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Effect of organochlorine insecticides on nicotinic acetylcholine receptor-rich membranes

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Abstract

The so-called generalized polarization (GP) of the fluorescent probe Laurdan and the steady-state fluorescence anisotropy of the probe diphenylhexatriene (DPH) and its phenylpropionic derivative (PA-DPH) were used to study the effects of several organochlorine insecticides of the chlorophenylethane, chlorinated cyclohexane and chlorinated cyclodiene families on the *Torpedo* nicotinic acetylcholine receptor (AChR)-rich native membrane. All insecticides, with the exception of Lindane, augmented Laurdan GP both in the native membrane and in model lipid systems. Most organochlorine compounds produced a concentration-dependent decrease of DPH and PA-DPH anisotropy in the AChR-rich membrane. These compounds exhibited a dual behavior vis-à-vis the native AChR-rich membrane, exerting disordering effects at the bilayer core while ordering and/or excluding water molecules from the lipid–protein interface region, as sensed by DPH anisotropy and Laurdan GP, respectively. Furthermore, all insecticides decreased the efficiency of fluorescence resonance energy transfer between the intrinsic protein and Laurdan, albeit to different extents. On the basis of all these observations, the existence of potential target sites for insecticides in the protein–lipid interface region is postulated.

Keywords: Organochlorine insecticides; Nicotinic acetylcholine receptor; Laurdan; Membranes; Lipid–protein interactions

1. Introduction

The nicotinic acetylcholine receptor (AChR) is one of the best-characterized members of the ligand-gated ion channel superfamily (see reviews in Barrantes, 1998). The AChR of the *Torpedo* sp. electric organ is a pentamer, composed of four different but homologous subunits in the stoichiometry $\alpha_2\beta\gamma\delta$. Each subunit contains four hydrophobic segments referred to as M1–M4 and proposed to be membrane-spanning segments (Blanton and Cohen 1992, 1994). The M2 segment from each subunit is thought to contribute structurally to form the ion channel proper (see review in Barrantes, 1998) whereas M1, M3 and M4 have some contact with lipid, since they effectively incorporate membrane-partitioning photoactivatable probes (Blanton and Wang, 1990; Blanton and Cohen 1992, 1994).

In line with its proposed transmembrane organization, the AChR has been shown to be quite sensitive to the lipid environment (see, e.g., review in Barrantes, 1993). It is still not certain whether an appropriate membrane fluidity is necessary for receptor state transitions (Fong and McNamee, 1986; Sunshine and McNamee, 1994), although it is clear that the presence of both sterol and negatively charged phospholipids is required for the maintenance of AChR state transitions and ion-channel gating (Criado et al. 1982, 1984; Fong and McNamee, 1987; Rankin et al., 1997; Addona et al., 1998). In our laboratory the close association between sterols and fatty acids with the *Torpedo* AChR was first demonstrated by using electron spin resonance techniques (Marsh and Barrantes, 1978; Marsh et al., 1981). More recently, the modulatory effects of steroids on AChR function have been studied in intact cells using patch-clamp single-channel recording (Bouzat and Barrantes 1993, 1996).

During the last two decades there has been increasing interest in the nervous system as a target organ for toxicity. It is now well established that exposure to organochlorine insecticides (OCI) used in agriculture and industry produces neurotoxic effects characterized by

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motor, sensory, cognitive, and autonomic nervous system dysfunction (Longnecker et al., 1997). Several investigations implicate chlorinated hydrocarbons as affecting the Na^+ and K^+ permeability of axons during the propagation of the action potential (Arhem and Frankenhaeuser, 1974) or modifying the electrical properties of the cell membrane in general (Schefczik and Buff, 1984). It has also been shown that these compounds inhibit ion transport, as exemplified by the effect of DDT on Na^+/K^+ ATPase (Doherty, 1979). The highly lipophilic nature of the OCI, characterized by high partition coefficients in both native and model membranes (Antunes-Madeira and Madeira, 1986), together with the temperature sensitivity of some of their effects, suggest that the interaction of these compounds with biological systems consists not only in the direct interaction of the pesticides with membrane proteins but also in their effect on the physicochemical properties of the lipid bilayer. However, the considerable structural diversity of this class of compounds has so far hindered a detailed description of their mechanism of action and neurotoxicity at the molecular level.

Our current interest is to study the effects that OCI may possibly exert on the AChR and to elucidate their mechanisms of action. In the present work we exploit the spectroscopic properties of Laurdan and other environmentally sensitive fluorescent probes [diphenylhexatriene (DPH) and its phenylpropionic derivative (PA-DPH)] to study the effects of various OCI on the physical properties of the native *Torpedo* AChR-rich membrane. Almost all the insecticides tested were found to cause a progressive decrease in the lipid order of the AChR-rich membrane, and to decrease the efficiency of the energy transfer between the membrane proteins and Laurdan. From the experimental data we conclude that some of the OCI perturb both the bulk membrane and the protein-associated lipid microenvironment. Potential target sites for insecticides are postulated to occur either in the lipid-exposed transmembrane domains of the AChR protein and/or in its immediate lipid microenvironment.

2. Materials and methods

2.1. Materials

The insecticides 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane (DDT), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD), 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane (Methoxychlor), 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE), the γ-isomer of 1,2,3,4,5,6-hexachlorocyclohexane (Lindane), 1,2,3,4,10,10-hexachloro-1,4,4a,5,8, 8a-hexahydro-1,4-endo-5,8-exo-dimethaminonaphthalene (Aldrin), 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,8,8a-hexahydro-1,4-endo-5,8-exo-dimethanonaphthalene (Dieldrin), 1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene (Heptachlor), 1,2,4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene (Chlordane) and 1,2,3,4,5,5-hexachlorobicyclic(2,2,1)hepteno-5,6-bisoximethylene sulfite (Endosulfan) were obtained from Supelco (Bellefonte, PA). Laurdan and 3-[*p*-(6-phenyl)-1,3,5-hexatrienyl]phenylpropionic acid (PA-DPH) were purchased from Molecular Probes (Eugene, OR). 1,6-Diphenyl-1,3,5-hexatriene (DPH), dimyristoyl-L-α-phosphatidylcholine (DMPC), dioleoyl-L-α-phosphatidylcholine (DOPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC) and all other drugs were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Methods

2.2.1. Preparation of AChR-rich membranes

AChR-rich membranes were prepared from *Torpedo marmorata* electric organ (obtained either from Roscoff marine station in France or from the Mediterranean coast off Alicante, Spain) as described previously (Barrantes, 1982). Fish were killed by pithing after mild anesthesia. The specific activity of the membranes, as measured by [^{125}I]-α-bungarotoxin binding, was usually in the range of 600–1200 pmol/mg protein. Non-specific binding was measured using boiled and/or carbamoylcholine-preincubated membranes. Protein concentration was determined by the method of Lowry et al. (1951) using bovine albumin as standard. For fluorescent measurements, AChR-rich membranes were resuspended in buffer A (20 mM HEPES buffer, 150 mM NaCl and 0.25 mM $MgCl_2$, pH 7.4) at a final concentration of 50 μg protein/ml. The optical density (OD) of the membrane suspension was kept below 0.1 to minimize light scattering.

2.2.2. Preparation of multilamellar liposomes

Briefly, multilamellar liposomes were prepared by mixing different amounts of synthetic lipids (DOPC, DMPC, DPPC and cholesterol) with or without OCI (DDD, Aldrin and Lindane) and Laurdan at a constant final concentration of 0.6 μM. The lipid-to-probe ratio was similar to that in AChR-rich membranes. The organic solvents were evaporated under nitrogen, and the lipids resuspended in buffer A and sonicated until clarity to yield a liposome preparation with a final phospholipid concentration of 35.7 μM, similar to that used in AChR-rich membrane preparations.

