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Interactions of pyrethroids with gramicidin-containing liposomal membranes

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Fluorescence steady-state anisotropy and phase-modulation lifetime techniques have been utilized to study the interactions of pyrethroid compounds with fluid-phase phosphatidylcholine membranes containing the polypeptide gramicidin. This polypeptide is considered to be a model of hydrophobic regions of cellular integral membrane proteins. The pyrethroids disorder lipid packing in cellular membranes and gel-phase liposomes but do not disorder lipid packing in fluid-phase lipid (Stelzer, K.J. and Gordon, M.A. (1984) J. Immunopharmacol. 6, 381–410; (1985) Biochim. Biophys. Acta 812, 361–368) Irrespective of liposomal size, gramicidin incorporation resulted in a substantial increase in anisotropy of the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), in fluid phase lipid. In the absence of gramicidin, permethrin and three other pyrethroids, allethrin, cypermethrin and fenpropathrin, increased DPH anisotropy. In these fluid phase systems, as the protein:lipid ratio was increased, the extent of the pyrethroid-mediated increase in fluorescence anisotropy diminished. Also, the pyrethroids shortened DPH fluorescence lifetimes. At high gramicidin:lipid ratios, permethrin substantially lowered anisotropy in the fluid phase lipid, relative to controls. The data suggest that pyrethroids disturb fluid-phase lipids which have been promoted to a relative state of order by proximity to an integral membrane protein. This type of order is one which is represented by DPH fluorescence anisotropy. A model based on these results is proposed to explain the effects of pyrethroids on lipid packing order in cellular membranes, as determined by DPH fluorescence anisotropy.

Introduction

Pyrethroids are hydrophobic compounds which have been shown to perturb lipid packing order in cellular and liposomal membrane systems [1,2]. The type of lipid packing order referred to is one which is represented by DPH fluorescence anisotropy. The disordering effects of pyrethroids on phosphatidylcholine (PC) packing order in liposomes could only be demonstrated when the lipid was in the gel phase [1,2]. The inability of pyrethroids to perturb the packing order of fluid-phase PC liposomal membranes would appear to contradict the results observed in cellular membranes. Although cellular membrane lipids are considered to be relatively fluid at physiological temperature, pyrethroids disordered lipid packing in mouse splenic lymphocytes at 37 °C [1].

A possible explanation for this apparent discrepancy involves the presence of integral proteins

Abbreviations: PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; tPnA, *trans*-parinaric acid; SUV, small unilamellar vesicle; T_c, phase transition temperature; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene.

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within cellular membranes. According to this explanation, these integral membrane proteins increase a type of order in proximal lipids [3], creating local or 'annular' domains which may be disordered by pyrethroids. To examine this possible explanation of pyrethroid actions on cellular lipids, gramicidin was incorporated into fluid phase PC vesicles which were labeled with the fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH).

Gramicidin, a pentadecapeptide, is considered to be a model for the hydrophobic regions of integral membrane proteins found in cells. Gramicidin has been shown to increase lipid packing order in fluid phase PC, using Raman spectroscopy as a measure of order [4]. Gramicidin dimerizes to form membrane-spanning channels which conduct cations [5]. Cationic channels are some of the most important integral membrane proteins which are functionally altered by pyrethroids [6–12], further supporting the usefulness of the gramicidin-PC model for studying the effects of pyrethroids on annular lipid domains.

Materials and Methods

Chemicals

Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), gramicidin (approx. 86% gramicidin A, 7% gramicidin B, 5% gramicidin C and 0.3% gramicidin D), valinomycin, monensin and nystatin were purchased from Sigma, St. Louis, MO. DPH and *trans*-parinaric acid (tPnA) were purchased from Molecular Probes, Inc., Junction City, OR. The following analytical grade pyrethroids were donated by the indicated companies: permethrin and cypermethrin (FMC Corp., Princeton, NJ), fenpropathrin (Chevron Chemical Co., Richmond, CA), and allethrin (McLaughlin Gormley King Co., Minneapolis, MN).

Vesicle preparation

All lipids were kept in 20 mg/ml stock solutions of chloroform. Gramicidin in chloroform was added to aliquots of the PC stock solutions in screw cap test tubes to give the desired ratio, mol gramicidin : mol lipid. The solvent was evaporated under nitrogen.

