Biochimica et Biophysica Acta, 1024 (1990) 345–351
Elsevier

BBAMEM 74864

Plant sterol inhibition of abscisic acid-induced perturbations in phospholipid bilayers

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(Received 14 December 1989)

Key words: Abscisic acid; Sterol; Sitosterol; Campesterol; Phospholipid bilayer; Membrane

Abscisic acid (ABA)-induced phospholipid bilayer perturbations (permeability and lipid vesicle aggregation) are shown to be reversed by incorporation of a commercially available mixture of plant sterols (60% β-sitosterol, 27% campesterol and 13% dihydrobrassicasterol) into the membranes. As little and 5 membrane mol% plant sterol inhibits ABA-stimulated permeability of both saturated and unsaturated mixed phosphatidylcholine/phosphatidylethanolamine bilayers to the fluorescent anion carboxyfluorescein by more than 50%. The same conclusion was reached by an osmotic swelling technique for the uncharged permeant solute erythritol. Hormone-induced carboxyfluorescein permeability to mixed acyl chain phosphatidylcholine bilayers was similarly inhibited by the sterols, but only if the membranes were tested at a temperature where liquid crystal and gel states coexist. The plant sterols were also shown to prevent the ABA-induced fusion of mixed phosphatidylcholine/phosphatidylethanolamine bilayers. The ABA effect on membranes is inhibited equally by plant sterols as well as cholesterol. From these experiments a possible role is suggested for plant sterols in controlling the mode of action of ABA.

The nature of the initial site of action of ABA on plant membranes remains a matter of conjecture. The continued failure to conclusively identify any proteinaceous binding proteins or receptors for ABA [1] in responsive tissues (e.g., guard cells [2]) has led us to suggest that ABA affects membranes through interactions with membrane lipids [3]. Experiments with phospholipid bilayers have demonstrated that ABA can indeed severely alter the basic membrane properties of permeability [3–8], lipid vesicle aggregation [9] and fusion [10], without substantially modifying fluidity and order within the membranes [3]. From analysis of the phospholipid compositional dependency of the ABA responses, we have proposed that the hormone's initial site of action is at regions of membrane defects which result from either two different phospholipid head group types or single component head group lipids containing different acyl chains in which liquid crystal and gel states coexist [3]. Although these lipid bilayer experiments have tested a wide variety of phospholipids, to date no attempts have been made to measure the influence of plant sterols on the ABA–lipid interactions.

From a wide variety of experiments primarily on lipid bilayers, it has been shown that the animal sterol cholesterol can greatly alter membrane properties such as bilayer fluidity, compressibility, permeability and hydration, acyl chain motion, phospholipid phase transitions and lipid domain formation [11]. Because of the plethora of sterol effects, it has been predicted that cholesterol may play a 'dynamic regulatory function in biological membranes' [11]. Plant sterols have received far less attention but the obvious similarity to the

Abbreviations: ABA, abscisic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPC, dimyristoylphosphatidylcholine; DMPE, dimyristoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; CF, carboxyfluorescein; DPH, 1,6-diphenyl-1,3,5,-hexatriene; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylethanolamine; Rho-PE, *N*-(lissamine rhodamine-B-sulfonyl)dioleoylphosphatidylethanolamine; MLV, large multilamellar vesicle; SUV, small unilamellar vesicle.

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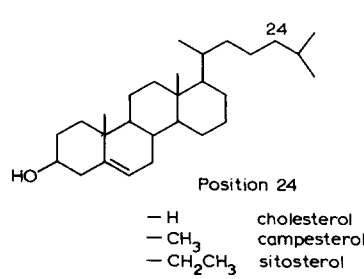

Structure I.

structure of cholesterol and the parallel effects of both plant and animal sterols on membrane properties such as molecular packing [12,13] and permeability [14,15], interaction with phospholipids [11] and polyene antibiotics [16], and uptake into natural membranes [11] indicates that many of the observations and conclusions made for cholesterol would also hold for sitosterol, stigmasterol and campesterol, the predominant plant sterols [17]. Here we demonstrate that the ABA-induced enhancement in bilayer permeability and vesicle fusion can be inhibited by plant sterols. This is consistent with the possibility that plant sterols may affect the existence or extent of membrane defects we propopse are required for ABA action.