2.2.3. Fluorescence measurements

All fluorimetric measurements were performed with an SLM model 4800 fluorimeter (SLM Instruments, Urbana, IL) using the vertically polarized light beam from a Hannovia 200 W Hg/Xe arc obtained with a Glan-Thompson polarizer (4 nm excitation and emission slits) and 5 mm×5 mm or 10 mm×10 mm quartz

cuvettes. Emission spectra were corrected for wavelength-dependent distortions. The temperature was set at 20°C with a thermostated circulating water bath.

2.2.3.1. Laurdan generalized polarization (GP) measurements Laurdan was added to AChR-rich membrane samples from an ethanol solution, to give a final probe concentration of 0.6 μM. The amount of organic solvent in these and all other experiments was kept below 0.2%. The samples were incubated in the dark for 60 min at room temperature. Insecticides were added to the samples from a stock solution in ethanol (5 mM) and incubated in the dark for 30 min at 20°C. The OD of the sample was recorded for each insecticide concentration and used to calculate the correction factors (CF) using the following formula:

$$CF = 10^{(ODexc.+ODem.)/2}, \quad (1)$$

where ODexc. corresponds to the OD of the sample at 290 and 360 nm in the Förster resonance energy transfer (FRET) and direct excitation conditions, respectively. ODem. is the absorbance at 330, 434 and 490 nm in the FRET experiments, and 434 and 490 nm in the direct excitation measurements, respectively.

Laurdan localizes itself at the hydrophilic/hydrophobic interface of the bilayer, with the naphthalene fluorescent moiety placed at the level of the glycerol backbone and its hydrophobic tail, the lauric acid, in the phospholipid acyl chain region (Parasassi et al. 1990, 1991). This probe possesses an exquisite spectral sensitivity to the phase state of the membrane, owing to its capacity to sense the polarity and molecular dynamics of the solvent dipoles in its environ, which arise from solvent dipolar relaxation processes. A 50 nm red shift is observed in Laurdan emission by passing from the gel to the liquid-crystalline phase, as is also observed in polar solvents with respect to non-polar solvents. The emission spectra of Laurdan presents two characteristic peaks — one at 434 nm, corresponding to probe molecules located in gel-phase regions of the membrane where the solvent dipolar relaxation process is not possible (the excited-state lifetime of the probe is very short compared with the solvent relaxation time), and the other one at 490 nm, corresponding to Laurdan molecules located in liquid-crystalline regions, where solvent relaxation can be completed before emission (see Parasassi et al. 1990, 1991).

Excitation GP (Parasassi et al. 1990, 1991) was calculated according to:

$$\text{excGP} = (I_{434} - I_{490})/(I_{434} + I_{490}), \quad (2)$$

where I_{434} and I_{490} are the emission intensities at the characteristic wavelength of the gel phase (434 nm) and the liquid-crystalline phase (490 nm), respectively. ExcGP values were obtained from emission spectra at different excitation wavelengths (320–420 nm) or at only one excitation wavelength where indicated. The emission GP was calculated according to the following formalism:

$$\text{emGP} = (I_{410} - I_{340})/(I_{410} + I_{340}), \quad (3)$$

where I_{410} and I_{340} are the excitation intensities [after correction following Eq. (1) above] at the wavelengths corresponding to the gel (410 nm) and the liquid-crystalline (340 nm) phases, respectively (Parasassi et al. 1990, 1991). The emGP values were obtained from excitation spectra at different emission wavelengths (420–520 nm).

2.2.3.2. Measurement of steady-state anisotropy of DPH and PA-DPH Aliquots of PA-DPH in dimethylsulfoxide, or DPH in tetrahydrofuran, were added to the AChR-rich membrane suspension to give a final concentration of 1 μM. Samples were incubated at 25°C for 30 min. Insecticides were added from concentrated ethanolic solutions (1.75 mM), keeping the ethanol concentration below 0.05%. The excitation and emission wavelengths used were 365 and 425 nm, respectively. Fluorescence anisotropy measurements were done in the T format with Schott KV418 filters in the emission channels and corrected for optical inaccuracies and for background signals. The anisotropy value, r_s, was obtained from the classical expression:

$$r_s = \frac{(I_v/I_h)_v - (I_v/I_h)_h}{(I_v/I_h)_v + 2(I_v/I_h)_h}, \quad (4)$$

where $(I_v/I_h)_v$ and $(I_v/I_h)_h$ are the ratios of the emitted vertical or horizontally polarized light to the exciting, vertical or horizontally polarized light, respectively. r_s values can range between −0.2 and 0.4. A high value of fluorescence anisotropy is interpreted as corresponding to high lipid order and/or low membrane fluidity. Whereas DPH senses the general order at the bilayer core, PA-DPH is located closer to the polar region and therefore reports on lipid order in more superficial regions of the membrane.

2.2.3.3. Förster resonance energy transfer (FRET) measurements Measurements of the extent of quenching of donor fluorescence by Förster energy transfer (Förster, 1948) were carried out by titration with insecticides in the absence and presence of Laurdan (Antollini and Barrantes, 1998). The energy transfer efficiency (E) was calculated as:

$$E = 1 - (\phi/\phi_D) \approx 1 - (I/I_D), \quad (5)$$

where ϕ and ϕ_D are the fluorescence quantum yields of donor in the presence and absence of the acceptor, respectively, and I and I_D are the corresponding emission intensities in any given measurement. Here I and I_D correspond to the maximum intrinsic protein emission intensities at a wavelength of 330 nm.

When E was measured in the presence of insecticides, a further correction was introduced to compensate for the modifications of the intrinsic fluorescence introduced by the presence of OCI:

$$I_{corr}=I_{meas}+(I_0-I_{OCI}), \quad (6)$$

where I_0 is the intrinsic fluorescence intensity in the absence of Laurdan and OCI; I_{OCI} is the corresponding intensity in the presence of OCI; I_{meas} is the fluorescence emission intensity in the presence of Laurdan and OCI. I_{corr} is the intensity corrected for the intensity decrease caused by the presence of OCI, which was in turn used to calculate E_{corr}.

3. Results

3.1. Effects of organochlorine insecticides on Laurdan GP in AChR-rich native membranes

Organochlorine insecticides comprise a diverse group of agents that can be categorized into three distinct chemical groups, namely the chlorodiphenylethane, the chlorinated cyclohexane and the chlorinated cyclodiene families (Fig. 1).

In order to study the influence of organochlorine insecticides on the physical characteristics of native AChR-rich membranes from *Torpedo* sp., we first resorted to the known steady-state spectral properties of the fluorescent probe Laurdan (Parasassi et al. 1990, 1991). Fig. 2 shows Laurdan excGP values calculated from emission spectra recorded under direct excitation and energy transfer (FRET) conditions, in the latter case using the intrinsic fluorescence of the membrane as donor and Laurdan as acceptor (Antollini et al., 1996; Antollini and Barrantes, 1998). The intrinsic fluorescence of the AChR protein is assumed to arise mainly from Trp residues with high quantum yield (Narayanaswami and McNamee, 1993).

