Kidney International, Vol. 57 (2000), pp. 1382–1389

Nongenomic effects of steroids on the nicotinic acetylcholine receptor

FRANCISCO J. BARRANTES, SILVIA S. ANTOLLINI, CECILIA B. BOUZAT, INGRID GARBUS, and RAMIRO H. MASSOL

Instituto de Investigaciones Bioquímicas, UNS/CONICET, Bahía Blanca, Argentina

Nongenomic effects of steroids on the nicotinic acetylcholine receptor. A fast signaling mode of natural and synthetic steroids is exerted on some ion channels and cell-surface receptors. This activity contrasts with their classic mode of action, via intracellular receptors. Early studies from our laboratory demonstrated that spin-labeled androstanol and cholestane interact with the nicotinic acetylcholine receptor (AChR) and that lipid mobility at the lipid belt surrounding the AChR is reduced relative to that of the bulk membrane lipid. The occurrence of discrete and independent sites for phospholipids and sterols, both accessible to fatty acids, was subsequently disclosed in the native membrane. Synthetic and natural glucocorticoids were found to act as noncompetitive inhibitors of AChR function. The influence of different substituent groups in the cyclepentane perhydrophenanthrene ring on the channel-shortening potency of various steroids has also been assayed in muscle-type AChR, and we found a certain selectivity of this effect. Some organochlorine pesticides are xenoestrogens, that is, environmental agents capable of disrupting endocrine system signaling. We determined their effects on the AChR membrane using novel fluorescence techniques.

Steroid hormone action is mainly exerted through the modulation of protein transcription. In addition to this mode of action, in the past few years, steroids have also been postulated to exert rapid, nongenomic action, mediated by specific interactions with membrane components present at the cell surface.

The nongenomic action of steroids was first described for the γ-aminobutiric acid receptor ($GABA_AR$), one of the members of the ligand-gated ion channel (LGIC) superfamily, of which the nicotinic acetylcholine receptor (AChR) is the paradigm. Electrophysiological studies on the $GABA_AR$ in the presence of the synthetic steroidal general anesthetic alphaxalone unequivocally demonstrated the rapid nongenomic actions on this receptor [1]. These ion channel-active steroids behave as positive allosteric modulators of the $GABA_AR$ [2, 3]. Moreover, at high concentrations, they can activate the receptor protein even in the absence of the agonist [4–7].

Subsequent work on the $GABA_AR$ demonstrated that steroids interact directly with the receptor protein through recognition sites different from those for barbiturates and benzodiazepines [7]. The heteromeric assembly of different $GABA_AR$ isoforms containing different subunit types results in multiple steroid recognition sites on these receptors. This structural diversity is thus responsible for the different modulatory effects exerted by neuroactive steroids on different $GABA_AR$ isoforms. For example, the subtypes of $GABA_AR$ containing α and γ subunits have the greatest sensitivity to pregnanolone modulation of receptor function, whereas those containing β and γ subunits appear to be more sensitive to the general anesthetic alphaxalone [8].

During the last two decades, we have studied some structural–functional aspects of the interactions between the AChR and steroids. This receptor is a neurotransmitter-gated ion channel responsible for the rapid propagation of electrical signals between cells at the neuron–neuron and nerve–muscle synapse. The binding of the neurotransmitter acetylcholine (ACh) to sites located in the extracellular moiety of the AChR causes a conformational change leading to the opening of its associated cation-permeable channel. This receptor is a pentameric integral membrane protein in which the subunit composition varies according to the particular neuroneal or muscular subtype. The AChR in skeletal muscle cells is a heterologous pentamer composed of four different but highly homologous subunits in the stoichiometry of $\alpha_2\beta\gamma\delta$ (embryonic receptor) or $\alpha_2\beta\varepsilon\delta$ (adult receptor). Models for the transmembrane organization of each subunit have been proposed based on cDNA sequence analysis. The current consensus view on the organization of the AChR assumes the occurrence of four transmembrane segments, referred to as M1 through M4, each 20 to 30 amino acid residues in length (Fig. 1).

Electrophysiological, biochemical, and mutagenesis studies have provided strong evidence that the M2 trans-

Key words: nicotinic acetylcholine receptor, lipid-protein interactions, steroids, annular lipids, xenoestrogens, Laurdan.

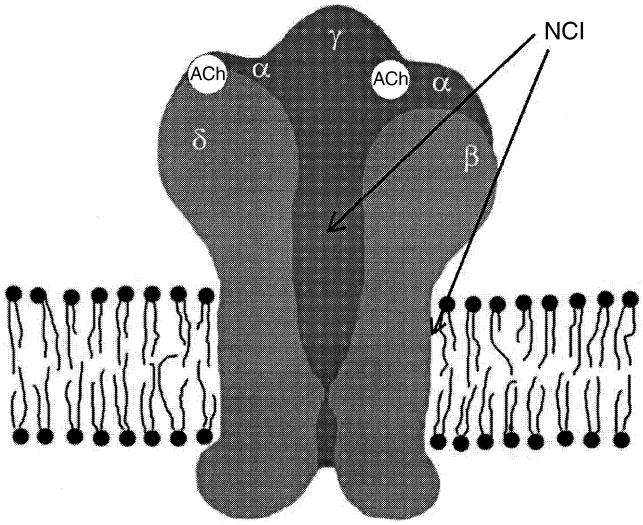

Fig. 1. Schematic representation of the acetylcholine receptor (AChR) molecule and binding sites for the natural neurotransmitter acetylcholine (ACh) and for noncompetitive inhibitors (NCI).

membrane domain is the main contributor to the walls of the ion channel proper. M4 is the firmest candidate among the four transmembrane segments to be in contact with membrane lipids, given its high hydrophobicity and the fact that it constitutes the predominant target for hydrophobic photoaffinity labels [9]. Although this segment is not part of the ion pore, it is involved in channel-gating kinetics [10].