Materials and Methods

Materials. The phospholipids DMPC, DMPE, DOPC, DOPE, DPPC, soy PC and soy PE and the fluorescent membrane probes NBD-PE and Rho-PE were purchased from Avanti Polar Lipids, Pelham, AL. The plant sterol mixture (composed of 60% β-sitosterol, 27% campesterol and 13% dihydrobrassicasterol [18]) and the hormone (±)-*cis-trans*-ABA were supplied by Sigma Chemical Co., St Louis, MO. Carboxyfluorescein was obtained from Eastmen Kodak Co., Rochester, NY and the fluorescent membrane probe DPH from Molecular Probes, Eugene, OR.

Lipid vesicles. Three types of lipid vesicles were used: large multilamellar vesicles (MLV) for the erythritol permeability studies; small unilamellar vesicles (SUV) for the fluidity and fusion assays; and large unilamellar vesicles (LUV) for the carboxyfluorescein release experiments. Appropriate phospholipids, sterols and fluorescent membrane probes were mixed in organic solution. They were then dried under nitrogen and vacuum pumped overnight to remove traces of any carrier solvent. All vesicles were first made as MLVs by hydrating the dried lipids above their phase transition temperatures. SUVs were made from the MLVs by sonicating under nitrogen, on ice, with a Heat Systems W-380 Cell Disruptor until the milky MLV suspension cleared (about 5 min). LUVs were made by forcing the MLVs ten times through 1.0 and 0.1 μm Nucleopore filters under high nitrogen pressure using a temperature controlled 'extruder' (Lipex Biomembranes, Vancouver, BC, Canada) [19].

Erythritol permeability. MLVs are known to behave as almost perfect osmometers [20] and their swelling rates can be easily followed with a spectrophotometer [21]. MLVs were made in 40 mM glucose, 10 mM acetate (pH 5.0) and were then rapidly mixed into the swelling buffer, 40 mM erythritol, 10 mM acetate (pH 5.0). Final mixtures in the cuvettes contained about 0.5 mM lipid and ABA/lipid ratios of either 0, 1:4, 1:2, or 1:1. Included into the membrane lipids were 0, 1, 3, 5 or 10 membrane mol% sterol. Initial vesicle swelling velocities, determined from changes in absorbance (350 nm) and expressed as $d(1/A)/dt\%$ [22], were measured on a temperature controlled (±0.1°C) Perkin-Elmer Lambda 4C UV/Vis Spectrophotometer controlled by a Perkin-Elmer Model 7700 Professional Computer. Results are the average of five determinations with standard errors of $\leq 5\%$.

Carboxyfluorescein release. LUVs were made from 10 mM lipid in 60 mM CF, 30 mM KCl, 10 mM acetate (pH 5.0). Non-sequestered CF was removed on Sephadex G-80 with 90 mM KCl, 10 mM acetate (pH 5.0). Sterol concentrations were either 0, 1, 5 or 10 membrane mol%. CF-loaded lipid vesicles were mixed with the buffer containing ABA to produce ABA/lipid ratios of either 0 or 1:1. The high concentration of sequestered CF self quench [23]. As the dye leaked out the fluorescent intensity increased (excitation 490 nm, emission 520 nm), which was followed on a Perkin-Elmer MPF-66 Fluorescence Spectrophotometer controlled by a Perkin-Elmer Model 7700 Professional Computer. After 15 min the total amount of sequestered CF was determined by lysing the vesicles with Triton X-100. Results are expressed as the percentage of initially sequestered CF leaking out over time.

Fusion. Two populations of SUVs were made identical in lipid composition (5 mM total lipid) with the exception that one contained the fluorescent 'donor' NBD-PE and the other the fluorescent 'acceptor' Rho-PE, each at 0.3 membrane mol%. The vesicle populations were mixed 1:1 and excited at 464.2 nm, while emission was monitored at 593.9 nm on the temperature controlled Perkin-Elmer MPF-66. By this fluorescence resonance energy transfer assay [24], an increase in fluorescence emission is indicative of vesicle fusion. To determine the percentage of total possible fusion, the vesicles were removed from the cuvettes after 10 min and briefly resonicated. A final emission value was determined and results are expressed as the percentage of total possible fusion over time (25)].