Within the concentration range used ($\leq$20 μM) all insecticides, except for Lindane and Dieldrin, produced a significant dose-dependent increase in excGP (Fig. 2). The OCI from the chlorodiphenylethane family induced the most intense effects on Laurdan excGP, albeit with significant differences among its members. These results indicate that the OCI were incorporated in the AChR-rich membrane and that they decreased Laurdan–solvent dipolar relaxation, an observation that has been previously interpreted as reflecting a decrease in membrane molecular dynamics (Parasassi et al. 1990, 1991). Similar variations of GP values in the presence of OCI were obtained under direct and FRET conditions (Fig. 2), indicating that these insecticides exert similar effects both on the bulk membrane lipid and on the lipid microenvironment of the donor protein, although with relatively greater intensity in the former case.

3.2. Effects of insecticides on the efficiency of FRET

We have recently introduced a new strategy exploiting Laurdan FRET measurements in AChR-rich membranes to localize sites for different lipid classes on the surface of the AChR protein (Antollini and Barrantes, 1998). Here we extend this approach in an attempt to determine whether OCI are localized and/or exert effects in/on the vicinity of emitting protein donor fluorophores. FRET experiments were carried out at an excitation wavelength of 290 nm (Table 1).

All insecticides used decreased the efficiency of FRET between the membrane protein and Laurdan, albeit to different extents. Chlorodiphenylethane derivatives produced a marked decrease in FRET efficiency, most of them within a range between ~30% and 50%. Lindane and Dieldrin decreased FRET efficiency by only 9% and 25%, respectively, whereas the rest of the cyclodiene compounds decreased FRET efficiency by 45–60%. The data further reinforce the conclusion from excGP experiments (Fig. 2), firmly establishing that these compounds localize in the immediate lipid microenvironment of the membrane protein.

3.3. Effect of OCI on fluorescence anisotropy of DPH and related compounds

Fluorescence anisotropy of DPH and its more polar derivative, PA-DPH, was used to study the interaction of OCI with the native AChR-rich membrane and model lipid systems. At room temperature, the AChR-rich membrane was found to be in a highly ordered liquid-crystalline phase, in accordance with its very high ($>$40%) cholesterol content (see review in Barrantes, 1993). Nearly all the insecticides tested produced intense disordering effects in AChR-rich DPH-doped membranes, but Lindane exerted a very small disordering effect (1.6%). Dieldrin produced only a slight decrease (0.6%) in anisotropy. PA-DPH detected far less important changes for all the compounds tested, with trends similar to those sensed by DPH. Lindane was the exception, showing a very small ordering effect (0.2%). Dieldrin produced a small diminution of the anisotropy (2.6%), although greater than that observed with DPH (Fig. 3).

The effects exerted by the chlorodiphenylethane OCI were more pronounced than those produced by members of the chlorinated cyclodiene family (Fig. 3). In order to better understand these effects, we studied the fluorescence anisotropy of DPH in synthetic liposomes made of DOPC–40 mol% cholesterol, a mixed system exhibiting a highly ordered liquid-crystalline phase (Table 2).

In this model system, all the insecticides decreased DPH anisotropy, an effect similar to that observed in the native AChR-rich membrane. At variance with the latter results, the cyclodiene family induced higher disordering

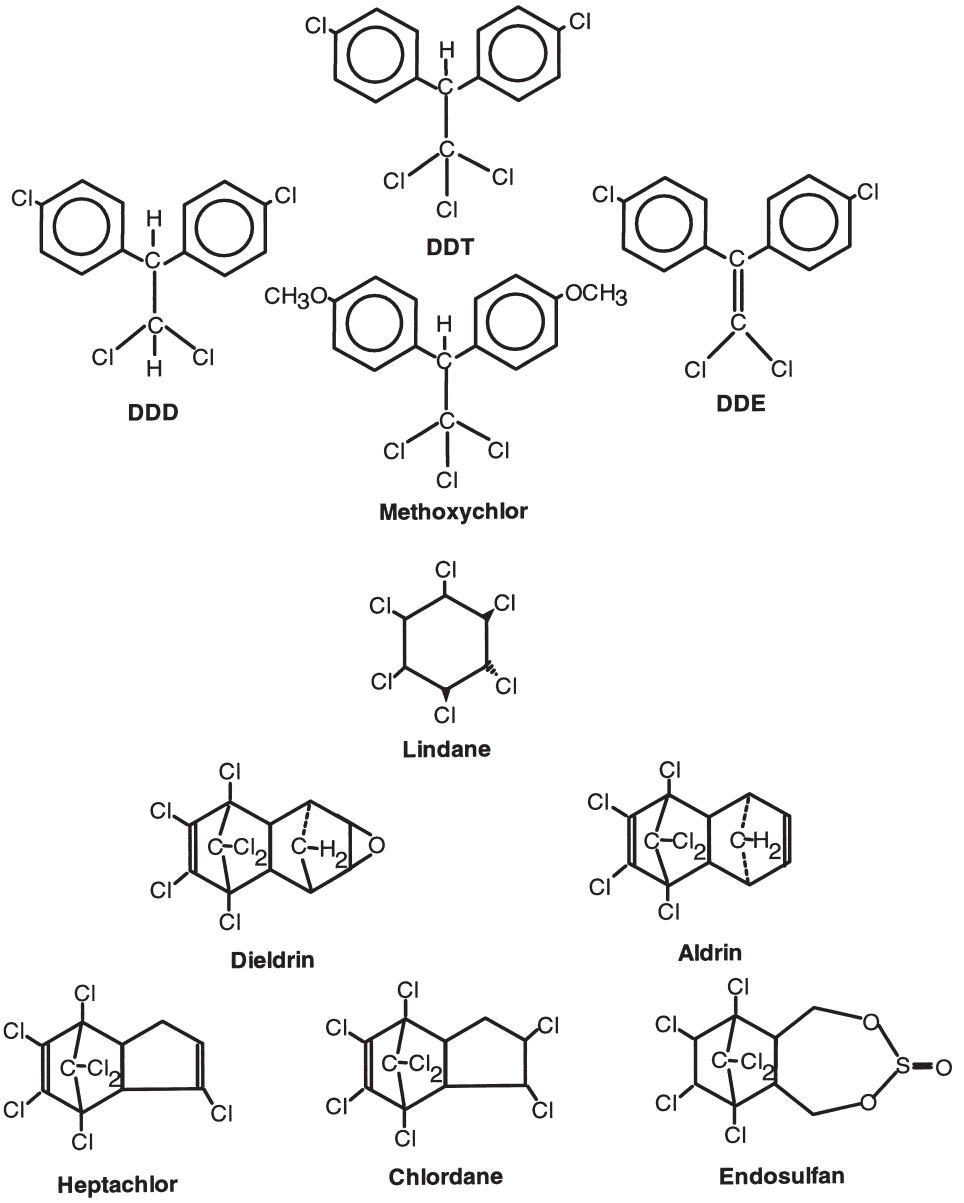

Fig. 1. Schematic formulae of some organochlorine insecticides.

effects than the chlorophenylethane family. Lindane and Dieldrin exerted only minor changes in this model system.

3.4. Effects of OCI on thermotropic properties of native and model membranes sensed by Laurdan GP

We next studied the thermotropic behavior of Laurdan GP in the presence of insecticides in both native AChR-rich membranes and pure DMPC liposomes (Fig. 4).

All organochlorine compounds decreased the gel to liquid-crystalline phase transition temperature midpoint in DMPC bilayers and broadened the temperature range of the transition profile, an observation in agreement with previous reports (Antunes-Madeira and Madeira, 1989; Chefurka et al., 1987; Buff and Berndt, 1981; Buff et al., 1982; Sabra et al., 1996). The data of Fig. 4 also indicate that the chlorophenylethane insecticides increased GP values with respect to the control condition (DMPC without insecticides) throughout the temperature range explored, whereas only a slight diminution in GP was observed in the presence of Lindane and the chlorinated cyclodienes in the phase transition region. In the gel and liquid-crystalline phases of DMPC bilayers Lindane showed higher GP values, whereas the cyclodiene derivatives did not produce any significant effect.