For all PC-gramicidin samples, either in the form of large multilamellar vesicles or small unilamellar vesicles (SUVs), the final PC concentration was $6 \cdot 10^{-5}$ M, regardless of the gramicidin to lipid ratio. Large multilamellar vesicles were prepared by adding phosphate-buffered saline (pH 7.4) to the dried sample and heating in a water bath to at least 15 C° above the transition temperature (T_c) of the lipid. The sample was then shaken vigorously by vortex for 2 min. For preparation of SUVs, the dried lipid was dissolved in ethanol, and the ethanol solution was injected while vortexing into phosphate-buffered saline heated at least 15 C° above the T_c. The vesicles were labeled with either DPH or tPnA by direct injection of these fluorophores into the suspensions while vortexing. The lipid : probe mole ratios were 300 : 1 and 350 : 1 for DPH and tPnA, respectively. For experiments in which the samples were treated with pyrethroids, the pyrethroids were dissolved in dimethyl sulfoxide, and were added in a volume of 1 μl directly to cuvettes containing 3 ml of liposomal suspension. The final concentration of pyrethroids was 10 μM in all cases. Control samples received an equivalent volume of dimethyl sulfoxide.

Fluorescence measurements

Phase shift and demodulation parameters were measured with an SLM 4800 fluorometer, and fluorescence lifetimes were subsequently calculated [13]. The excitation wavelength was set by a monochromator at 360 nm when DPH was used as the fluorescent probe and at 320 nm when tPnA was used. A Schott KV-389 cut-on filter was placed in the path of the emitted light. A solution of the quantum-counter rhodamine was placed in the reference chamber. Phase-modulation fluorometeric determinations were made using intensity modulation frequencies of 30, 18 and 6 MHz. The extent of phase delay and demodulation of the emitted fluorescence light relative to the incident light was measured by the fluorometer for the sample and for a dimethyl POPOP standard of known fluorescence lifetime (1.45 ns). The sample and standard were measured alternately, four times each, with each measurement being the average of ten readings. Phase lifetime (T_p) were then calculated from the phase shift (θ) relative to that of

the standard of known fluorescence lifetime, and modulation lifetimes were calculated analogously from demodulation (M) as follows:

$$T_p = w^{-1} \tan \theta$$

$$T_m = w^{-1} (M^{-2} - 1)^{\frac{1}{2}}$$

where w is the angular modulation frequency (rad/s) of incident light.

Fluorescence steady-state anisotropy measurements were made with an SLM 8000 fluorometer in the T configuration. Excitation wavelengths were set as described above, and emission wavelengths of 420 nm and 410 nm for DPH and tPnA, respectively, were set by a monochromator.

Temperature in the sample chambers was regulated by a circulating water bath.

Results

In fluid-phase DMPC large vesicles, gramicidin caused large increases in DPH fluorescence anisotropy as the gramicidin : lipid ratio was increased (Fig. 1a). Gramicidin also caused modulation lifetimes to increase and phase lifetimes to decrease with these increasing ratios (Fig. 1b and c). At a gramicidin : lipid ratio of 0, permethrin increased the fluorescence anisotropy of DPH. This increase could be a result of the lower lifetimes caused by permethrin in the absence of further disordering of lipid packing at the low value of control anisotropy. With the incorporation of gramicidin up through a gramicidin : lipid ratio of 0.35, permethrin substantially decreased DPH fluorescence anisotropy. The decreased anisotropy is indicative of permethrin-induced disordering of gramicidin-enhanced lipid packing, since permethrin shortened lifetimes to a greater degree as the gramicidin : lipid ratio was increased. Qualitatively similar results were obtained for DMPC SUVs and for DPPC in the form of both large multilamellar vesicles and SUVs. The most dramatic effects of gramicidin (alone or with permethrin) were noted in the DMPC large vesicle system with less prominent effects noted with DPPC or in SUV systems.

It has been proposed that the increase in DPH anisotropy observed as an integral membrane pro-