Fluidity. SUVs containing 1.0 membrane mol% of DPH were made with 3 mg lipid per ml of 20 mM sodium chloride, 10 mM sodium acetate (pH 5.0). Fluorescence polarization values were measured from the parallel and perpendicular intensities [26]:

$$P = \frac{\langle VV\rangle - G\langle VH\rangle}{\langle VV\rangle + G\langle VH\rangle}$$

where V and H refer to the fluorescent intensities with the polarizers in the vertical and horizontal orientations, respectively. G is a 'grating factor' which corrects for instrument artifacts and is equal to $\langle HV\rangle/\langle HH\rangle$. Excitation was at 351 nm and emission at 430 nm. Temperatures were decreased from 52°C to 10°C and the reported values are the average of six determinations.

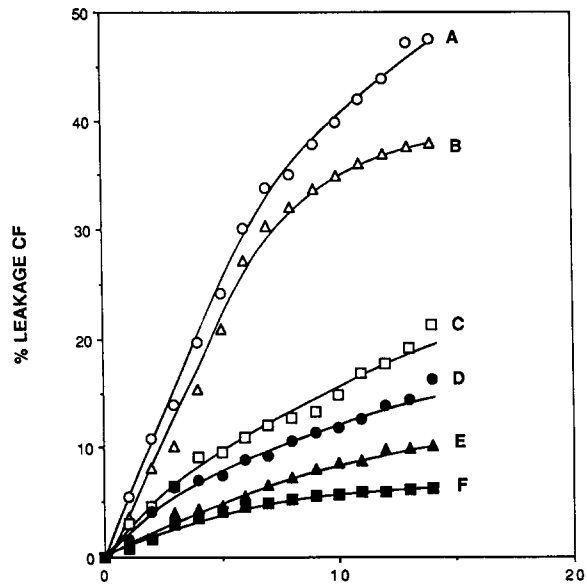

Fig. 1. Effect of plant sterols on the ABA-induced permeability of carboxyfluorescein to DMPC/DMPE (4:1) bilayers. ABA/lipid, 1:1, pH 5.0, 50°C. A, +ABA, 0% sitosterol; B, +ABA, 1% sitosterol; C, +ABA, 5% sitosterol; D, +ABA, 10% sitosterol; E, +ABA, 20% sitosterol; F, no ABA, 0% sitosterol.

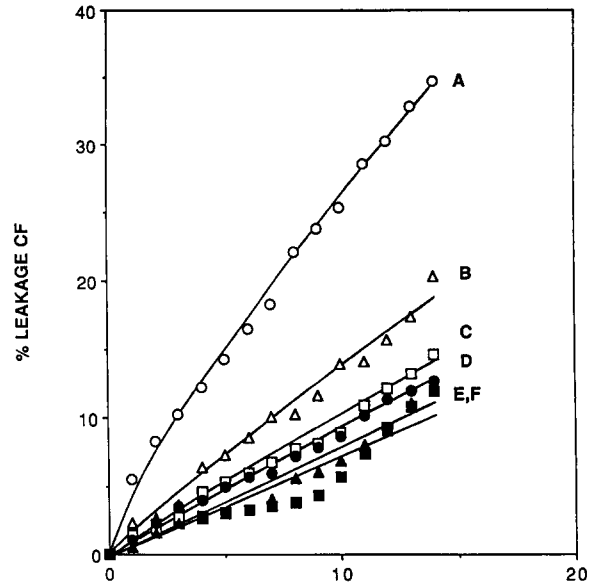

Fig. 2. Effect of plant sterols on the ABA-induced permeability of carboxyfluorescein to DOPC/DOPE (4:1) bilayers. ABA/lipid, 1:1, pH 5.0, 30°C. A, +ABA, 0% sitosterol; B, +ABA, 1% sitosterol; C, +ABA, 5% sitosterol; D, +ABA, 10% sitosterol; E, +ABA, 20% sitosterol; F, no ABA, 0% sitosterol.

ABA concentration was 1:1, hormone/lipid and sterol concentrations were 0, 10, 20 and 50 membrane mol%.

