of plant cells (Bagni et al. 1981). Endogenous levels are normally high in young, actively growing plant tissues and decline with age (Bagni et al. 1981; Kaur-Sawhney et al. 1982; Palavan and Galston 1982), and it has been suggested that they may constitute a new class of hormones (Galston and Kaur-Sawhney 1982). They elicit a variety of physiological responses ranging from promotion of growth and cell division (Bachrach 1973; Huhtinen et al. 1982) to retardation of senescence (Altman 1982). Most of the physiological properties presently ascribed to polyamines have been inferred from experiments in which these compounds were applied exogenously to tissues. It has been reported, for example, that exogenous di- and polyamines retard protein degradation, inhibit chlorophyll loss and minimize the rise in ribonuclease activity normally accompanying senescence of detached leaves maintained in darkness (Kaur-Sawhney et al. 1977; Galston et al. 1978; Shih et al. 1982). Exogenous polyamines have also been shown to reduce the surge in ethylene production observed during dark- or wound-induced senescence (Apelbaum et al. 1981; Suttle 1981; Ben-Arie et al. 1982; Even-Chen et al. 1982).

Recently, Birecka et al. (1984) posed the possibility that exogenous polyamines may exhibit nonspecific effects that are perhaps unrelated to the physiological roles of their endogenous counterparts. This may be particularly true of those properties of polyamines attributed to an effect at the level of membranes including reduced leakage, amelioration of lipid peroxidation, retardation of senescence and inhibition of ethylene production (Naik and Srivastava 1978; Kitada et al. 1979; Altman 1982; Ben-Arie et al. 1982), for at physiological pH, polyamines are fully protonated, polycationic (Morris and Harada 1980) and presumably able to associate non-specifically with negatively charged phospholipids. In the present study, we provide biophysical data demonstrating that concentrations of polyamines known to elicit physio-

Abbreviations and symbols: DPH = diphenylhexatriene; Hepes = 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; I-1,14 = 16-doxylstearic acid; TMA-DPH = trimethylammonium diphenylhexatriene; 18-NP = 3-(octadecylaminomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl; τ_c = rotational correlation time

logical effects in plant tissue selectively rigidify the surface of membranes. This information strengthens the possibility that some proportion of polyamines added exogenously to tissue may be inducing a physiological effect by acting non-specifically on the plasmalemma without gaining access to the cell cytoplasm.

Material and methods

Plant material. Seeds of *Phaseolus vulgaris* L. cv. Kinghorn (Ontario Seed Co., Waterloo, Ont. Can.) were germinated in a mixture of sand, peat and soil (1:1:2, by vol.) and grown under greenhouse conditions at 22–27° C with photoperiods extended to 16 h using Westinghouse (Ellis-Howard Ltd., Kitchener, Ont. Can.) fluorescent Agro-lights to give an irradiance of 20 $W \cdot m^{-2}$ at plant height.

Isolation of microsomal membranes. Primary leaves of bean seedlings (9 d old) were rinsed with distilled water, blotted dry and cut into strips $\approx$1 cm in width. Approximately 100 *g* of cut leaf tissue were homogenized at 4° C for three 5-s bursts, separated by 1-min cooling periods, in a Sorvall Omnimixer (Dupont Instruments, Newtown, Conn., USA) containing 150 ml of 50 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (Hepes; Sigma Chemical Co., St. Louis Mo., USA) buffer (pH 7.0), 1 mM polyvinylpyrrolidone (PVP-10; Sigma), 0.1 mM dithiothreitol (DTT; Sigma) and 0.4 M sucrose. The slurry was passed through four layers of cheesecloth and centrifuged at 10000 *g* for 10 min.The supernatant was centrifuged at 113000 *g* for 1 h to obtain microsomal membranes. The pelleted membranes were washed by resuspension in 2 mM Hepes buffer (pH 7.0) and centrifugation at 136000 *g* for 1 h. The final pellet was resuspended in an equivalent weight of 2 mM Hepes buffer at pH 7.0 and used directly for the biophysical measurements.