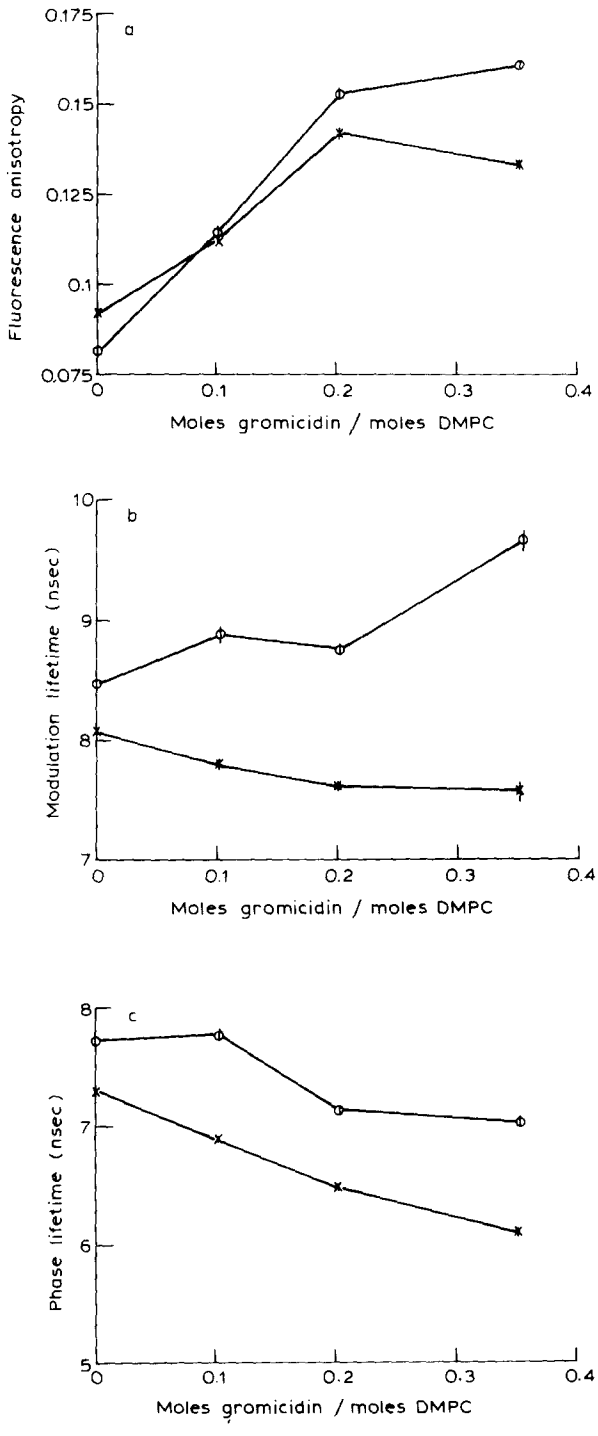

Fig. 1. Effects of 10^{-5} M permethrin on fluid-phase DMPC large multilamellar vesicles containing gramicidin, as determined by DPH fluorescence anisotropy (a), 18 MHz modulation lifetimes (b) and 18 MHz phase lifetimes (c). O, Control; ×, permethrin. The temperature was 40 °C.

tein, such as gramicidin A, is incorporated into fluid-phase lipid is due to direct effects upon DPH itself, without requiring constraints on lipid motility in the vicinity of the polypeptide [14,15]. To determine if the effects of gramicidin upon DPH fluorescence anisotropy in fluid-phase lipid were the result of exclusive interaction with the DPH probe, measurements were made in fluid-phase DPPC large vesicles with another fluorescent probe, tPnA. This second probe is a fatty acid which should be positioned in the bilayer in a similar manner to the acyl side chains of the lipid [3]. Increasing the gramicidin : lipid ratio resulted in increased tPnA fluorescence anisotropy, following the same pattern observed when DPH was used as the probe (Fig. 2a). Fig. 2b shows the