The transitions between the functional states of the AChR are affected by a broad class of pharmacological agents termed noncompetitive inhibitors (NCIs). These compounds comprise a wide range of structurally different chemicals, ranging from the neuroleptic chlorpromazine to the hallucinogen drug phencyclidine, ethidium, local anesthetics related to lidocaine, derivatives of the general anesthetics, and alcohols. Endogenous ligands include free fatty acids [11], steroids [12–14], the neuropeptide substance P [15], and the neurotransmitter serotonin [16]. Their modulatory action is based on the inhibition of the ion flux by sterical or allosterical channel blockade or by modification of the rate of desensitization. Based on their affinity and location of binding sites, NCI have been classified into high- and low-affinity compounds. The high-affinity NCIs bind with a stoichiometry of 1:1 to a site presumably located at the lumen of the ion channel. Low-affinity NCIs comprise a diverse group of hydrophobic agents, which probably affect ion conduction by allosteric mechanisms. The precise site of action of these compounds and the mechanism of channel inhibition are still a matter of controversy.

Although there is little doubt about the location of the binding sites for high-affinity NCIs at the channel lumen, the mechanism and site of action of low-affinity NCIs, such as neuroactive steroids, have proved to be particularly elusive. Early work from our and other laboratories has shown that the AChR protein associates preferentially with some sterols [9, 17–20]. In addition, photoaffinity labeling studies established the close proximity of cholesterol to the AChR protein in *Torpedo* membranes [21].

The previously mentioned biochemical and biophysical findings provided the necessary background to study the interaction of steroids with the AChR in living cells, an issue that is the focus of intense investigation in our laboratory. Our initial studies made use of the clonal cell line BC3H-1, which expresses endogenous embryonic-type muscle AChR. We studied the action of the prototype glucocorticoid hydrocortisone (HC) at the single-channel level (Fig. 2). We first compared the effect of HC on embryonic and adult-type AChR. Cell-attached patches were made in the presence of different concentrations of HC in the pipette solution. The main change in channel kinetics was a dose-dependent decrease in the duration of the open state. Thus, single-channel recordings of BC3H-1 cells in the presence of the synthetic glucocorticoid dexamethasone and the natural glucocorticoids HC and 11-desoxycortisone enabled us to demonstrate that steroids act as NCIs of the muscle AChR [12, 13]. Furthermore, we showed this to be a rapid and direct effect on the ACh-activated channel that was not mediated by steroid receptors: The action was similar on both intact cells and isolated patches [22], and no delay was apparent in their action on the AChR.

These results were first interpreted considering that the action of the drug followed a simple blocking mechanism of the type:

$$C \underset{\alpha}{\overset{\beta}{\Leftrightarrow}} O \underset{b}{\overset{f[B]}{\Leftrightarrow}} OB \qquad \text{(Scheme 1)}$$

where C is closed, O is open, OB is the blocked state of the AChR, and f and b are the forward and backward rate constants for the blocking effect. The values obtained for f were 2.04×10^6 and 1.78×10^6 mol/L$^{-1} \cdot$ s^{-1} for fetal and adult AChR, respectively. These values are similar to those previously reported for other low-affinity NCIs, such as general anesthetics (isoflurane, alphaxalone) and alcohols, and indicate that HC does not distinguish between the γ and ϵ subunits or, alternatively, that the site(s) on which HC acts comprises regions of the AChR protein well conserved among the two subunits. The fact that the effects of the drug on the AChR are consistent with a protein-blocking scheme supports the view of direct action of the drug with protein domains and discards an indirect, lipid-mediated mechanism.

In another series of experiments, we observed that the burst duration decreased as a function of HC concentration [14]. A burst is defined as a series of opening events