Results

Our previous experiments have determined that several types of PC/PE mixed bilayers can be affected by ABA [3–7,9,10]. This is confirmed in Figs. 1–3 where we report that ABA enhances permeability to the anion carboxyfluorescein with saturated (DMPC/DMPE, 4:1, Fig. 1) and unsaturated (DOPC/DOPE, 4:1, Fig. 2) phosholipid membranes. Other membrane systems including both synthetic and natural, saturated and unsaturated PC/PE mixed bilayers produced simi-

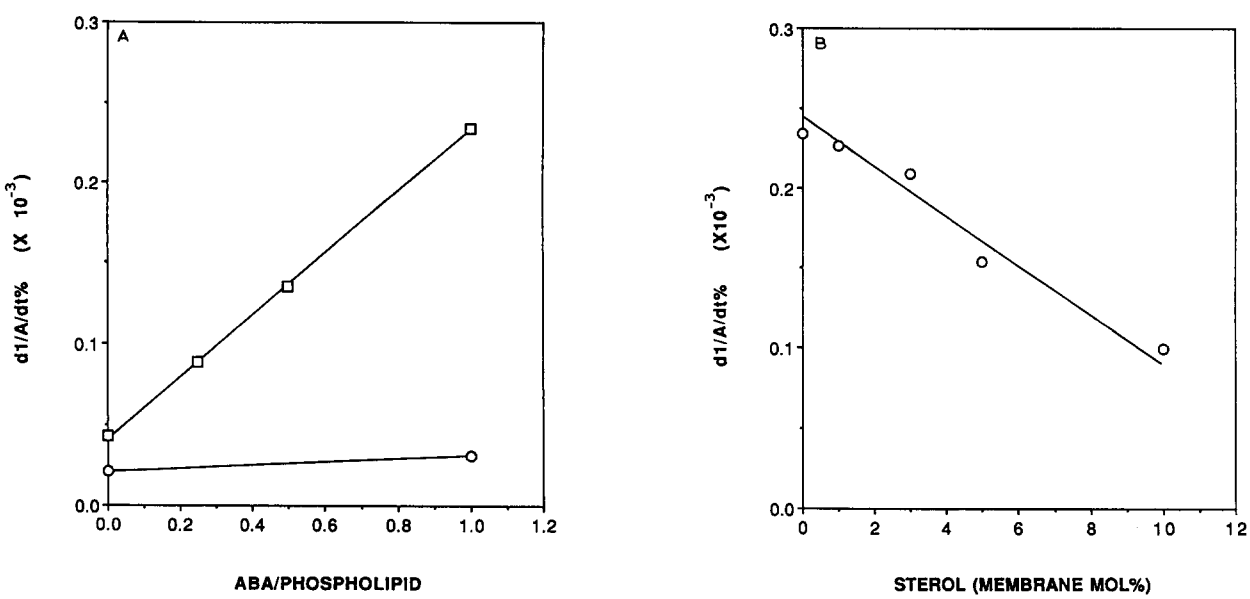

Fig. 3. Effect of plant sterols on the ABA-induced permeability (expressed as the swelling velocity $d(1/A)/dt\%$) for DMPC and DMPC/DMPE (9:1) bilayers (30°C). (A) Swelling velocity vs. ABA/phospholipid ratio for bilayers composed of: □———□, DMPC/DMPE (9:1); ○———○, DMPC. (B) Swelling velocity vs. sterol concentration expressed as membrane mol% for DMPC/DMPE (9:1) bilayers in the presence of ABA (ABA/phospholipid, 1:1).

lar results (data not shown). In each case the enhancement was shown to be ABA dependent between the ABA/phospholipid levels of 1 : 4 to 1 : 1, although only the highest level of ABA is depicted. Permeability of membranes composed of PC/PE ratios of 9 : 1 was also shown to be simlarly increased by ABA, while single component DMPC, DOPC and soy PC bilayers were shown to be almost uneffected by the hormone (results not shown). The influence of plant sterols was investigated for each mixed component membrane type only of the highest level of ABA-induced permeability measured (ABA/phospholipid, 1 : 1). Figs. 1 and 2 show that when the sterols were incorporated into the membranes between 1 and 20 membrane mol%, ABA-induced permeability for each membrane was depressed. Sterol reduction of the ABA-induced enhancement of permeability of DMPC/DMPE mixed bilayers was confirmed using a completely different technique. By measuring multilamellar vesicle swelling in isomolar erythritol (a neutral solute, Fig. 3), ABA is shown to enhance permeability to the mixed component DMPC/DMPE bilayers with ABA/lipid ratios of 0, 1 : 4, 1 : 2 and 1 : 1. The highest level of ABA tested (ABA/lipid, 1 : 1) had almost no effect on the permeability of single component DMPC bilayers. As little as 10% sterol is enough to reverse the ABA-induced permeability (Fig. 3) by more than 75%.