It has long been known that cholesterol concentrations above 30 mol% abolish phase transitions in liposomes (Ladbrooke et al., 1968). Taking into account the high cholesterol concentration of the AChR-rich membrane

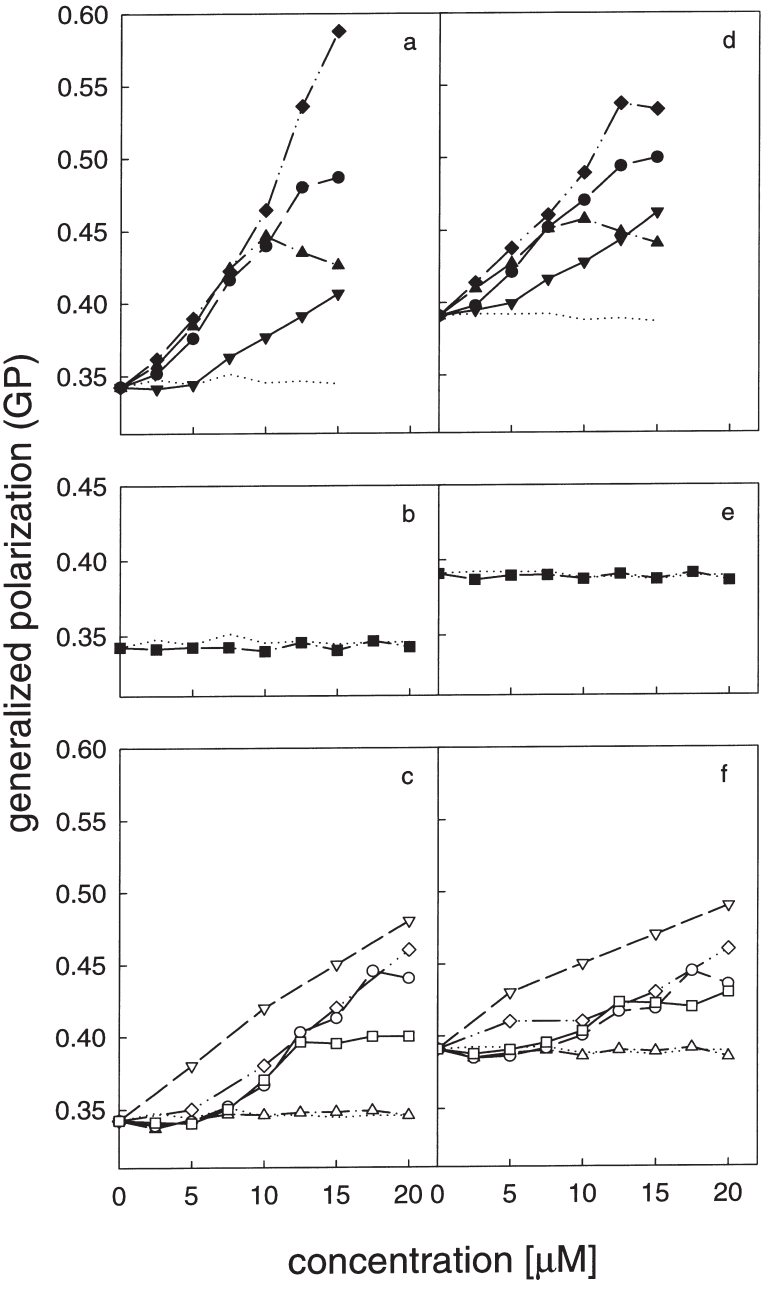

Fig. 2. Laurdan excGP in *Torpedo marmorata* AChR-rich membrane measured using direct excitation of the probe [360 nm, panels (a)–(c)] or FRET [290 nm, panels (d)–(f)] from the protein emission, in the presence and absence of increasing concentrations of OCI. Panels (a) and (d): DDT (▲), DDD (◆), Methoxychlor (●) and DDE (▼). Panels (b) and (e): Lindane (■). Panels (c) and (f): Dieldrin (△), Aldrin (◇), Heptachlor (○), Chlordane (▽) and Endosulfan (□). The effect produced by solvent (ethanol) is also shown (dotted lines). Each point corresponds to the mean of more than three different determinations. Average standard deviations were <0.01 and are not shown for the sake of clarity.

(>40 mol%), it is not surprising that the thermotropic profiles of Laurdan GP showed no phase transition in the presence of the aforementioned insecticides. The GP values and trends obtained are in good agreement with those found in titration experiments at a fixed temperature (20°C, cf. Fig. 2).

3.5. Comparative effects of cholesterol and OCI

In order to understand further the effects of OCI we measured the wavelength dependence of Laurdan GP incorporated into several phospholipid model systems, with different amounts of cholesterol and/or insecticide. GP measurements at more than one excitation or emis-

Table 1
Effect of OCI on the efficiency of FRET in AChR-rich native membranes

Insecticide	Δ(Eff. FRET) (%)
DDT	−49.1
DDD	−41.8
Methoxychlor	−45.6
DDE	−57.8
Lindane	−9.9
Dieldrin	−24.5
Aldrin	−32.9
Heptachlor	−58.4
Chlordane	−63.9
Endosulfan	−42.6

Table 2
Effect of OCI on DPH anisotropy in liposomes of DOPC–40 mol% cholesterol

Insecticide	Δ*r* (%)
DDT	−5.3
DDD	−7.3
Methoxychlor	−3.8
DDE	−7.6
Lindane	−1.8
Dieldrin	−2
Aldrin	−7.1
Heptachlor	−9.2
Chlordane	−4.8
Endosulfan	−9.5