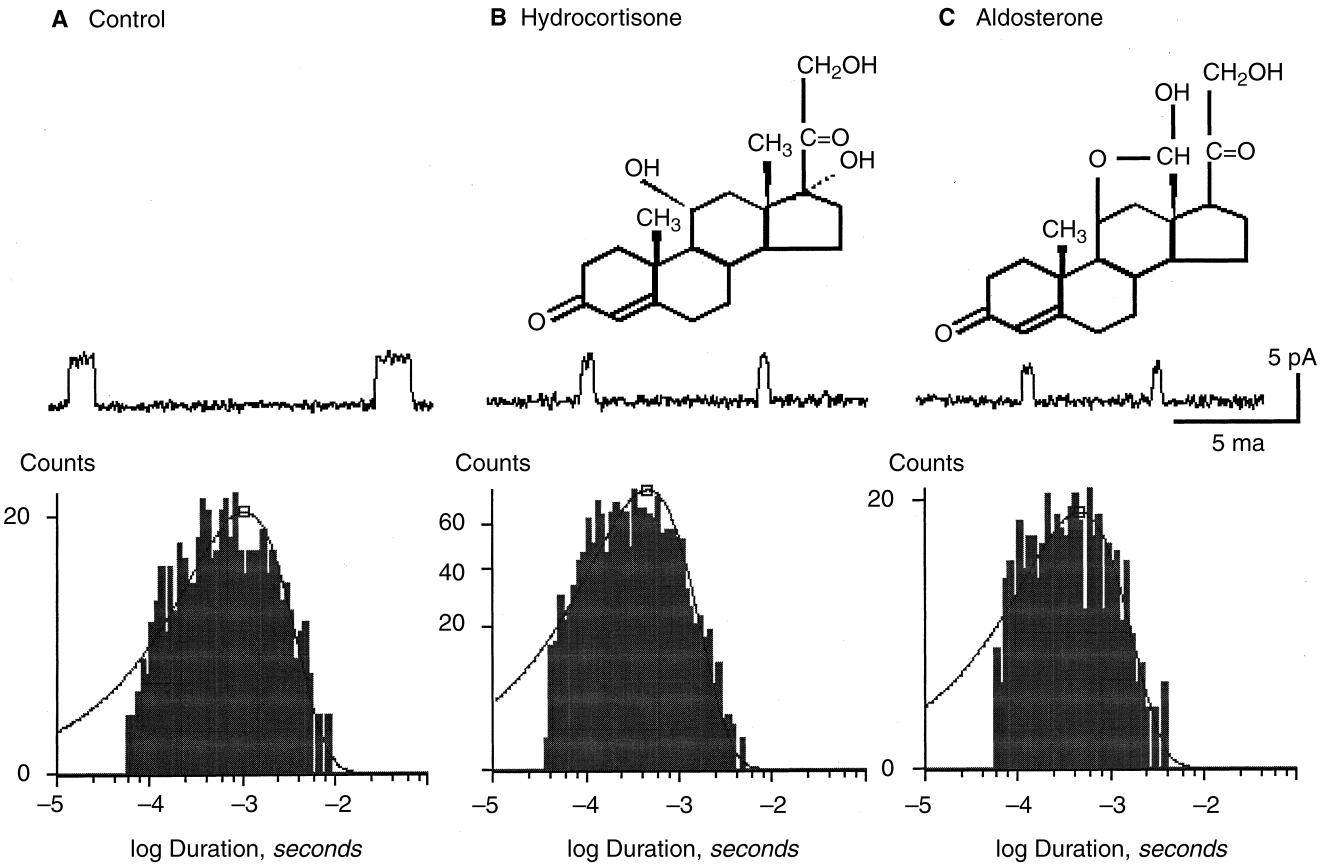

Fig. 2. Effect of the glucocorticoid hydrocortisone and the mineralocorticoid aldosterone on the AChR, as observed by patch-clamp recording. Below, the chemical formulas of the two compounds single-channel traces are observed. The opening of a channel is observed as an upward deflection. The bottom panels show histograms corresponding to the mean open time of the adult-type AChR in the absence (*A*) or in the presence of 200 μmol/L hydrocortisone (*B*) or 200 μmol/L aldosterone (*C*). AChR channels were activated in all cases by 1 μmol/L ACh present in the patch pipette.

corresponding to the same AChR molecule. According to scheme 1, the burst duration in the presence of HC is expected to increase because blockages of the channel delay the open-close channel transition, that is, the ending of the burst. Thus, the observed reduction in the burst duration [14] is not compatible with a classic open-channel blocking mechanism, but can be explained by the closure of the open-blocked channel in accordance with the following scheme that has also been suggested for the action of isoflurane on the AChR [23]:

$$C \underset{\alpha}{\overset{\beta}{\Leftrightarrow}} O \underset{b}{\overset{f[B]}{\Leftrightarrow}} OB \underset{\beta'}{\overset{\alpha'}{\Leftrightarrow}} CB \qquad \text{(Scheme 2)}$$

Knowing that HC acted at AChR domains, we investigated whether these domains were located inside or outside of the membrane. To determine if HC was acting on the ion pore at the same site as open-channel blockers, we tested the effect of HC in the presence of the open-channel blocker QX-222. We found that the reduction in channel open time induced by QX-222 was independent of the presence or absence of HC, indicating that the compounds act on different sites. In another series of experiments, we took advantage of the different configurations afforded by the patch-clamp technique, allowing the addition of drugs from either side of the AChR-containing membrane. Similar effects on the mean open time were observed whether HC was applied to the extracellular or to the intracellular membrane leaflet. Experiments with dexamethasone produced similar results. Moreover, dexamethasone affected the AChR channel mean open time when added from the extracellular side of the membrane, but in a region distant from the patch pipette. For this to occur, the drug had to diffuse along the membrane to reach its site of action on the AChR protein. These data support the hypothesis that the site of action of HC is located at the level of the lipid bilayer and that this steroid reaches this site through a membrane pathway.

Analyses at the single-channel level allow the following conclusions: (*1*) glucocorticoids act as NCIs of the AChR; (*2*) they block embryonic and adult AChRs in a similar manner; (*3*) the blocking phenomenon differs from that of classic open-channel blockers; and (*4*) the

blockade does not depend on agonist concentration. Our experiments located the HC site in domains of the receptor probably in contact with the membrane lipid. HC might thus enter the membrane and move to a site(s) from which it allosterically blocks the AChR ion channel.

We are currently analyzing the relationship between substrates of the cyclepentaneperhydrophenantrene ring and the modulatory action of steroids by performing single-channel patch-clamp recordings in the presence of different concentrations of various steroids. One of the steroids being assayed is the mineralocorticoid aldosterone, a natural steroid synthesized by the adrenal cortex. Its chemical structure is related to HC (Fig. 2), and it plays a key role in the regulation of sodium and potassium equilibrium. Although aldosterone and HC differ in their main physiological action, their capacity to modify AChR function was found to be similar. Analysis of the inhibitory potency of a wide range of steroids allowed us to conclude that the modulatory effect is structurally specific. The presence of an OH group in position 11 appears to be a crucial factor in conferring steroid potency on the AChR (Garbus, Bouzat, and Barrantes, unpublished observations).

PRESENCE OF DISTINCT SITES FOR CHOLESTEROL AT THE ACETYLCHOLINE RECEPTOR–LIPID INTERFACE

The presence of immobilized lipid molecules in the vicinity of the AChR led to development of the concept of the receptor-associated lipid belt region or "annulus" (Fig. 3) [17]. This region, located in the immediate microenvironment of the AChR, possesses certain biophysical characteristics that distinguish it from the bulk lipid in the rest of the membrane. Moreover, it has been demonstrated that different classes of lipids have different affinities for the AChR lipid belt region [19, 24]. The original suggestion that this region could be relevant in functional terms, for example, for the modulation of receptor function by lipophilic substances like local anesthetics [17], has led to a variety of in vitro studies aimed at characterizing the dependence on lipid composition.