Previously we reported that significant ABA-enhanced permeability could be measured not only for mixed head group bilayers (such as the PC/PE membranes reported in Figs. 1 and 2) but also for lipids with the same head group but different acyl tails in which liquid crystal and gel states coexist [3]. In Fig. 4 sterols are shown to block permeability to CF for a mixed component bilayer containing the same head group (PC) and different acyl chains (DMPC/DPPC, 1 : 1). By differential scanning calorimetry the DMPC/DPPC system is known to have a phase transition temperature midway between that of DMPC (23.6° C) and DPPC (41.3° C) [26]). We confirm this in Fig. 5 using fluorescence polarization of DPH in DMPC/DPPC vesicles. A sharp increase in polarization at 30° C occurs upon cooling, corresponding to the liquid crystal to gel transition. This is the temperature at which the permeability experiments in Fig. 4 were run, so that liquid crystal and gel states would coexist. In Fig. 5 the plant sterols are also shown at low levels to decrease, and at 50 membrane mol% to completely obliterate, the phase transition for DMPC/DPPC bilayers. Thus, there is a parallel between the ability of the sterols to reduce both the phase transition (Fig. 5) and ABA-induced permeability to carboxyfluorescein (Fig. 6).

Because of the obvious structural similarity of sitosterol, campesterol and cholesterol, we would not anticipate any general membrane bilayer role for the plant sterols which would not also be provided by cholesterol. This is supported in Fig. 6 where cholesterol similarly quenches the ABA-induced CF permeability for DMPC/DPPC mixed acyl chain membranes.

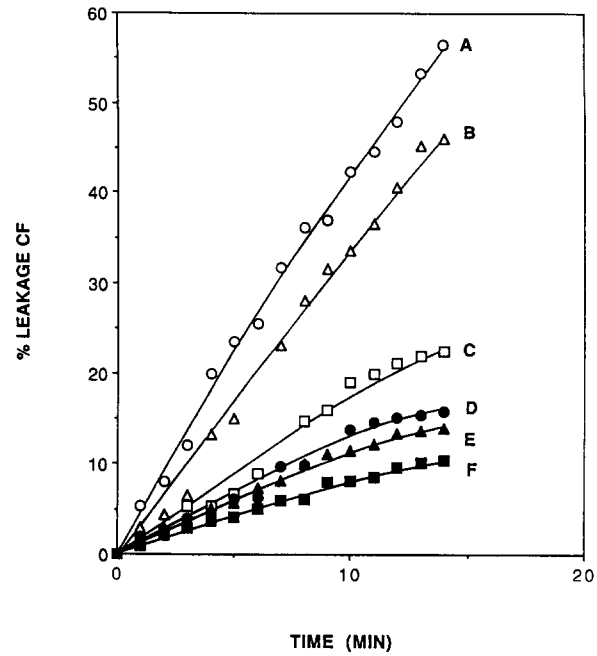

Fig. 4. Effect of plant sterols on the ABA-induced permeability of carboxyfluorescein to DMPC/DPPC (4 : 1) bilayers. ABA/lipid, 1 : 1, pH 5.0, 30° C. A, +ABA, 0% sitosterol; B, +ABA, 1% sitosterol; C, +ABA, 5% sitosterol; D, +ABA, 10% sitosterol; E, +ABA, 20% sitosterol; F, no ABA, 0% sitosterol.