Lipid extraction and preparation of liposomes. Total lipids were extracted from the isolated microsomal membranes in chloroform:methanol (2:1, v/v) as described by Bligh and Dyer (1959), evaporated to dryness, and taken up in chloroform. To prepare liposomes, 200 µl of the chloroform solution (50 mg lipid/ml) were placed in a test tube (18 mm diameter, 150 mm high), the chloroform was evaporated with nitrogen at 30° C, and the tube placed under vacuum overnight. At the end of this period, 10 ml of 2 mM Hepes buffer (pH 7.0) were added, and the lipids were hydrated under nitrogen overnight and suspended by vortexing. The resulting liposomes were pelleted by centrifugation at 136000 *g* for 1 h and used directly for biophysical studies.

Measurements of fluidity. Electron-spin-resonance spectra were recorded on a Varian (Palo Alto, Cal., USA) E-12 spectrometer equipped with a Varian temperature control accessory. To label the isolated membranes, 5 µl of 2 mM 16-doxyl stearic acid (I-1,14), 5-doxylstearic acid (I-12,3) or 3-(octadecylaminomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyl-oxyl (18-NP) in 100% ethanol were added to a small test tube (6 mm diameter, 50 mm long) and evaporated to dryness. Seventy-five µl of microsomal suspension were added, and the contents were gently mixed by vortexing for 10–20 s. Specified amounts of polyamines (Sigma) or $CaCl_2$ were included in the microsomal suspensions as required. For membranes labelled with I-1,14 or 18-NP, values for the rotational correlation time (τ_c) were calculated according to the following equation:

$$\tau_c = 6.9 \cdot 10^{-10}\, w_1[(h_1/h_{-1})^{1/2} - 1]\, s$$

where w_1 and h_1 are the width and height of the low-field spectral line and h_{-1} the height of the high-field spectral line (Rule et al. 1979). For membranes labelled with I-12,3, an order parameter (S) was calculated according to the following equation:

$$S = \frac{A_{\parallel} - A_{\perp}}{A^c_{zz} - (A^c_{xx} + A^c_{yy})}$$

where $A_{\parallel}$ equals 1/2 A_{max}, and $A_{\perp}$ equals 1/2 A_{min} + 0.86 (Hubbell and McConnell 1971). A_{max} and A_{min} are the maximum and minimum hyperfine splittings in gauss and were calculated as illustrated in Fig. 1.

The labelling conditions used in this study were similar to those used previously (Pauls et al. 1980; Legge et al. 1982) and yielded a membrane phospholipid:spin label ratio of 200:1. No spin-spin interactions were detected at this concentration of spin label. This was confirmed by experiments in which lower (100:1) and higher (500:1) ratios of phospholipid:spin label were used. The line shapes of spectra obtained at these ratios were indistinguishable from those for spectra obtained at a phospholipid:spin label ratio of 200:1, and values of τ_c (in the case of I-1,14 and 18-NP) and S (in the case of I-12,3) were virtually identical for all three ratios.

Liposomes and isolated microsomes were also labelled with diphenylhexatriene (DPH) and trimethylammonium diphenylhexatriene (TMA-DPH) in order to measure fluidity by fluorescence polarization. Stock solutions of DPH (2 mM) and TMA-DPH (2 mM) were prepared in tetrahydrofuran and dimethylsulfoxide, respectively. Samples for polarization measurements were prepared by diluting appropriate aliquots of the stock solutions 1:1000 (v/v) with 2 mM Hepes buffer (pH 7.0), which were mixed 1:1 (v/v) with 2 ml of membrane suspension (100 µg protein in 2 mM Hepes buffer, pH 7.0) or liposome suspension (50 µg lipid in 2 mM Hepes buffer, pH 7.0). When required, the microsome and liposome suspensions also contained putrescine, spermidine, spermine or $CaCl_2$ at specified concentrations. Polarization measurements were made with an SLM 8000 polarizing spectrofluorometer (SLM, Urbana, Ill., USA) as previously described (Thompson et al. 1982; Mayak et al. 1983).