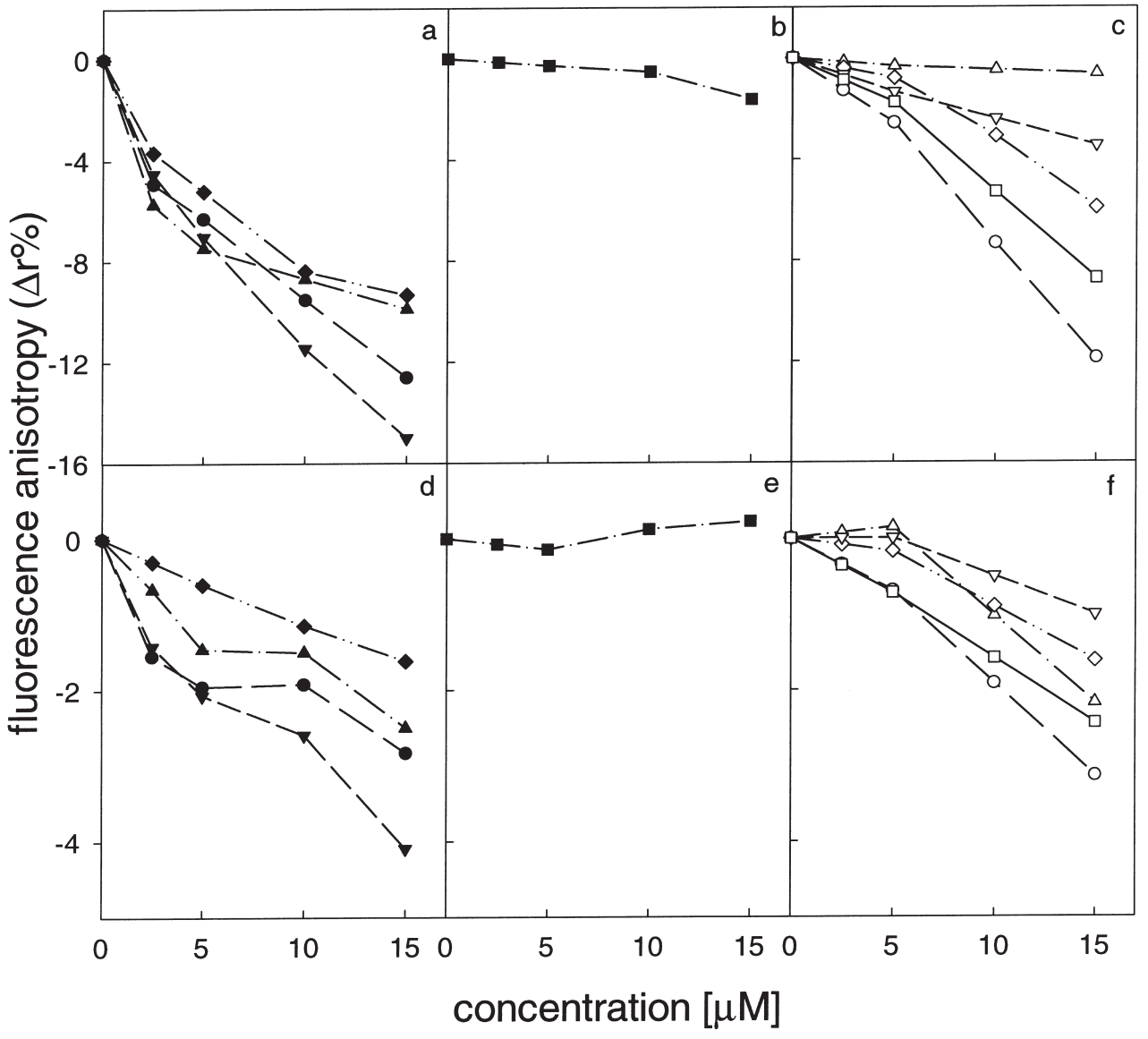

Fig. 3. Relative changes in fluorescence anisotropy of DPH (a)–(c) and PA-DPH (d)–(f) in *Torpedo marmorata* AChR-rich membrane at 20°C, in the presence and absence of increasing concentrations of OCI. Each point corresponds to the mean of more than three different determinations. Average standard deviations were <0.01 and are not shown for the sake of clarity. Symbols as in Fig. 2.

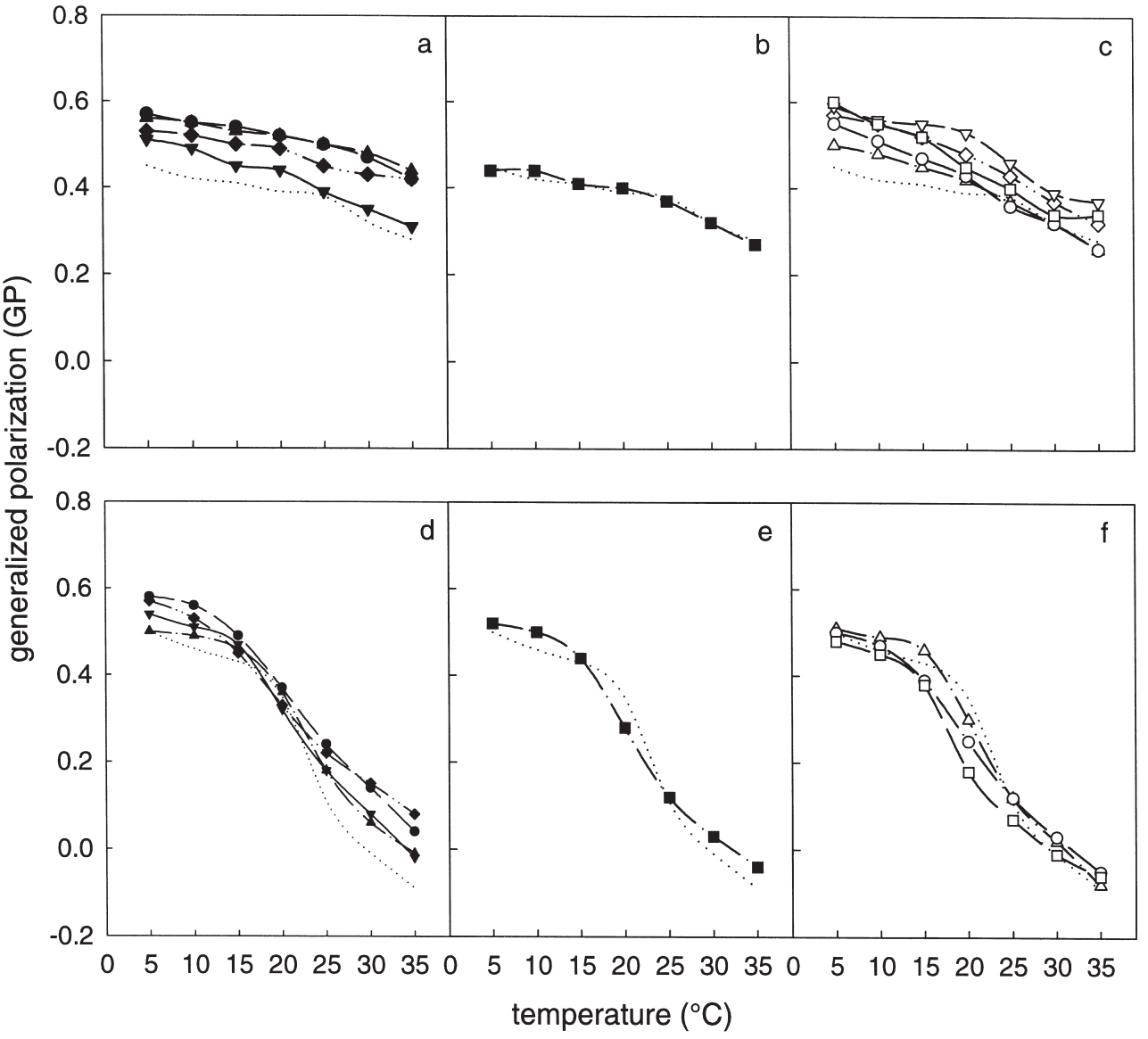

Fig. 4. Temperature dependence of Laurdan excGP in *Torpedo marmorata* AChR-rich membrane (upper panel) and in multilamellar liposomes of DMPC (lower panel), by direct excitation of the probe (360 nm). The concentration of the chlorodiphenylethane derivatives was 10 μM [(a) and (d)], and that of Lindane [(b) and (e)] and the chlorinated cyclodienes [(c) and (f)], 20 μM. Each point corresponds to the mean of more than three different independent determinations. Average standard deviations were <0.01 and are not shown for the sake of clarity. Symbols as in Fig. 2.

sion wavelength allowed us to determine whether domains of different phase properties coexisted in the membrane in the presence of the insecticides. We chose one representative insecticide from each family, and their influence on the physical state of synthetic phospholipids in gel, liquid-crystalline, and coexisting gel/liquid-crystalline phases was investigated (Figs. 5 and 6).