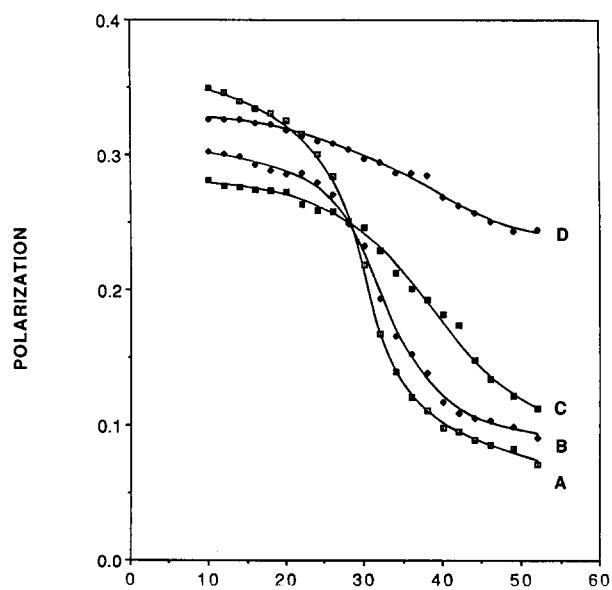

Fig. 5. Effect of plant sterols on the phase transition of DMPC/DPPC (1 : 1) small unilamellar vesicles as measured by fluorescence polarization of the probe DPH. A, 0% sitosterol; B, 10% sitosterol; C, 20% sitosterol; D, 50% sitosterol.

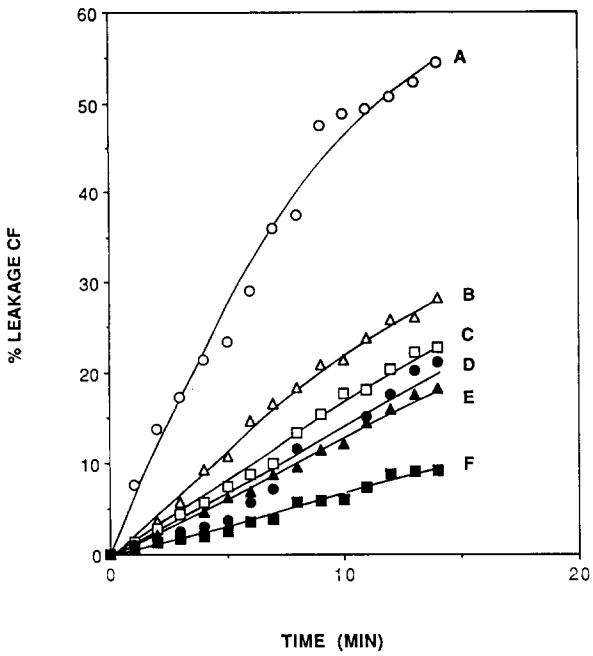

Fig. 6. Effect of cholesterol on the ABA-induced permeability of carboxyfluorescein to DMPC/DPPC (1 : 1) bilayers. ABA/lipid, 1 : 1, pH 3.0, 50 °C. A, +ABA, 0% cholesterol; B, +ABA, 1% cholesterol; C, +ABA, 5% cholesterol; D, +ABA, 10% cholesterol; E, +ABA, 20% cholesterol; F, no ABA, 0% cholesterol.

Earlier we reported that ABA can induce the aggregation [9] and fusion [10] of lipid vesicles composed of the same phospholipid types whose permeability was also affected by the hormone. In Fig. 7 ABA is shown to enhance the fusion of DMPC/DMPE (9 : 1) mixed bilayers while being ineffective with single component DMPC bilayers. As with the permeability measurements, plant sterols substantially decrease the ABA-induced fusion with 10 membrane mol% reducing the ABA fusion by about 70%.