Early work established that the presence of cholesterol and acidic phospholipids was necessary to maintain agonist-induced state transitions of the AChR in vitro [25–27]. It was suggested that the lipid annulus needed to be "fluid" for a correct AChR activity [27, 28]. Sunshine and McNamee subsequently demonstrated that the AChR channel properties were maintained on reconstitution of the AChR protein in synthetic lipid systems exhibiting different degrees of fluidity, concluding that for a proper AChR activity, the presence of some specific lipids ought to be more important than membrane fluidity [29, 30]. This led to several hypotheses on the role of the aforementioned lipids in AChR function.

The effects of cholesterol and acidic phospholipids on AChR structure have also been reported. Both types of lipid were postulated to contribute to the stabilization of the secondary structure of AChR transmembrane segments [28, 31, 32]. Furthermore, specific sites have been determined for these lipids at the lipid–AChR interface [33, 34]. These hypotheses are useful in explaining the mechanism and sites of action of some NCIs. These compounds could alter AChR function by: (*1*) modifying the fluidity of the receptor lipid annulus, (*2*) displacing specific endogenous lipids from the AChR–lipid interface, and/or (*3*) interacting with the AChR at sites located at the protein–lipid interface.

To address the characterization of specific lipid sites on the AChR protein, we have recently employed several fluorescence spectroscopy techniques in a well-characterized model system, the (native) AChR-rich membrane from *Torpedo* electric tissue. We have extended the use of the fluorescent probe Laurdan (6-dodecanoyl-2-(dimethylamino)-naphthalene) [35], introduced in the field of biological membranes by Parasassi et al to study the AChR in its native membrane environment by means of (*1*) the so-called generalized polarization (GP) technique and (*2*) measurement of the efficiency of the Förster resonance energy transfer (FRET) process in the presence of exogenous ligands, including lipids, sterols, and xenosteroids [36, 37].

The fluorophore Laurdan localizes at the level of the polar head groups of phospholipids, and it is extremely sensible to the polarity and dynamics of water dipoles in its surroundings. Laurdan GP reports on the polarity and the phase state of the membrane [36, 37]. In order to study the AChR lipid microenvironment specifically, we designed a new strategy that measures GP under FRET conditions between the intrinsic fluorescence of the AChR protein (donor) because of its tryptophan residues, and Laurdan as the acceptor. This approach allowed us to determine that the lipid annulus in the native AChR membrane has a lower polarity (that is, a lower degree of water penetration) than the bulk lipid membrane. This was interpreted as a reflection of the higher order of the lipid molecules surrounding the AChR protein [35].

Using the same strategy, we determined that when exogenous lipids are added to the AChR-rich membrane, they perturb the polarity and order of both bulk and AChR-vicinal lipids [38]. Dioleoylphosphatidylcholine (DOPC) and oleic acid (18:1) decrease the polarity and the order of these two lipid environments. The water-soluble ester of cholesterol, cholesterol hemisuccinate (CHS), induces only a minor decrease in the polarity of both lipid regions, without significantly modifying the lipid order.

We have used the efficiency of the energy transfer process, $\boldsymbol{E}$, as a sensitive tool to measure the influence