No significant changes in Laurdan excGP (360 nm excitation) or in the GP versus wavelength profile (data not shown) were observed in DPPC liposomes in the gel phase for any of the insecticides studied, either in the presence or absence of cholesterol. In a different lipid system in the liquid-crystalline phase (pure DOPC liposomes), DDD and Aldrin significantly augmented the excGP at 360 nm [Fig. 5(b)]. In the presence of cholesterol, DDD and Aldrin effects on Laurdan GP were only ~60% and 20%, respectively, of those produced in the absence of cholesterol. Conversely, the cholesterol-mediated increase in Laurdan GP also decreased up to ~35% in the presence of insecticides (data not shown). Thus, the effects of OCI and cholesterol do not appear to be additive.

Furthermore, when we compared the effect of OCI with that of cholesterol on the wavelength dependence of Laurdan GP in DOPC–DPPC liposomes, we found that all the chlorophenylethane and chlorinated cyclodienes behaved in a manner strikingly similar to choles-

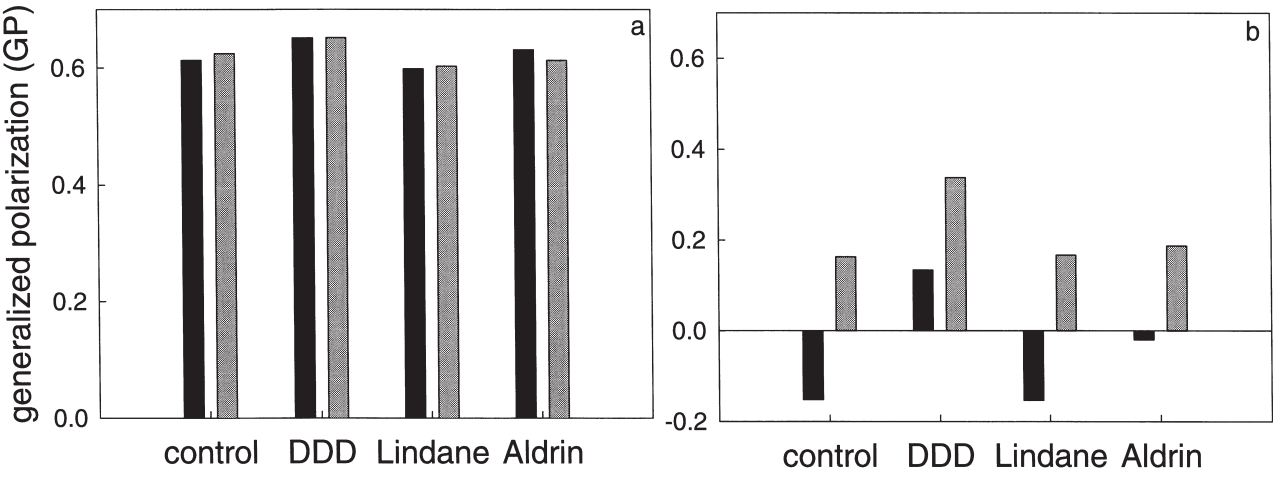

Fig. 5. Maximum Laurdan excGP attained in titration experiments with DDD, Lindane and Aldrin in DPPC (a) and in DOPC (b) multilamellar liposomes at 20°C, in the absence (black columns) and presence (gray columns) of cholesterol. All insecticides were used at a final concentration of 15 μM. Control experiments were done in parallel with those of OCI, by ethanol titration of pure liposomes.

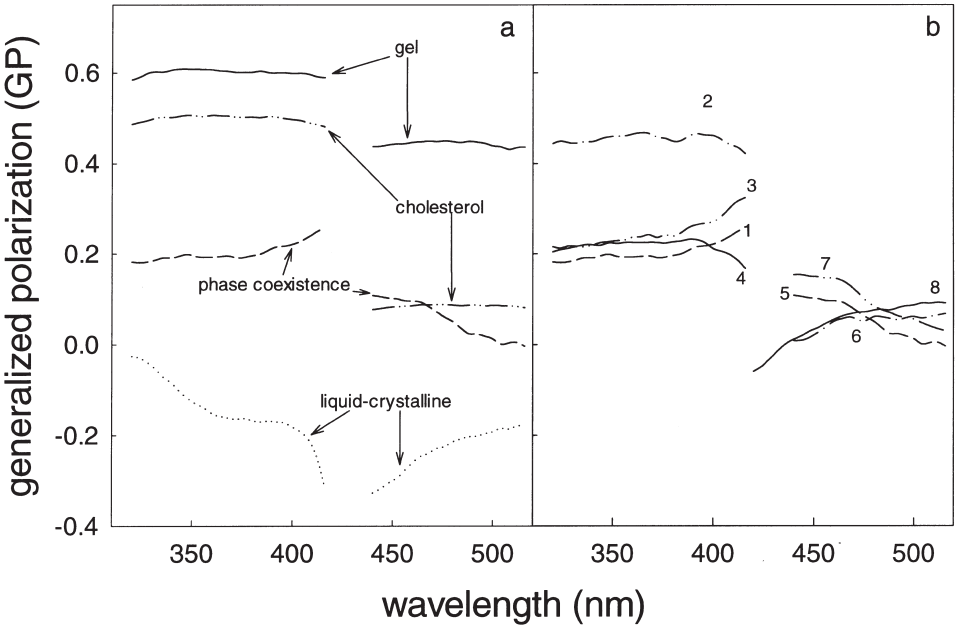

Fig. 6. Wavelength dependence of Laurdan excGP and emGP measured in different lipid model systems at a temperature of 20°C in the absence or presence of OCI at a final concentration of 15 μM. (a) DPPC in the gel phase (———), DOPC–DPPC (—— ——), DOPC (· · ·), DOPC–DPPC–50% mol cholesterol (– · · –). (b) Curves 1 and 5, DOPC–DPPC; curves 2 and 6, DDD; curves 3 and 7, Lindane; curves 4 and 8, Aldrin.

terol, with far more intense effects observed for DDD than for Aldrin: the presence of these compounds profoundly perturbed the biophysical properties of the phase coexistence. In the liquid-crystalline/gel phase, cholesterol is considered to average or abolish the differences in motion and order of lipids belonging to different phase domains, such that the liquid-crystalline/gel phase can no longer be detected (Parasassi et al., 1993). Lindane did not significantly affect the wavelength dependence of Laurdan GP.

4. Discussion

Our current interest in studying organochlorine insecticides is to establish whether this class of hydrophobic compounds has direct effects on the lipid microenvironment of the AChR protein and to elucidate their possible mechanism of action. In the present work we exploited the spectroscopic properties of the fluorescent probe Laurdan to investigate the effects of several OCI on the lipid molecular dynamics using the paradigm experi-

mental system, i.e., native AChR-rich membranes from *Torpedo* sp.

The chemical characteristics of OCI enable their classification into three separate groups (Fig. 1). Our present results, shedding light on physical parameters of the AChR-rich membrane, appear to concur with this classification, since OCI of the same family produce similar effects. The solvent dipolar relaxation process, as measured by Laurdan GP in the AChR-rich membrane, was decreased to different extents by the chlorophenylethane and chlorinated cyclodiene derivatives, respectively. The largest increase in GP values was observed for the DDD family; Chlordane, Heptachlor, Aldrin and Endosulfan produced intermediate changes, and Lindane and Dieldrin produced no statistically significant changes at all (Fig. 2).

Silvestroni et al. (1997), who studied the effects of Lindane on human sperm cells, observed very small changes in Laurdan excGP and emGP ($\leq$1%) within a broad concentration range; we observe similarly small but not statistically significant changes in the AChR-rich membrane.