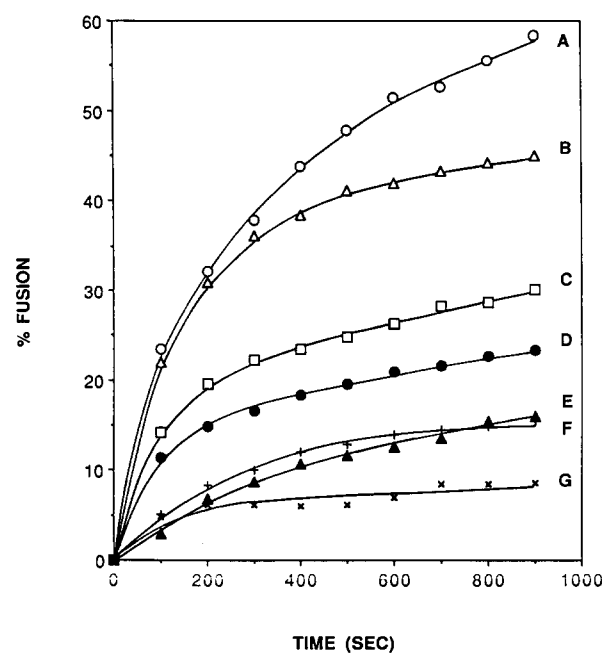

Fig. 7. Effect of plant sterols on the ABA-induced fusion of DMPC and DMPC/DMPE (9 : 1) small unilamellar vesicles. pH 5.0, ABA/lipid (1 : 1), 30 °C. A, DMPC/DMPE, +ABA; B, DMPC/DMPE, +ABA, +5% sitosterol; C, DMPC/DMPE, +ABA, +10% sitosterol; D, DMPC/DMPE, +ABA, +20% sitosterol,; E, DMPC/DMPE, no ABA, F, DMPC, +ABA; G, DMPC.

Discussion

The effect of sterols on membranes is substantial and complicated. They may function through two distinct mechanisms. At very low concentrations they may directly alter some metabolic event [28] or at higher levels they may partition into the bilayer portion of membranes modifying the general membrane lipid environment [29]. This latter role is studied here.

Sterols are known to affect many membrane properties including permeability, fluidity, acyl chain packing, phase behavior and lipid domain structure and stability [11,29]. The sterol-induced alteration of basic membrane properties is then further reflected by changes in physiological events such as transport and enzymatic activity [11]. Sterol levels can vary dramatically. Cholesterol, for example can be totally absent from some membranes in a cell while comprising well over 50% of the lipids in others. Plant sterols present an even more challenging problem than do animal sterols. While animals have only a few sterols, with cholesterol being by far the major one, plant sterols come in a wide variety of structures [17,30]. Sitosterol and campesterol, the major lipids used in the present study, are also major plant sterols [17,30]. No plant sterol, however, has been directly linked to a specific membrane type or function. Indeed, the relative levels of each kind of sterol remains fairly constant in plants from membrane to membrane but the total amount of sterol may vary considerably [30]. Analogous to the animal cell case, sterol levels in plants are high in the plasma membrane and much lower in membranes of the endoplasmic reticulum and mitochondria [30]. Although details are not yet at hand, it is likely that plant sterols play an important but ill-defined regulatory role in the function of membranes. This paper represents a first attempt to associate plant sterols with a well defined membrane function.

Our previous work with ABA and phospholipid bilayers has linked the hormone with substantial alterations in the bilayer properties of permeability [3–7] and lipid vesicle aggregation [9] and fusion [10]. The hormone effect is pH dependent, being observable primarily with undissociated ABA [7] ($pK_a = 4.85$) and is dependent on the bilayer phospholipid composition. Single-component PC membranes were unaffected by ABA whether the membranes were in the liquid crystal or gel states [5]. However, mixed head group component

PC/PE, PC/PG, PC/PA, PC/CL, PC/PS, PC/SA (all 4 : 1) membranes were affected by ABA [3]. In addition PC membranes containing mixed acyl chains (e.g., DMPC/DPPC) were affected by ABA if the membranes were tested at a temperature midway between the phase transition of either PC. At that temperature liquid crystal and gel states co-exist. There is specificity associated with the ABA–phospholipid interactions as the biologically active *cis-trans*-ABA isomer is effective with the lipid bilayer systems while the biologically inactive geometric isomer *trans-trans*-ABA is not [7,8]. The large effect of ABA on gross membrane properties prompted us to search for specific molecular interactions using the spectroscopic techniques of ^{1}H; ^{13}C- and ^{31}P-NMR, ESR and fluorescence polarization. These experiments, however, have continually failed to detect any significant ABA–phospholipid interactions at the molecular level [3]. Specifically, significant changes in molecular ordering or fluidity due to the hormone, either within the membrane interior or in the head group region, were not discernible even in membranes with phospholipid compositions which displayed enhanced permeability. A problem arises in explaining how ABA can enhance bilayer permeability to anions, cations, neutral solutes and water and dramatically increase lipid vesicle aggregation and fusion without displaying any measurable interaction with the only components of the bilayers, namely the phospholipids. We concluded that ABA must be acting at only a small proportion of the membrane surface and its effect is lost in the population weighted average response monitored for the entire membrane. We hypothesized that ABA is acting at regions of membrane defect occurring between different head group components or between liquid crystal and gel states. One possible method of further elucidating these interactions is with a membrane probe, such as a sterol, which is known to perturb phospholipid interactions and accumulate at liquid crystal/gel interfaces affecting lipid domain size and stability [31]. The experiments reported here support the idea that ABA and plant sterols may be competing for the same site on the membrane, perhaps regions of bilayer defects.