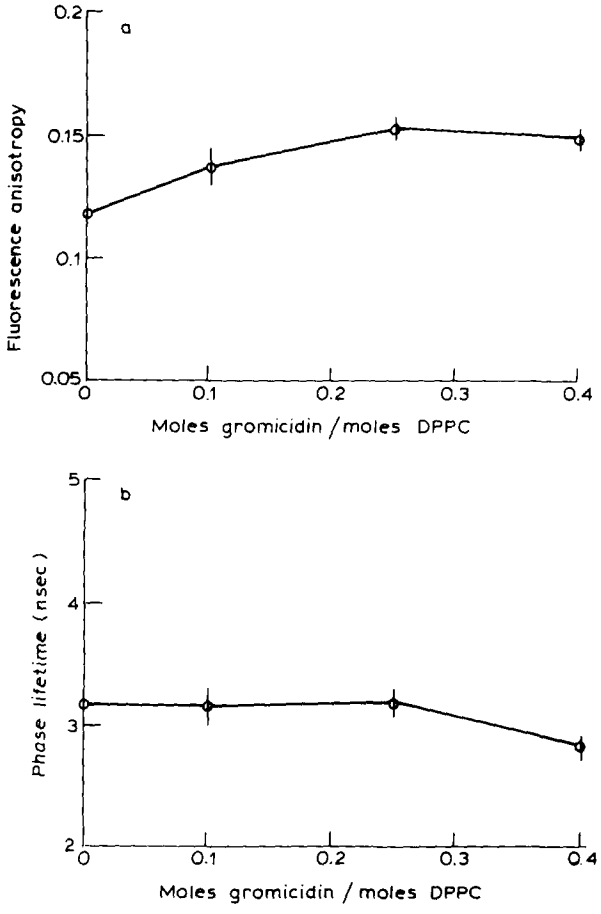

Fig. 2. Effects of gramicidin on fluid-phase DPPC large multilamellar vesicles, as determined by tPnA fluorescence anisotropy (a) and 18 MHz phase lifetimes (b). The temperature was 55 °C.

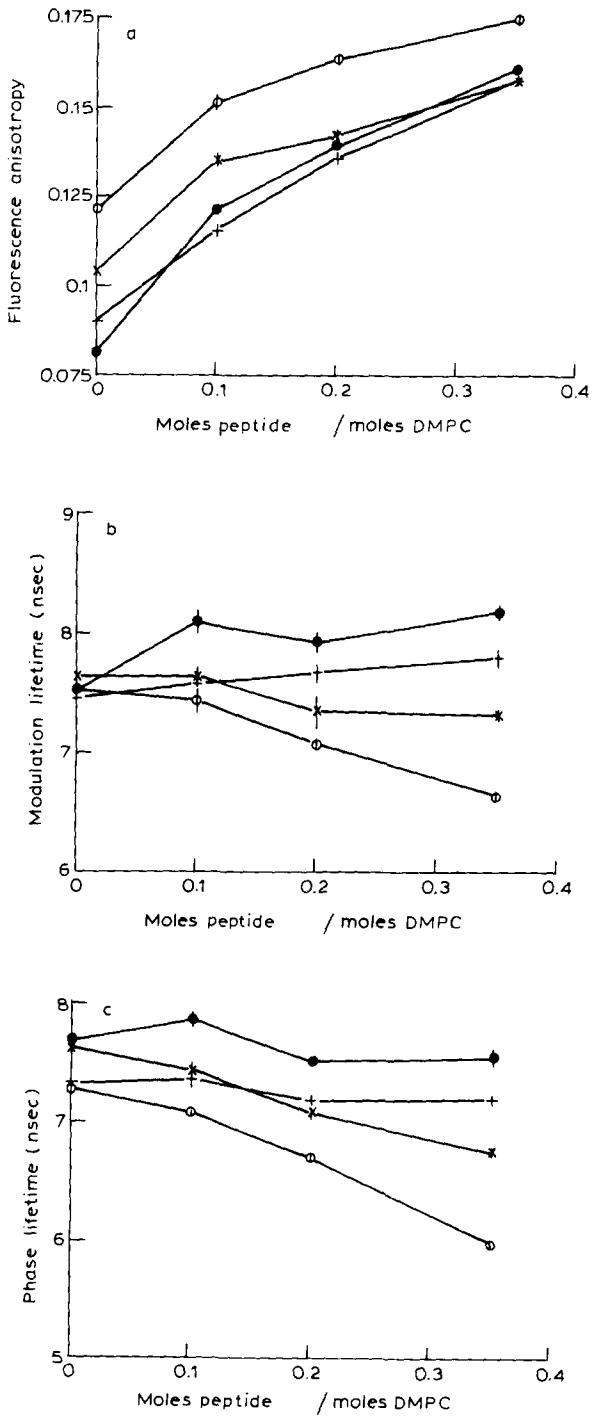

Fig. 3. Effects of 10^{-5} M pyrethroids on fluid-phase DMPC large multilamellar vesicles, as determined by DPH fluorescence anisotropy (a), 18 MHz modulation lifetimes (b) and 18 MHz phase lifetimes (c). ●, Control; ○, cypermethrin; ×, fenpropathrin; +, allethrin. The temperature was 40 °C.

phase lifetimes obtained at 18 MHz. Neither phase nor modulation lifetimes at any intensity modulation frequency were altered by gramicidin. The results demonstrate that the gramicidin-induced increase in fluorescence anisotropy is not restricted to the DPH probe and that motional constraints are likely to occur with PC acyl side chains located near the polypeptide.