The most obvious effect produced by nearly all the pesticides studied is the decrease in DPH and PA-DPH fluorescence anisotropy in a concentration-dependent manner, in both the native AChR-rich membrane and model liposomes. The DPH steady-state anisotropy is primarily related to the restriction in rotational motion of the probe molecule owing to the acyl chain packing order. The decrease observed in this parameter can therefore be explained by a significant structural alteration, induced by OCI, of the bilayer hydrophobic region and to a lesser extent of the more superficial or polar regions, an observation in agreement with previous reports (Antunes-Madeira and Madeira, 1989; Buff et al., 1982; Suwalsky et al., 1997).

All OCI produced a shift of the phase transition midpoint to lower temperatures and a broadening of the transition range (Fig. 4). These changes in thermotropic behavior are traditionally explained by assuming that the insecticide molecules intercalate between fatty acyl chains of adjacent lipids, thus weakening the hydrophobic forces that are the main contributors to stabilization of the bilayer (Buff and Berndt, 1981). The broadening of the transition reflects a disordering of the gel phase and, to a lesser extent, an ordering of the fluid phase. According to Jain and Wagner (1980), the broadening and shifting of the transition profile can be attributed to the location of the foreign molecules in the vicinity of the so-called phase cooperativity region — the first eight carbons of the phospholipid acyl chains — where cholesterol is supposedly preferentially localized. The slight decrease in the GP values observed in the phase transition region in DMPC bilayers (Fig. 4) for Lindane and the chlorinated cyclodienes differs from the temperature-dependent GP profile in the AChR-rich membrane. Nevertheless, Lindane does not perturb the cooperativity extensively, since only a small shift in transition temperature was observed in DMPC bilayers. Suwalsky et al. (1997) studied the effect of Heptachlor on Laurdan GP in large unilamellar DMPC liposomes and also found that this compound exerted a significant decrease in GP in parallel with a decrease in DPH anisotropy. Thus, Laurdan GP would appear to sense the insecticide-induced increase in the dynamics and/or the number of water molecules in the membrane, mediated by an increase in membrane fluidity.

The GP values observed in the phase transition region of DMPC liposomes could be due to the stronger interaction of Lindane and chlorinated cyclodienes with the membrane, especially favored in the phase coexistence region where the oscillations between the two phases augment the packing defects and thereby facilitate the incorporation and/or activity of foreign molecules. This increase in insecticide partition, and consequently the increased capacity of these compounds to perturb the lipid phase, is most likely what Laurdan GP senses in the membrane. This parameter reflects here a disordering effect induced by these two insecticide families. In the case of the chlorophenylethane insecticides, Laurdan GP appears to conceal the physical nature of the effects exerted by these compounds in the phase transition region. This masking effect in the presence of OCI is in accordance with the complex behavior of Laurdan fluorescence in sphingomyelin and glycosphingolipid model systems, in which Laurdan GP appears to reflect the dipolar relaxation process better than the state of order or "fluidity" of the membrane (Bagatolli et al., 1998).

The somewhat paradoxical observation of high Laurdan GP with low DPH anisotropy in the AChR-rich membrane can be interpreted in the light of the observations on the thermal dependence of Laurdan GP in multilamellar DMPC liposomes. Laurdan GP reflects the molecular dynamics and number of solvent dipoles. Thus the increase in GP induced by the OCI in the native AChR-rich membrane may be due to the rearrangement and ordering of solvent dipoles in Laurdan's lipid environment and, indirectly, around pesticide molecules. Since the latter populate preferentially fluid regions of the bilayer (Antunes-Madeira and Madeira, 1989), those molecules located at the hydrophobic core exert disordering or "fluidizing" effects, sensed by DPH fluorescence anisotropy, and those acting at more superficial regions may produce the decrease of the solvent dipolar relaxation process, i.e., the ordering effect, sensed by Laurdan GP.

The presence of cholesterol modified the effects produced by the representative insecticides of the chlorodiphenylethane and cyclodiene families on the liquid-crystalline phase of DOPC liposomes [Fig. 5(b)]. Cholesterol increased the molecular order of fluid-phase bilayers

[Fig. 5(b)] and abolished the phase coexistence of model systems (Fig. 6). One possibility is that cholesterol inhibits the insecticide–membrane interaction either by decreasing the partition coefficient (Antunes-Madeira and Madeira, 1989), and hence the saturation capacity (Omann and Lakowicz, 1982) of OCI in the membrane, or by increasing the lipid structural order, resulting in a diminution of the insecticide's capacity to increase Laurdan GP.

The remarkably similar ability of DDD, Aldrin and cholesterol to abolish phase coexistence in DOPC–DPPC liposomes (Fig. 6) suggests that: (1) both chlorophenylethane and chlorinated cyclodiene pesticides perturb the membrane by a mechanism similar to that of cholesterol and (2) their potency in affecting Laurdan GP may be due to differences in their chemical structure and hydrophobicity. Despite such similarities, OCI induce intense disordering effects at deep regions of the bilayer, whereas cholesterol exerts opposite, rigidizing effects.

From the higher Laurdan GP values under FRET conditions and from the diminution of the efficiency of the FRET process in the presence of OCI, we postulate the existence of potential target sites for these compounds in the AChR microenvironment. The most likely target regions where OCI could exert their effects are the immediate lipid microenvironment of the AChR and/or the lipid-exposed regions of the protein. Neurotoxic chlorinated cyclodienes (Dieldrin) and cyclohexane (Lindane) have been shown to act as non-competitive $GABA_A$ or $GABA_C$ receptor antagonists interacting with the picrotoxinin site, either in membranes or in intact cultured neurons, thereby inhibiting the GABA-induced Cl^- flux following agonist activation (Pomes et al., 1994). Since both GABA receptors and AChR are highly homologous members of the ligand-gated ion channel superfamily (Ortells and Lunt, 1995), it is possible that sites of action for OCI are evolutionarily conserved in both receptor families. Experiments are currently underway in our laboratory to learn about the consequences of OCI effects on AChR function.

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References

Addona, G.H., Sandermann, H. Jr., Kloczewiak, M.A., Husain, S.S., Miller, K.W., 1998. Where does cholesterol act during activation of the nicotinic acetylcholine receptor? Biochimica et Biophysica Acta 1370, 299–309.

Antollini, S.S., Soto, M.A., Bonini De Romanelli, I., Gutiérrez Merino, C., Sotomayor, P., Barrantes, F.J., 1996. Physical state of bulk and protein-associated lipid in nicotinic acetylcholine receptor-rich membrane studied by laurdan generalized polarization and fluorescence energy transfer. Biophysical Journal 70, 1275–1284.

Antollini, S.S., Barrantes, F.J., 1998. Disclosure of discrete sites for different lipids at the protein–lipid interface in native acetylcholine receptor-rich membrane. Biochemistry 37, 16653–16662.

Antunes-Madeira, M.C., Madeira, V.M., 1986. Partition of DDT in synthetic and native membranes. Biochimica et Biophysica Acta 861, 159–164.

Antunes-Madeira, M.C., Madeira, V.M., 1989. Membrane fluidity as affected by the insecticide lindane. Biochimica et Biophysica Acta 982, 161–166.

Arhem, P., Frankenhaeuser, H., 1974. DDT and related substances: effects on permeability properties of myelinated *Xenopus* nerve fibre. Potential clamp analysis. Acta Physiologica Scandinavica 91, 502–511.

Bagatolli, L.A., Gratton, E., Fidelio, G.D., 1998. Water dynamics in glycosphingolipid aggregates studied by LAURDAN fluorescence. Biophysical Journal 75, 331–341.