The basic structure of many sterols is similar being comprised of a membrane anchoring polar hydroxyl group attached at C-3 to an extended rigid ring system [30]. Attached at the other end of the ring system is a flexible acyl tail and it is here that the major difference between cholesterol and the plant sterols resides [30]. The plant sterols used in these experiments differ from cholesterol primarily by having either an additional ethyl (sitosterol) or methyl (campesterol) at C-24. Since the major portion of the sterol molecule that provides the bilayer perturbation is the ring structure and this is the same with cholesterol and the plant sterols, we anticipated that similar effects on the ABA-induced membrane properties would be measured for both types of sterols. This is born out in Fig. 6 where cholesterol is shown to be about as effective as the plant sterols at abolishing the ABA-induced CF permeability with the DMPC-DPPC mixed chain bilayer system. The phospholipids employed in this study are all relatively saturated. Van Ginkel et al. [32], using angle-resolved fluorescence polarization, have recently reported differences between cholesterol and stigmasterol on the molecular order and dynamics of higly unsaturated bilayers. Only minor differences however were measured between the sterols with more saturated membranes.

By DSC, cholesterol has been shown to broaden and decrease the phase transition of synthetic saturated phosphatidylcholines resulting in complete obliteration of the transition at 50 membrane mol% [33]. In Fig. 5 fluorescence polarization of DPH demonstrates that the plant sterols also broaden and decrease the phase transition of the mixed acyl chain DMPC/DPPC (1 : 1) bilayer system, with complete destruction of the transition at 50 membrane mol%. This behavior parallels the reversal of ABA-induced permeability produced by the plant sterols. It may be reconciled with our proposal that the hormone acts at membrane defects. By analogy with cholesterol [34] the plant sterols are expected to disorder the gel phase and order the liquid crystalline phase, thereby reducing the miss-match in molecular packing at the interface between regions of different phase.

These experiments are based on the premise that perhaps the reason ABA binding proteins or proteinaceous receptors have not yet been identified is that ABA initially acts instead on the lipid bilayer component of membranes altering in some way membrane properties. Changes in these properties may directly affect physiological events (by increasing ion permeability, for example) or else may alter the activity of some membrane-bound protein which in turn alters cellular processes. Although it is clearly demonstrated that ABA can affect simple bilayer membranes composed of two different phospholipids, it has yet to be demonstrated that ABA can affect biological membranes by a similar mechanism. The composition and structure of ABA-responsive (e.g., guard cell) plasma membranes has not been studied in any detail. To determine the possible physiological significance of ABA–lipid interactions on biological membranes, the phospholipid and acyl chain compositions, sterol levels, lipid asymmetries and lipid domains must be defined. Detectable differences should be evident between the responsive and non-responsive membranes.

The concentration of ABA used in these studies are generally between 0.5 and 1.0 mM and represent levels required to measure significant changes by the different biophysical techniques. While the absolute concentra-

tion of ABA in these studies is therefore not relevant, the ABA/lipid ratios are significant and so are reported. But even the ABA concentrations used in this model stuidy may not be unrealistic. Recent reports by Behl and Hartung [35] and Lahr and Raschke [36] have indicated that the local concentration of ABA in guard cells may be quite high, perhaps into the millimolar range.

These experiments indicate that plant sterols may modulate the conditions necessary for the action of ABA on the lipid bilayer component of membranes. Perhaps the membrane perturbation caused by ABA resulting in a rapid increase in permeability may be reversed by redistribution of sterols to the area of ABA action, namely regions of membrane defects.

Acknowledgements

This work was supported by National Science Foundation Grants DMB-860701 and DCB-8715558.

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