Three other pyrethroids, cypermethrin, fenpropathrin and allethrin, were examined with regard to their effects on DPH fluorescence parameters in fluid-phase DMPC large multilamellar vesicles reconstituted with gramicidin. This particular lipid system was chosen because of its sensitivity in the fluid phase to the lipid ordering effects of gramicidin.

In fluid-phase DMPC large vesicles (Fig. 3) allethrin, fenpropathrin, and cypermethrin behaved in a qualitatively similar manner to permethrin. In pure fluid-phase DMPC the three pyrethroids all increased DPH fluorescence anisotropy with the following order of effectiveness: cypermethrin > fenpropathrin > allethrin. The higher anisotropy values are most likely the result of lipid ordering properties of these pyrethroids in fluid-phase DMPC, as lifetimes are not significantly altered in the pure lipid. As the gramicidin : lipid ratio was increased, however, the magnitude of anisotropy elevation was decreased. Relative to control, allethrin and fenpropathrin eventually decreased DPH anisotropy as the gramicidin : lipid ratio was increased. These results are again consistent with pyrethroid induced disordering of lipids in the vicinity of gramicidin, as increasing gramicidin : lipid ratios resulted in enhancement of fluorescence lifetime shortening by the pyrethroids.

In order to examine the ability to generalize the results with gramicidin A, experiments with other ionophores were carried out. Valinomycin and monensin behaved similarly to gramicidin by increasing anisotropy as the ionophore : lipid ratio was increased, but to a lesser extent (Fig. 4). Similarly, the increase in anisotropy caused by permethrin in pure fluid-phase DMPC was attenuated as the ionophore : lipid ratio was increased, but to a lesser degree than with gramicidin. Furthermore, nystatin did not increase anisotropy up to a nystatin : lipid ratio of 0.4, and the

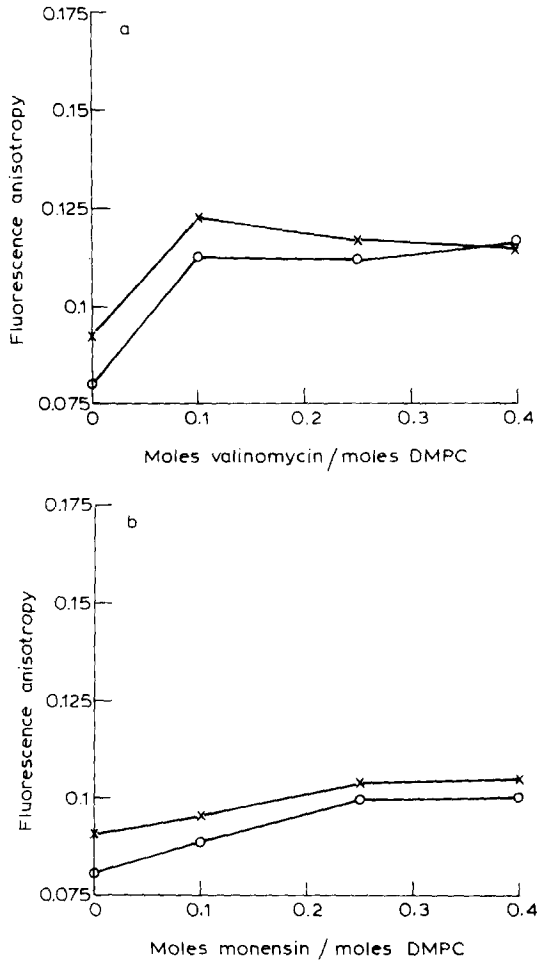

Fig. 4. Effects of 10^{-5} M permethrin on fluid-phase DMPC large multilamellar vesicles containing valinomycin (a) or monensin (b), as determined by DPH fluorescence anisotropy. ○, Control; ×, permethrin. The temperature was 52° C.

increase in anisotropy caused by permethrin remained constant at all nystatin : lipid ratio. Apparently, the effect of permethrin on DPH anisotropy depends upon the degree that anisotropy is increased with increasing ionophore : lipid ratio.

Discussion

The effects of integral membrane proteins on lipid order are controversial, with variable results occurring between fluorescence anisotropy, ESR, IR spectroscopy, Raman spectroscopy and ^{2}H-NMR [3,4,14–17]. In interpreting the results of

these studies, it is important to realize that the type of order being measured varies among the techniques [3]. It is difficult to define precisely the type of lipid order which manifests as motional constraints on DPH, as reflected by fluorescence anisotropy measurements. One must question whether the type of order represented by DPH anisotropy is of functional significance. Studies of the effects of pyrethroids on the aggregation state of a membrane protein, bacteriorhodopsin in model membranes indicate that the lipid disordering detected by DPH anisotropy is closely associated with disaggregation of the protein assemblies [18]. Apparently, the type of lipid order that DPH anisotropy represents has a physical meaning in terms of membrane-associated bacteriorhodopsin oligomer-monomer equilibria.