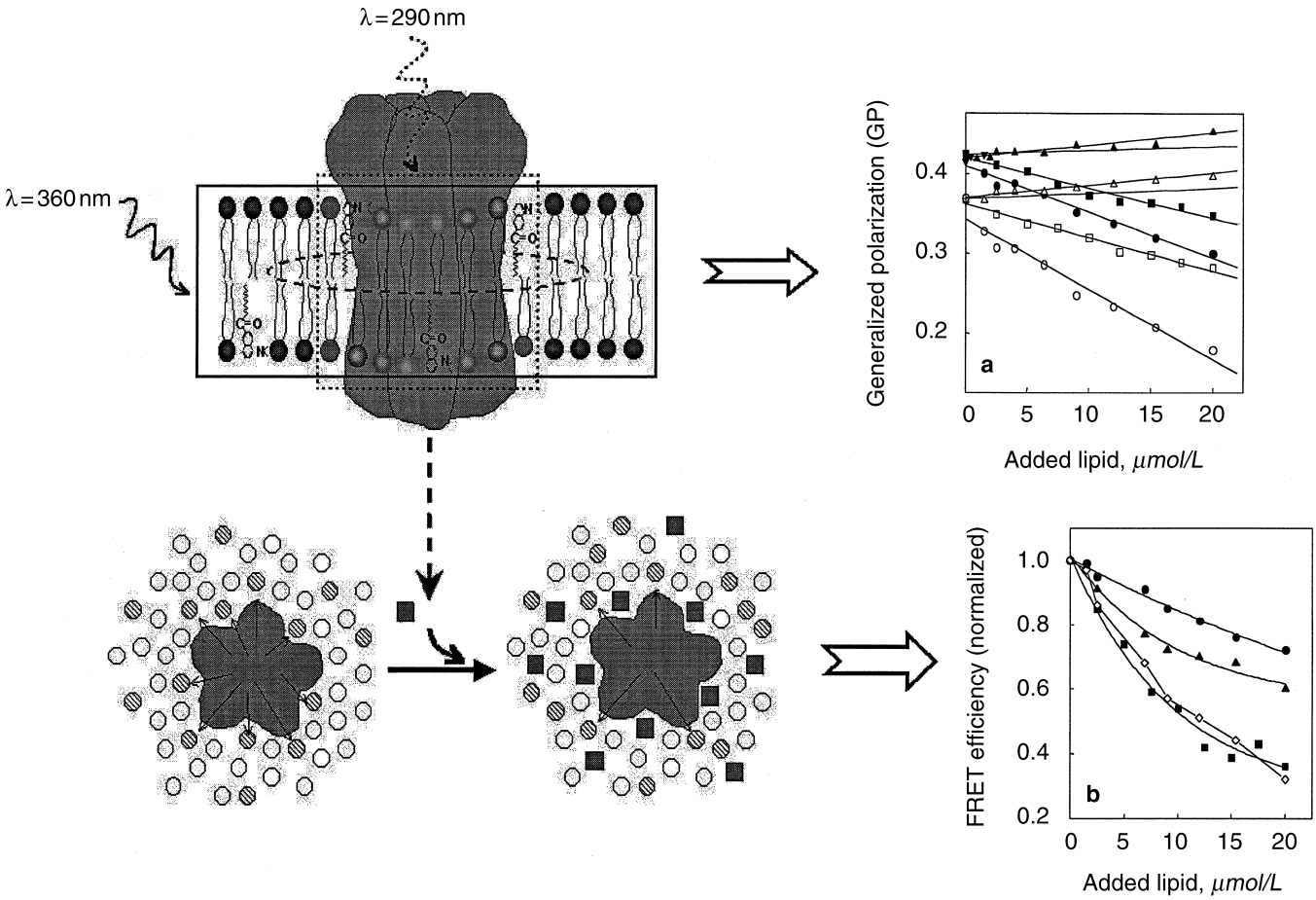

Fig. 3. Schematic representation of the AChR, its lipid microenvironment, and the fluorescent probe Laurdan, side view (top left) and cross-section (bottom left). (Top left) Laurdan molecules reach the excited state following direct excitation (360 nm) or by energy transfer upon excitation of donor tryptophan residues in the AChR at 290 nm. The cartoon shows these two conditions in the solid-line square and in the dash-line square, respectively. (Bottom left) Displacement of Laurdan molecules (dashed circles) from the AChR annulus by addition of exogenous lipid (squares). (Top right) Actual titration curves of Laurdan generalized polarization (GP) under direct excitation (open symbols) and FRET conditions (solid symbols) in AChR-rich native membranes. The control (buffer) curve is shown without symbols; symbols are: 18:1 (circles), DOPC (squares), CHS (triangles). (Bottom right) Efficiency of energy transfer of Laurdan in AChR-rich native membranes. Added lipids are: 18:1 (dark squares), DOPC (dark circles), CHS (dark triangles), and CHS + DOPC (open diamonds). (Parts a and b reprinted with permission from *Biochemistry* 37:16653–16662.)

of compounds on the parameters that determine this phenomenon, the distance between donors and acceptor molecules being especially sensitive. The addition of exogenous lipids to AChR-rich membranes decreased the ***E*** between AChR and Laurdan, the extent of the decrease depending on the specific chemical class of lipid. The maximal decrease in ***E*** was obtained with the fatty acid 18:1 (60%), whereas additions of either cholesterol hemisuccinate or DOPC diminished ***E*** by 35 and 25%, respectively. Furthermore, when a saturating concentration of one class of lipids was added, followed by one of the other classes, the total effect amounted to approximately 60%. However, if 18:1 was added first, a total reduction of approximately 60% was obtained without a further decrease when cholesterol hemisuccinate or DOPC was subsequently added. We interpret these results by considering that the diminution of ***E*** reflects the displacement of Laurdan molecules from the AChR microenvironment by exogenous lipids that increase the distance between donor and acceptor molecules. Such displacements are independent of one another and additive, whereas the effect of fatty acid alone amounts to the sum of the effects caused by DOPC and the cholesterol ester together. The different extents of decrease in ***E*** induced by different classes of lipids could be explained by the fact that each type of lipid displaces a finite number of Laurdan molecules from the AChR–lipid interface. This study allowed us to postulate the occurrence of different sites for DOPC and the cholesterol ester [38]. Thus, two classes of lipid sites appear to occur in the native AChR membrane, one for phospholipid (but not sterol) and the other for sterol (but not