Barrantes, F.J., 1982. Interactions between the nicotinic acetylcholine receptor and the non-receptor, peripheral nu-peptide (M_r 43,000). In: Hucho, F. (Ed.) Neuroreceptors. W. De Gruyter, Berlin/New York, pp. 315–328.

Barrantes, F.J., 1993. The lipid annulus of the nicotinic acetylcholine receptor as a locus of structural–functional interactions. In:. Watts, A. (Ed.), New Comprehensive Biochemistry, vol. 26. Elsevier, Amsterdam, pp. 231–257.

Barrantes, F.J., 1998. The Nicotinic Acetylcholine Receptor: Current Views and Future Trends. Springer Verlag and Landes Publishing Co, Berlin/Heidelberg and Georgetown, TX226 pp.

Blanton, M.P., Wang, H.H., 1990. Photoaffinity labeling of the *Torpedo californica* nicotinic acetylcholine receptor with an aryl azide derivative of phosphatidylserine. Biochemistry 29, 1186–1194.

Blanton, M.P., Cohen, J.B., 1992. Mapping the lipid-exposed regions in the *Torpedo californica* nicotinic acetylcholine receptor. Biochemistry 31, 3738–3750.

Blanton, M.P., Cohen, J.B., 1994. Identifying the lipid–protein interface of the *Torpedo* nicotinic acetylcholine receptor: secondary structure implications. Biochemistry 33, 2859–2872.

Bouzat, C.B., Barrantes, F.J., 1993. Acute exposure of nicotinic acetylcholine receptor to the synthetic glucocorticoid dexamethasone alters single-channel gating properties. Molecular Neuropharmacology 3, 109–116.

Bouzat, C.B., Barrantes, F.J., 1996. Modulation of muscle nicotinic acetylcholine receptors by the glucocorticoid hydrocortisone. Possible mechanism of channel blockade. Journal of Biological Chemistry 271, 25835–25841.

Buff, K., Berndt, J., 1981. Interaction of DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane with liposomal phospholipids. Biochimica et Biophysica Acta 643, 205–212.

Buff, K., Brundl, A., Berndt, J., 1982. Differential effects of environmental chemicals on liposomal bilayers. Fluorescence polarization and pesticide–lipid association studies. Biochimica et Biophysica Acta 688, 93–100.

Chefurka, W., Chatelier, R.C., Sawyer, W.H., 1987. Perturbation of phospholipid bilayers by DDT. Biochimica et Biophysica Acta 896, 181–186.

Criado, M., Eibl, H., Barrantes, F.J., 1982. Effects of lipid on acetylcholine receptor: essential need of cholesterol for maintenance of agonist-induced state transitions in lipid vesicles. Biochemistry 21, 3622–3629.

Criado, M., Eibl, H., Barrantes, F.J., 1984. Functional properties of the acetylcholine receptor incorporated in model lipid membranes.

Differential effects of chain length and head group of phospholipids in receptor affinity states and receptor-mediated ion translocation. Journal of Biological Chemistry 259, 9188–9198.

Doherty, J.D., 1979. Insecticides affecting ion transport. Pharmacology and Therapeutics 7, 123–151.

Fong, T.M., McNamee, M.G., 1986. Correlation between acetylcholine receptor function and structural properties of membranes. Biochemistry 25, 830–840.

Fong, T.M., McNamee, M.G., 1987. Stabilization of acetylcholine receptor secondary structure by cholesterol and negatively charged phospholipids in membranes. Biochemistry 26, 3871–3880.

Förster, TH., 1948. Intermolecular energy migration and fluorescence. Annalen der Physik (und Chemie, Leipzig) 2, 55–75.

Jain, M.K., Wagner, R.C., 1980. Introduction to Biological Membranes. John Wiley and Sons, New York.

Ladbrooke, B.D., Williams, R.M., Chapman, D., 1968. Studies on lecithin–cholesterol–water interactions by differential scanning calorimetry and X-ray diffraction. Biochimica et Biophysica Acta 150, 333–340.

Longnecker, M.P., Rogan, W.J., Lucier, G., 1997. The human health effects of DDT (dichlorodiphenyl trichloroethane) and PCBS (polychlorinated biphenyls) and an overview of organochlorines in public health. Annual Review of Public Health 18, 211–244.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Marsh, D., Barrantes, F.J., 1978. Immobilized lipid in acetylcholine receptor-rich membranes from *Torpedo marmorata*. Proceedings of the National Academy of Sciences of the USA 75, 4329–4333.

Marsh, D., Watts, A., Barrantes, F.J., 1981. Phospholipid chain immobilization and steroid rotational immobilization in acetylcholine receptor-rich membranes from *Torpedo marmorata*. Biochimica et Biophysica Acta 645, 97–101.

Narayanaswami, V., McNamee, M.G., 1993. Protein–lipid interactions and *Torpedo californica* nicotinic acetylcholine receptor function. 2. Membrane fluidity and ligand-mediated alteration in the accessibility of gamma subunit cysteine residues to cholesterol. Biochemistry 32, 12420–12427.

Omann, G.M., Lakowicz, J.R., 1982. Interactions of chlorinated hydrocarbon insecticides with membranes. Biochimica Biophysica Acta 684, 83–95.

Ortells, M.O., Lunt, G.G., 1995. Evolutionary history of the ligand-gated ion-channel superfamily of receptors. Trends in Neuroscience 18, 121–127.

Parasassi, T., De Stasio, G., D'Ubaldo, A., Gratton, E., 1990. Phase fluctuation in phospholipid membranes revealed by laurdan fluorescence. Biophysical Journal 57, 1179–1186.

Parasassi, T., De Stasio, G., Ravagnan, G., Rusch, R.M., Gratton, E., 1991. Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of laurdan fluorescence. Biophysical Journal 60, 179–189.

Parasassi, T., Loiero, M., Raimondi, M., Ravagnan, G., Gratton, E., 1993. Absence of lipid gel-phase domains in seven mammalian cell lines and in four primary cell types. Biochimica et Biophysica Acta 1153, 143–154.

Pomes, A., Frandsen, A., Sunol, C., Sanfeliu, C., Rodriguez-Farre, E., Schousboe, A., 1994. Lindane cytotoxicity in cultured neocortical neurons is ameliorated by GABA and flunitrazepam. Journal of Neuroscience Research 39, 663–668.

Rankin, S.E., Addona, G.H., Kloczewiak, M.A., Bugge, B., Mille, K.W., 1997. The cholesterol dependence of activation and fast desensitization of the nicotinic acetylcholine receptor. Biophysical Journal 73, 2446–2455.

Sabra, M.C., Jorgensen, K., Mouritsen, O.G., 1996. Lindane suppresses the lipid-bilayer permeability in the main transition region. Biochimica et Biophysica Acta 1282, 85–92.

Schefczik, K., Buff, K., 1984. The insecticide DDT decreases membrane potential and cell input resistance of cultured human liver cells. Biochimica et Biophysica Acta 776, 337–339.

Silvestroni, L., Fiorini, R., Palleschi, S., 1997. Partition of the organochlorine insecticide lindane into human sperm surface induces membrane depolarization and Ca^{2+} influx. Biochemical Journal 321, 691–698.

Sunshine, C., McNamee, M.G., 1994. Lipid modulation of nicotinic acetylcholine receptor function: the role of membrane lipid composition and fluidity. Biochimica et Biophysica Acta 1191, 59–64.

Suwalsky, M., Benites, M., Villena, F., Aguilar, F., Sotomayor, C.P., 1997. The organochlorine pesticide heptachlor disrupts the structure of model and cell membranes. Biochimica et Biophysica Acta 1326, 115–123.