Protein was measured as described by Bradford (1976).

Results and discussion

Two spin labels, I-1,14 and 18-NP, which probe the interior and headgroup regions of the membrane bilayer, respectively, were used to measure the effects of polyamines on the fluidity of microsomal membranes. Once partitioned into membranes, these spin labels give rise to a 3-line ESR spectrum (Fig. 1), which is indicative of relatively isotropic motion and from which a rotational correlation time (τ_c) can be calculated (Pauls et al. 1980). Values of τ_c for membranes labelled with I-1,14, which reports from deep in the bilayer, were not affected when putrescine, spermidine or spermine were present in the membrane suspension at a concentration of 50 mM (Table 1). However, the values of τ_c for membranes labelled with 18-NP, which probes the headgroup region of the bilayer, were raised substantially in the presence of polyamines or 50 mM Ca^{2+} (Table 1). Moreover, sper-

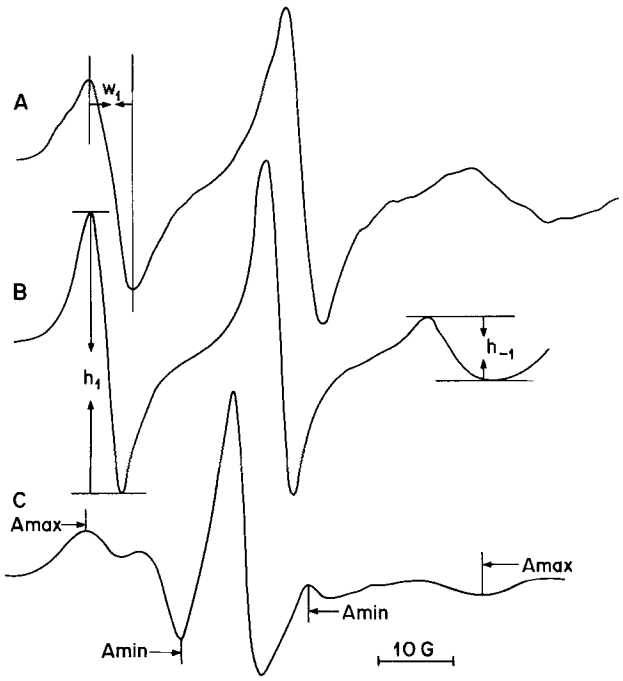

Fig. 1 A–C. Electron-spin-resonance spectra of lipid-soluble probes partitioned into microsomal membranes isolated from primary leaves of bean. **A** 18-NP; **B** I-1,14; **C** I-12,3. Spectra were recorded at 25° C

midine and spermine, which have three and four amino groups respectively, as well as Ca^{2+}, had a significantly greater effect on τ_c than the diamine, putrescine. Thus it is clear that polyamines are able to rigidify membranes, but like Ca^{2+} (Table 1 and Legge et al. 1982) they have their greatest effect at the membrane surface. This is consistent with the fact that polyamines are fully protonated and cationic at pH 7.0 (Morris and Harada 1980) and thus presumably able to associate with negatively-charged phospholipid headgroups.

The effects of amines on microsomal membranes that had been labelled with 5-doxylstearic acid (I-12,3) were also determined. This spin label probes the hydrocarbon region just beneath the phospholipid headgroups and gives rise to a 2-line spectrum from which an order parameter (S) can be calculated (Fig. 1). Values for S were also increased in the presence of amines, and again the effects were greater for the polyamines, spermidine and spermine, than for the diamine, putrescine (Table 1). However, the degree of rigidification detected by I-12,3 was not as great as that detected by 18-NP (Table 1). 5-Doxylstearic acid probes just beneath the headgroup region of the bilayer, whereas 18-NP probes right in the headgroup region. Accordingly, the relative effects of the amines on the motion parameters determined with I-12,3 and 18-NP, respectively, support the contention that the amines are associating with the phospholipid headgroups and causing rigidification primarily at the bilayer surface.