Gramicidin increased fluorescence anisotropy in fluid-phase DPPC and DMPC in the form of both large vesicles and SUVs. However, the degree of anisotropy increase was dependent upon the type of PC and upon the vesicle morphology. The present results support the model in which gramicidin increases lipid packing order in fluid-phase lipid [3,4]. In this model, we suggest that permethrin may then disorder the lipid which has been promoted to a relative state of order by proximity to gramicidin.

The other pyrethroids tested behaved in a similar manner to permethrin, except that these agents exhibited greater ordering effects upon lipid packing in pure fluid-phase DMPC. These enhanced ordering effects in part prevented cypermethrin, fenpropathrin and allethrin from causing the large decreases in anisotropy at high gramicidin : lipid ratios as had been observed with permethrin in DMPC. Additionally, the results were not peculiar to gramicidin as similar results were obtained with valinomycin and monensin. Experiments with these other ionophores, including nystatin, demonstrated that the degree of lipid disordering caused by permethrin depended upon the initial state of order promoted by the ionophore.

Fluorescence anisotropy reflects an average order [3] and the pyrethroid-induced perturbation of the lipid which has been ordered by the presence of gramicidin will continue to be offset by the pyrethroid-induced ordering of fluid-phase PC. Put another way, the pyrethroids have an ordering effect upon fluid-phase PC. Addition of gramicidin increases the order of lipids; however, pyrethroids inhibit the gramicidin-induced ordering in the gramicidin-influenced lipid regions. The expected results would be a lower rate of rise in anisotropy with increasing gramicidin : lipid ratio in the presence of pyrethroids, as was observed.

A theory has been proposed to account for discrepancies between lipid ordering detected by fluorescent and spin label probes and disordering detected by ^{2}H-NMR in response to membrane incorporation of propteins [14,15]. In this theory, ordering effects are attributed to the hindrance of probe motility by adjacent intrinsic proteins, rather than effects on an annular lipid phase. Our results showing the decrease in DPH anisotropy with the addition of pyrethroids to liposomes containing gramicidin and other ionophores are probably inconsistent with this theory. The results presented here and elsewhere [2] are more in agreement with a disordering effect of pyrethroids on lipids which are initially in a relative state of order. It appears that pyrethroids may exert their disordering effects on lipids whether the initial state of relative order is brought about by lowering the temperature below the T_c [2] or by incorporation of an integral membrane protein. In order to be consistent with the theory that anisotropy changes reflect only a direct hindrance of DPH motion by proximity to the intrinsic protein [14,15], it would be necessary to propose that pyrethroids somehow prevent the direct interaction between probe and protein. This alternative seems unlikely in light of the evidence that the gel-phase disordering abilities of the individual pyrethroids were correlated with their apparent boundary lipid disordering properties. For instance, allethrin has been shown to be least effective of the four compounds in decreasing gel-phase anisotropy [2]. This compound also failed to cause substantially lower anisotropy values relative to controls, in fluid-phase DMPC at high gramicidin : lipid ratios.

Based on the results of this study a model is proposed in order to explain why pyrethroids disorder lipid packing in relatively fluid cellular membranes at 37 °C [1], but disorder only gel-phase lipids in vesicular systems [1,2]. In this model, the pyrethroids have little effect on the packing order of the more fluid bulk lipid domain

of cellular membranes. Conversely, pyrethroids disrupt the packing order of cellular lipids which are proximal to integral membrane proteins. These proximal or 'annular' lipids are 'ordered' by integral membrane proteins to varying degrees and are similar to gel-phase lipids in that they are vulnerable to lipid packing perturbations caused by pyrethroids. Therefore, the annular lipid domains in cellular membranes (those domains likely to be most important to the function of integral membrane proteins) are the most susceptible to disordering by pyrethroids. Furthermore, if a pyrethroid (or any other compound) fails to disorder a model membrane consisting purely of fluid phase lipid, it cannot be concluded that cell membranes at physiological temperature will also be unaffected. In fact, experiments in liposomes consisting of gel-phase lipid may actually be more representative of packing order changes which occur in the important lipid domains surrounding integral proteins of cellular membranes.

Acknowledgment

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