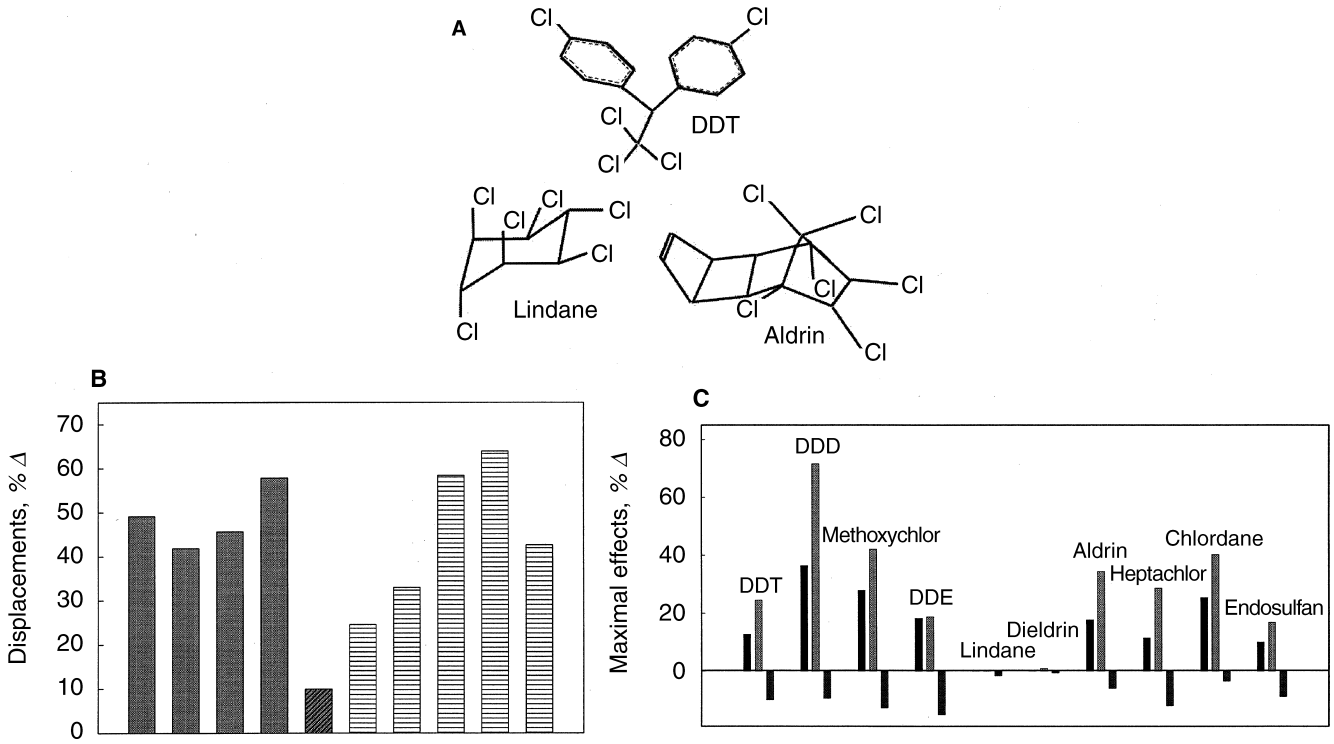

Fig. 4. (*A*) Schematic molecular structures of three representative organochlorine insecticides (OCI), namely DDT, Lindane, and Aldrin. (*B*) Effect of OCI on the efficiency of FRET in AChR-membranes (from left to right): 1. DDT, 2. DDD, 3. Methoxychlor, 4. DDE, 5. Lindane, 6. Dieldrin, 7. Aldrin, 8. Heptachlor, 9. Chlordane, 10. Endosulfan. (*C*) Maximal effects of OCI (15 μmol/L) on Laurdan excGP measured using direct excitation of the probe (360 nm, gray columns) or FRET from the protein emission (290 nm, ■), and on the fluorescence anisotropy of DPH (■) in AChR-rich membranes from *Torpedo marmorata.*

phospholipid), both accessible to fatty acid. These sites may be equivalent to the annular and nonannular sites characterized by Jones and McNamee [33] and Narayanaswami and McNamee [34] for AChR purified and reconstituted in model systems.

We are currently extending our studies to different classes of fatty acids (saturated, unsaturated—*cis* or *trans* double-bond configuration—and with different acyl chain lengths), in order to compare the polarity/order perturbation and the modification of ***E*** induced by their incorporation in AChR-rich membranes. As mentioned previously in this article, fatty acids act as NCIs, and in early studies they appeared to inhibit AChR ionic flux in a similar manner, independent of their structural properties [11]. More recent results indicate that the perturbation of the lipid order in the AChR microenvironment depends on fatty acid structural characteristics, whereas a similar decrease of ***E*** (between 55 and 60%) is induced by different fatty acids independently of their structure (Antollini and Barrantes, unpublished results).

XENOESTROGENS AND ACETYLCHOLINE RECEPTOR

Numerous chemicals with the potential to disrupt the endocrine system [39], particularly by interfering with estrogen signaling, have been identified and termed xenoestrogens [40]. Xenobiotics may alter endocrine function by affecting the availability of a hormone to the target tissue and/or by affecting the cellular response to the hormone.

Organochlorine insecticides (OCI) are a diverse group of agents belonging to four distinct chemical families, including the dichlorodiphenylethane derivatives (DDT, and its metabolites DDE and DDD, Methoxychlor), the chlorinated cyclohexane (Lindane), the chlorinated cyclodiene family (Aldrin, Dieldrin, Heptachlor, Endosulfan, Chlordane, Endrin, Chlordecone, and Mirex), and Toxaphene, which is a mixture of chlorinated terpenes (Fig. 4). Some organochlorine pesticides display xenoestrogenic activity. Among these are *o,p′*-DDT, several *o,p′*-dichloro-substituted analogues, Methoxychlor, and its metabolites, Chlordecone, Dieldrin, Endosulfan, and Toxaphene [41–44]. Most of these compounds are weakly estrogenic, but may nonetheless have significant effects because of their greater bioavailability in serum than natural hormones [41].

The highly lipophilic nature of these compounds together with the temperature sensitivity of some of their effects suggests that their interaction with biological systems may result not only from a direct interaction of the

pesticides with the protein, but also from the perturbation of several physicochemical properties of the lipid bilayer. Dieldrin, Lindane, and Endosulfan have been shown to act as NCIs of the $GABA_AR$ [45, 46]. Given that both the $GABA_AR$ and the AChR are members of the ligand gated ion channel superfamily [47], there is a possibility that some site(s) and/or mechanisms of action for this class of compounds are evolutionarily conserved in both receptor families.

To study the effects that organochlorine pesticides may exert on the AChR and to elucidate their mechanisms of action, we undertook a series of fluorescence studies exploiting the spectral properties of Laurdan. We have found that all of the insecticides tested (with the exception of Lindane and Dieldrin) exert a progressive decrease in the lipid order of the AChR membrane, and that they also decrease ***E*** between the membrane proteins and Laurdan (Fig. 4 and unpublished results). Based on our experimental findings, we hypothesize that these compounds are localized in the so-called phase cooperativity region [48], where cholesterol is assumed to occur. We also present evidence supporting the view that they are able to abolish the phase coexistence of liquid-crystalline/gel systems, a well-known effect of cholesterol [49]. From our experimental data, we conclude that some of the organochlorine pesticides perturb both the bulk membrane and the protein-associated lipid microenvironment. We also postulate the existence of potential target sites for insecticides in either the AChR microenvironment or the membrane-embedded transmembrane protein domains of the receptor protein. Whether the organochlorine pesticides localize and act at cholesterol sites remains to be determined. In addition, experiments are underway to characterize the possible functional effects that this group of pesticides exerts on the AChR.