Table 1. Effects of polyamines and calcium on rotational correlation times (τ_c) and an order parameter (S) for spin probes 18-NP, I-1,14 and I-12,3 in microsomal membranes isolated from primary leaves of bean

Treatment	18-NP		I-12,3		I-1,14
	$\tau_c \times 10^9$ (s)	Increase (%)	(S)	Increase (%)	$\tau_c \times 10^9$ (s)
Control	2.04[d]*	–	0.799[b]	–	1.58[a]
Putrescine (50 mM)	2.21[c]	8	0.827[a]	3	1.52[a]
Spermidine (50 mM)	2.50[b]	23	0.839[a]	5	1.59[a]
Spermine (50 mM)	2.46[b]	21	0.846[a]	6	1.56[a]
Calcium (50 mM)	2.62[a]	28	n.d.		n.d.

* Means not followed by the same letter are significantly different at $P \leq 0.05$ using Duncan's New Multiple Range Test (Ott 1977). n.d. = not determined

Table 2. Effects of polyamines and calcium on fluorescence polarization values (P) for DPH and TMA-DPH in microsomal membranes isolated from primary leaves of bean

Treatment	DPH		TMA-DPH	
	P	Increase (%)	P	Increase (%)
Control	0.29[d]*	–	0.31[d]	–
Putrescine (50 mM)	0.31[c]	6.3	0.33[c]	8.3
Spermidine (50 mM)	0.32[b]	10.7	0.36[b]	18.0
Spermine (50 mM)	0.33[b]	11.2	0.39[a]	24.0
Calcium (50 mM)	0.34[a]	16.4	0.36[b]	18.0

* Means not followed by the same letter are significantly different at $P \leq 0.05$ using Duncan's New Multiple Range Test (Ott 1977)

Two fluorescent probes, DPH and TMA-DPH, were also used to determine the effects of polyamines on microsomal membrane fluidity. The DPH assumes a uniform distribution throughout the bilayer, whereas TMA-DPH remains anchored at the membrane surface and selectively probes in the region of the phospholipid headgroups (Prendergast et al. 1981). It is clear from Table 2 that

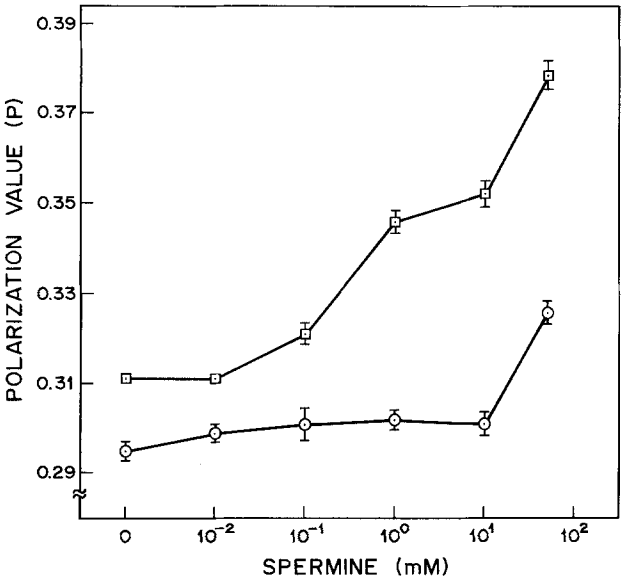

Fig. 2. Effects of spermine on the polarization values for TMA-DPH- and DPH-labelled microsomal membranes from primary bean leaves. (□), TMA-DPH; (○) DPH. The vertical bars represent means ±1 SE when it exceeds the size of the symbol

the polarization values for membranes labelled with TMA-DPH are markedly increased by treatment of the membranes with 50 mM putrescine, spermidine or spermine as well as by treatment with 50 mM Ca^{2+}, and the rigidifying effect was, again, greater for polyamines and Ca^{2+} than for the diamine, putrescine. Polyamines and Ca^{2+} also raised the polarization value for membranes labelled with DPH. However, for 50 mM spermidine and spermine the extent of rigidification registered by DPH, which assumes a uniform distribution throughout the bilayer, was only about half of that registered by the surface probe, TMA-DPH (Table 2). This finding supports the contention that the polyamines selectively rigidify the membrane surface. The observation that polyamines had a measurable effect on DPH polarization but no effect on τ_c of membranes labelled with I-1,14 (Tables 1, 2) reflects the fact that I-1,14 specifically probes deep in the membrane interior, whereas DPH assumes a uniform distribution throughout the bilayer (i.e. from the surface to deep in the bilayer) and thus provides an average measurement of fluidity.

The effects of varying concentrations of spermine over a range (0.01–10 mM) that embraces endogenous levels found in tissues as well as exogenous levels reported to elicit physiological effects are illustrated in Fig. 2. As the concentration of spermine was increased from 0.01 mM through 0.1, 1, 10 and 50 mM, the polarization values for membranes labeled with TMA-DPH rose progressively, whereas there was virtually no effect on DPH-labelled membranes except at the highest (50 mM) concentration (Fig. 2). Spermine at a concentration of 1 mM increased the polarization value for TMA-DPH-labelled membranes by 10% (Fig. 2) whereas the same concentrations of spermidine and putrescine were found to increase the polarization of TMA-DPH-labelled membranes by 8% and 3%, respectively. (The effects of 1 mM spermine and spermidine on polarization were significant at $P \leq 0.05$ based on Duncan's New Multiple Range Test (Ott 1977), but the effect of 1 mM putrescine was not.) Thus at physiological concentrations as well, the polyamines are better able to rigidify membranes than the diamine, putrescine.

In two recent reports, specific effects of polyamines on membranes through interactions with membrane proteins have been described. Ballas et al. (1983) have reported that polyamines stabilize erythrocyte membranes through an association with cytoskeletal proteins on the cytoplasmic surface of the membranes. Schindler et al. (1980) demonstrated that the lateral mobility of erythrocyte membrane proteins was decreased by polyamines. However, the decrease in mobility was not accompanied by a change in lipid microviscosity, and the effect was only observed when polyamines were inside the cells and had associated with cytoskeletal proteins (Schindler et al. 1980). In our study, the prospect that a protein component is required for induction of the selective rigidifying effects of polyamines on microsomal membranes was examined by treating heat-denatured membranes and liposomes prepared from total lipid extracts of microsomal membranes with polyamines. Polarization values obtained using TMA-DPH to probe native membranes, heat-denatured membranes and the liposomes from lipid extracts were all raised to an essentially comparable degree by treatment with 20 mM spermine (Table 3). These observations indicate that proteins are not required for polyamine-induced rigidification of the membrane surface. Rather, the change in fluidity appears to be a nonspecific effect of the polyamines incurred when they act as polyvalent cations and associate with the negatively charged headgroups of the bilayer phospholipid. The difference in fluidity between liposomes and the membranes from which they were derived (Table 3) can presumably be attributed to the removal of protein and possibly, as well, to some rearrangement of the lipids.

The liposome data also indicate that polyamines are able to nonspecifically alter the fluidity of various types of membranes, the plasmalemma

Table 3. Effects of spermine on fluorescence polarization values for TMA-DPH in microsomal membranes, denatured microsomal membranes and liposomes of total lipid extracts from microsomal membrane of primary bean leaves

Material and treatment	P^a	Increase (%)
Microsomal membranes		
Control	0.31	12.0
+20 mM spermine	0.35	
Denatured membranes:		
Control	0.34	9.5
+20 mM spermine	0.37	
Liposomes		
Control	0.35	13.0
+20 mM spermine	0.40	

[a] The effects of spermine on P were significant in each case at $P \leq 0.05$, using Duncan's New Multiple Range Test (Ott 1977)

as well as cytoplasmic membranes. Microsomes are small vesicles of membrane derived primarily from endoplasmic reticulum but also from other organelles including the plasmalemma. Thus a microsomal fraction could be expected to contain different types of membrane, which would have distinguishable lipid compositions that would be averaged when liposomes were formed from lipid extracts. The surface-rigidifying effect of polyamines on liposomes can be interpreted as reflecting an association with negatively charged headgroups of phospholipids. Cytoplasmic membranes as well as the plasmalemma contain phospholipids with negatively charged headgroups, and thus irrespective of their precise lipid compositions, these membranes would all be affected if polyamines were to associate with these negatively charged moieties.