Reprint requests to Dr. Francisco J. Barrantes, Instituto de Investigaciones Bioquímicas, UNS-CONICET, c.c. 857, 8000 Bahía Blanca, Argentina.
E-mail: rtfjb1@criba.edu.ar

APPENDIX

Abbreviations used in this article are: AChR, nicotinic acetylcholine receptor; CHS, cholesterol hemisuccinate; DOPC, dioleoylphosphatidylcholine; DPH, diphenylhexatriene; ***E***, energy transfer efficiency; FRET, Förster resonance energy transfer; $GABA_AR$, γ-aminobutiric acid receptor; GP, generalized polarization; HC, hydrocortisone; Laurdan, 6-dodecanoyl-2-dimethylamino naphthalene; and NCIs, noncompetitive inhibitors.

REFERENCES

1. Harrison NL, Simmonds MA: Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Res* 323:287–292, 1984
2. Xue BG, Whittemore ER, Park CH, Woodward RM, Lan NC, Gee KW: Partial agonism by 3alpha,21-dihydroxy-5beta-pregnan-20-one at the gamma-aminobutyric acid A receptor neurosteroid site. *J Pharmacol Exp Ther* 281:1095–1101, 1997
3. Hogenkamp DJ, Tahir SH, Hawkinson JE, Upasani RB, Alauddin M, Kimbrough CL, Acosta-Burruel M, Whittemore ER, Woodward RM, Lan NC, Gee KW, Bolger MB: Synthesis and in vitro activity of 3 beta-substituted-3 alpha-hydroxypregnan-20-ones: Allosteric modulators of the GABA A receptor. *J Med Chem* 40:61–72, 1997
4. Cottrell GA, Lambert JJ, Peters JA: Modulation of GABAA receptor activity by alphaxalone. *Br J Pharmacol* 90:491–500, 1987
5. Callachan H, Cottrell GA, Hather NY, Lambert JJ, Nooney JM, Peters JA: Modulation of the GABA A receptor by progesterone metabolites. *Proc R Soc Lond B Biol Sci* 21:359–369, 1987
6. Peters JA, Kirkness EF, Callachan H, Lambert JJ, Turner AJ: Modulation of the GABA A receptor by depressant barbiturates and pregnane steroids. *Br J Pharmacol* 94:1257–1269, 1988
7. Lambert JJ, Belelli D, Hill-Venning C, Peters JA: Neurosteroids and GABA A receptor function. *Trends Pharmacol Sci* 16:295–303, 1995
8. Maitra R, Reynolds JN: Modulation of GABA(A) receptor function by neuroactive steroids: Evidence for heterogeneity of steroid sensitivity of recombinant GABA(A) receptor isoforms. *Can J Physiol Pharmacol* 76:909–920, 1998
9. Barrantes FJ: Structural-functional correlates of the nicotinic acetylcholine receptor and its lipid microenvironment. *FASEB J* 7:1460–1467, 1993
10. Bouzat C, Roccamo AM, Garbus I, Barrantes FJ: Mutations at lipid-exposed residues of the acetylcholine receptor affect its gating kinetics. *Mol Pharmacol* 54:146–153, 1998
11. Bouzat CB, Barrantes FJ: Effects of long-chain fatty acids on the channel activity of the nicotinic acetylcholine receptor. *Receptors Channels* 1:251–258, 1993
12. Bouzat C, Barrantes FJ: Hydrocortisone and 11-desoxycortisone modify acetylcholine receptor channel gating. *Neuroreport* 4:143–146, 1993
13. Bouzat CB, Barrantes FJ: Acute exposure of nicotinic acetylcholine receptors to the synthetic glucocorticoid dexamethasone alters single channels gating properties. *Mol Neuropharmacol* 3:109–116, 1993
14. Bouzat C, Barrantes FJ: Modulation of muscle nicotinic acetylcholine receptors by the glucocorticoid hydrocortisone: Possible allosteric mechanism of channel blockade. *J Biol Chem* 271:25835–25841, 1996
15. Stallcup WB, Patrick J: Substance P enhances cholinergic receptor desensitization in a clonal nerve cell line. *Proc Natl Acad Sci USA* 77:634–638, 1980
16. García-Colunga J, Miledi R: Serotoninergic modulation of muscle receptors of different subunits composition. *Proc Natl Acad Sci USA* 93:3990–3994, 1996
17. Marsh D, Barrantes FJ: Immobilized lipid in acetylcholine receptor-rich membranes from *Torpedo marmorata. Proc Natl Acad Sci USA* 75:4329–4333, 1978
18. Marsh D, Watts A, Barrantes FJ: Phospholipid chain immobilization and steroid rotational immobilization in acetylcholine receptor-rich membranes from *Torpedo marmorata. Biochim Biophys Acta* 645:97–101, 1981
19. Ellena JF, Blazing MA, McNamee MG: Lipid–protein interactions in reconstituted membranes containing acetylcholine receptor. *Biochemistry* 22:5523–5535, 1983
20. Horváth LI, Arias HR, Hankovsky HO, Hideg K, Barrantes FJ, Marsh D: Association of spin-labeled local anesthetics at the hydrophobic surface of acetylcholine receptor in natives membranes from *Torpedo marmorata. Biochemistry* 29:8707–8713, 1990
21. Middlemas DS, Raftery MA: Identification of subunits of acetylcholine receptor that interact with a cholesterol photoaffinity probe. *Biochemistry* 26:1219–1223, 1987
22. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391:85–100, 1981
23. Dilger JP, Brett RS: Actions of volatile anesthetics and alcohols on cholinergic receptor channels. *Ann NY Acad Sci* 625:616–627, 1991
24. Dreger M, Krauss M, Herrmann A, Hucho F: Interactions of the nicotinic acetylcholine receptor transmembrane segments with

the lipid bilayer in native receptor-rich membranes. *Biochemistry* 36:839–847, 1997
25. Criado M, Eibl H, Barrantes FJ: Effects of lipids on acetylcholine receptor: Essential need of cholesterol for maintenance of agonist-induced state transitions in lipid vesicles. *Biochemistry* 21:3622–3629, 1982
26. Criado M, Eibl H, Barrantes FJ: Functional properties of the acetylcholine receptor incorporated in model lipid membranes: Differential effects of chain length and head group of phospholipids on receptor affinity states and receptor mediated ion translocation. *J Biol Chem* 259:9188–9198, 1984
27. Fong TM, McNamee MG: Correlation between acetylcholine receptor function and structural properties of membranes. *Biochemistry* 25:830–840, 1986
28. Fong TM, McNamee MG: Stabilization of acetylcholine receptor secondary structure by cholesterol and negatively charged phospholipids in membranes. *Biochemistry* 26:3871–3880, 1987
29. Sunshine C, McNamee MG: Lipid modulation of the nicotinic acetylcholine receptor function: The role of neutral and negatively charged lipids. *Biochem Biophys Acta* 1108:240–246, 1992
30. Sunshine C, McNamee MG: Lipid modulation of nicotinic acetylcholine receptor function: The role of membrane lipid composition and fluidity. *Biochim Biophys Acta* 1191:59–64, 1994
31. Fernández-Ballester G, Castresana J, Fernández AM, Arrondo J-LR, Ferragut JA, González-Ros JM: A role for cholesterol as a structural effect of the nicotinic acetylcholine receptor. *Biochemistry* 33:4065–4071, 1994
32. Méthot N, Demers C, Baenziger J: Structure of both the ligand- and lipid-dependent channel-inactive states of the nicotinic acetylcholine receptor probed by FTIR spectroscopy and hydrogen exchange. *Biochemistry* 34:15142–15149, 1995
33. Jones OT, McNamee MG: Annular and nonannular binding sites for cholesterol associated with the nicotinic acetylcholine receptor. *Biochemistry* 27:2364–2374, 1988
34. Narayanaswami V, McNamee MG: Protein-lipid interactions and Torpedo californica nicotinic acetylcholine receptor function. II. Membrane fluidity and ligand mediated alteration in the accessibility of gamma subunit cysteine residues to cholesterol. *Biochemistry* 32:12420–12427, 1993
35. Antollini SS, Soto MA, Bonini de Romanelli I, Gutierrez-Merino C, Sotomayor P, Barrantes FJ: Physical state of bulk and protein-associated lipid in nicotinic acetylcholine receptor-rich membrane studied by Laurdan generalized polarization and fluorescence energy transfer. *Biophys J* 70:1275–1284, 1996
36. Parasassi T, De Stasio G, d'Ubaldo A, Gratton E: Phase fluctuation in phospholipid membranes revealed by laurdan fluorescence. *Biophys J* 57:1179–1186, 1990
37. Parasassi T, De Stasio G, Ravagnan G, Rusch RM, Gratton EX: Quantitation of lipid phases in phospholipid vesicles by the generalized polarization of laurdan fluorescence. *Biophys J* 60:179–189, 1990
38. Antollini SS, Barrantes FJ: Disclosure of discrete sites for phospholipid and sterols at the protein-lipid interface in native acetylcholine receptor-rich membrane. *Biochemistry* 37:16653–16662, 1998
39. McLachlan JA: *Estrogens in the Environment.* New York, Elsevier Science, 1980, p 427
40. Colborn T, Vom Saal FS, Soto AM: Developmental effects of endocrine disrupting chemicals in wild life and humans. *Environ Health Perspect* 101:378–384, 1993
41. Arnold SF, Robinson MK, Notides A, Guillete LJ Jr, McLachlan JA: A yeast estrogen screen for examining the relative exposures of cells to natural and xenoestrogens. *Environ Health Perspect* 104:544–548, 1996
42. Steinmetz R, Young PC, Caperell-Grant A, Gize EA, Madhukar BV, Ben-Jonathan N, Bigsby RM: Novel estrogenic action of the pesticide residue beta-hexachlorocyclohexane in human breast cancer cells. *Cancer Res* 56:5403–5409, 1996
43. Safe S, Connor K, Gaido K: Methods for xenoestrogen testing. *Toxicol Lett* 102–103:665–670, 1998
44. Davidson NE: Environmental estrogens and breast cancer risk. *Curr Opin Oncol* 10:475–478, 1998
45. Pomes A, Rodriguez-Farre E, Sunol C: Disruption of GABA-dependent chloride flux by cyclodienes and hexachlorocyclohexanes in primary cultures of cortical neurons. *J Pharmacol Exp Ther* 271:1616–1623, 1994
46. Brannen KC, Devaud LL, Liu J, Lauder JM: Prenatal exposure to neurotoxicants dieldrin or lindane alters tert- butylbicyclophosphorothionate binding to GABA(A) receptors in fetal rat brainstem. *Dev Neurosci* 20:34–41, 1998
47. Ortells MO, Lunt GG: Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends Neurosci* 18:121–127, 1995
48. Jain MK, Wagner RC: *Introduction to Biological Membranes.* New York, John Wiley and Sons, 1980
49. Ladbrooke BD, Williams RM, Chapman D: Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and x-ray diffraction. *Biochim Biophys Acta* 150:333–340, 1968