The apparent ability of polyamines to stabilize membranes by inducing rigidification at the bilayer surface offers an explanation for some of their reported effects on plant tissues. For example, polyamines at concentrations within the range shown to selectively rigidify membrane surfaces have been reported to stabilize protoplasts against post-isolation lysis (Altman et al. 1977) and thermal damage (Hasnain et al. 1979) and to decrease betacyanin efflux from discs of beet root (Naik and Srivastava 1978). Each of these phenomena reflects membrane damage, and the mitigating effects of polyamines can presumably be directly attributed to their ability to rigidify the membrane surface, which may in turn retard membrane deterioration (Lieberman and Wang 1982). Exogenous polyamines have also been shown to delay the senescence of detached leaves (Popovic et al. 1979; Altman 1982; Fuhrer et al. 1982) and to delay fruit ripening (Ben-Arie et al. 1982), and it has been suggested that these anti-senescence effects also reflect stabilization of membranes (Popovic et al. 1979; Ben-Arie et al. 1982; Fuhrer et al. 1982). Polyamines also protect membranes against the degradative effects of phospholipase (Sechi et al. 1978) and delay the progressive rise in membrane microviscosity attributable to chemical changes in the bilayer that normally accompany senescence (Ben-Arie et al. 1982; Mayak et al. 1983). Indeed, the antisenescence effects of polyamines are not unlike those of Ca^{2+}, which is thought to delay fruit ripening and the onset of senescence in part by rigidifying membranes (Legge et al. 1982). The change in membrane fluidity accompanying natural senescence is to be distinguished from that induced by the association of exogenous polyamines with membranes in that the former is chemically mediated and occurs throughout the bilayer rather than selectively at the surface. Each of putrescine, spermidine and spermine is able to alter membrane fluidity, although the propensity of the polyamines, spermidine and spermine, which contain three and four amine groups, respectively, to rigidify the membrane surface is greater than that of the diamine, putrescine.

What remains in contention is whether such effects are true and specific physiological responses to polyamines. Amines at physiological concentrations selectively rigidify membrane surfaces (Fig. 2), and this indicates that endogenous polyamines, like Ca^{2+}, may contribute to the maintenance of membrane integrity in situ. There is considerable evidence based on measurements of polyamine biosynthetic enzymes and the use of inhibitors of polyamine biosynthesis that amines are involved in cell division (Cohen et al. 1982; Smith et al. 1985; Walker et al. 1985) and are required for somatic embryogenesis (Fienberg et al. 1984; Feirer et al. 1984). Although there is no evidence that these physiological properties of polyamines are attributable to their ability to alter membrane fluidity, this possibility is not precluded inasmuch as fluidity changes of this magnitude can alter the activity of membrane-associated enzymes (Salasse et al. (1982). However, the pronounced effects of polyamines on membrane fluidity also raise the possibility that physiological effects inferred exclusively from experiments in which they are applied exogenously may not reflect a true physiological response. To put this in perspective, unlike many metabolites added exogenously, which have no effect if they do not gain entry to the cell interior, polyamines could impact on physiological phenomena, whether they are absorbed or not, by as-

sociating with the plasmalemma and inducing bilayer rigidification. These findings indicate, therefore, that inferences concerning the physiological role of polyamines gleaned from experiments in which they are added exogenously to tissue should be buttressed by parallel in-situ experiements in which endogenous levels of polyamines are altered by, for example, the use of inhibitors.

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