Interactions of the Nicotinic Acetylcholine Receptor Transmembrane Segments with the Lipid Bilayer in Native Receptor-Rich Membranes†

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ABSTRACT: Proper ion channel function of the nicotinic acetylcholine receptor (nAChR) requires the interaction of the protein with distinct lipid species present in the receptor's membrane microenvironment. Two classes of lipid binding sites present at the protein−membrane interface have been postulated: annular binding sites primarily occupied by phospholipids and non-annular binding sites mainly occupied by cholesterol [Jones & McNamee (1988) *Biochemistry 27*, 2364−2374]. We investigated the binding of these lipids to the nAChR and potential dynamics of these interactions during events associated with signal transduction by electron spin resonance spectroscopy (ESR) using spin-labeled analogues of phospholipids, androstane, and stearic acid. Protein−lipid interactions were characterized in receptor-rich membranes prepared from *Torpedo californica* electric tissue preserving the native lipid environment of the nAChR. We found a strong preference of the receptor for the phosphatidylserine (PS) analogue as compared to the other probes. Up to 57% of PS were perturbed by the membrane protein, while the fraction of motionally restricted lipid for the other analogues was on the order of 30%. After removal of the extramembrane portions of the membrane-bound receptor, we observed a loss of binding sites for the spin-labeled analogue of androstane and for stearic acid, but not for phospholipids and sphingomyelin analogues. Our results demonstrate the existence of topologically distinct lipid binding sites for different lipid species. In the case of cholesterol, extramembrane portions of the receptor are involved, whereas the transmembrane segments meet the requirements for the binding of phospholipids. Tyrosine phosphorylation of the nAChR did not affect protein−lipid interactions in samples of intact nAChR. Similarly, no significant changes were observed in the presence of carbamoylcholine at concentrations that caused rapid and quantitative desensitization of the nAChR.

The nicotinic acetylcholine receptor (nAChR)[1] is a heteropentameric ligand-gated ion channel consisting of four different subunits arranged in a stoichiometry of $\alpha_2\beta\gamma\delta$ [reviewed by Galzi and Changeux (1994) and Karlin and Akabas (1995)]. From the tissue of the electric organ of *Torpedo californica*, the receptor can be prepared as receptor-rich membrane vesicles in which the receptor contributes up to 80% of the total amount of protein (Kasai & Changeux, 1971).

The presence of the nAChR leads to a motional restriction of lipids present in the plasmamembrane which can be monitored by ESR spectroscopy using spin-labeled lipid analogues (Marsh & Barrantes, 1978). It was demonstrated that the nAChR interacts preferentially with distinct lipid species, e.g., cholesterol, negatively charged phospholipids (such as phosphatidic acid), and fatty acids (Ellena et al., 1983). However, information concerning the specificity of protein−lipid interactions is limited to phosphatidic acid and fatty acids. In these cases, electrostatic forces seem to give rise to preferential interactions with the protein (Esmann & Marsh, 1985; Bhushan & McNamee, 1993; Raines & Miller, 1993).

Proper ion channel function of the nAChR requires the presence of distinct lipid species in the receptor's microenvironment, especially cholesterol and negatively charged phospholipids (Schiebler & Hucho, 1978; Fong & McNamee, 1986; Sunshine & McNamee, 1994), both of which are abundant in native receptor-rich membranes (Schiebler & Hucho, 1978; Gonzalez-Ros et al., 1982). It was shown that different lipid species stabilize the secondary structure of the receptor's transmembrane portions (Fong & McNamee, 1987; Fernandez-Ballester et al., 1993). In addition, it was demonstrated that the M4 segment of the receptor is the most lipid-exposed transmembrane region (Blanton & Cohen, 1992) and that point-mutations therein dramatically alter the ion channel properties of the receptor (Lee et al., 1994).

On the basis of competition studies, two different classes of lipid binding sites were postulated (Jones & McNamee, 1988): One population was termed "annular" binding sites occupied by phospholipids. The other was termed "non-annular" binding sites to which cholesterol is bound, whereas the binding of phospholipids is excluded. Applying fluorescence quenching techniques, Narayanaswami and McNamee (1993) demonstrated that cholesterol "senses" a conformational change in the nAChR at the water−lipid boundary upon desensitization of the receptor in the presence of the agonist carbamoylcholine. This observation prompted

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[1] Abbreviations: nAChR, nicotinic acetylcholine receptor; pnK, proteinase K; PSSL, spin-labeled phosphatidylserine analogue; PCSL, spin-labeled phosphatidylcholine analogue; ASL, spin-labeled androstane; I(1/14) FASL, 16-doxylstearic acid.

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us to investigate whether the protein−lipid interaction of the nAChR is sensitive to events associated with signal transduction.

Protein tyrosine phosphorylation has been shown to regulate many cellular events. Among the known substrates of protein tyrosine kinases are subunits of several members of the ligand-gated ion channel receptor superfamily (Swope et al., 1992). One of the most extensively investigated proteins in this family is the nAChR. It was shown that the receptor is phosphorylated on tyrosine residues *in vivo* and can be phosphorylated by an endogenous tyrosine kinase *in vitro* (Huganir et al., 1984). One tyrosine phosphorylation site is located on the major cytoplasmic loop between putative transmembrane regions M3 and M4 of the β-, γ-, and δ-subunits (Wagner et al., 1991).

Phosphorylation of ligand-gated ion channels may be a mechanism of synaptic plasticity [reviewed by Swope et al. (1992) and Raymond et al. (1993)] affecting the electrophysiological characteristics of the system under investigation. In the case of the nAChR prepared from *Torpedo californica*, a correlation between the extent of tyrosine phosphorylation and the kinetics of receptor desensitization in the presence of agonist was demonstrated using affinity-purified nAChR reconstituted in liposomes (Hopfield et al., 1988). This suggests a conformational change induced by tyrosine phosphorylation altering the ion channel and ligand binding properties of the receptor. However, no structural correlate for such a "retrograde" signal, i.e., from the cytoplasmic to the extracellular side of the plasma membrane, has been found yet.

At the neuromuscular junction, the nerve-derived extracellular matrix protein agrin induces tyrosine phosphorylation at least of the β- and δ-subunits (Wallace et al., 1991; Wallace, 1995). With nAChR in cultures of chick myotubes, it was demonstrated that tyrosine phosphorylation initiates the anchoring of the receptor to the cytoskeleton (Wallace, 1995). These results imply that tyrosine phosphorylation does not necessarily affect ion channel or ligand binding properties of the receptor, but may trigger conformational changes in the cytoplasmic portion of the receptor leading to alterations in the protein−protein interactions between the receptor and other proteins at the neuromuscular junction.

In the present study, we first characterized the protein−lipid interactions of native receptor-rich membranes prepared from the electric organ of *Torpedo californica* by conventional electron spin resonance (ESR) spectroscopy after incorporation of spin-labeled phospholipids, androstane or stearic acid. Spectra were compared to those obtained with samples after removal of the extracellular portions of the nAChR by proteinase K digestion. Furthermore, protein−lipid interactions in native receptor-rich membranes were monitored in the presence or absence of the agonist carbamoylcholine used at concentrations that cause the receptor to rapidly and quantitatively adopt the desensitized state. Finally, spectra of samples of native receptor-rich membranes with nAChR containing different amounts of phosphotyrosine were recorded. Parts of this work were presented at the 23rd FEBS Meeting at Basel, Switzerland, 1995.

MATERIALS AND METHODS

Preparation of nAChR-Rich Membranes. nAChR-rich membranes were prepared from the electric organ of *Torpedo californica* (the tissue was obtained frozen in liquid N_2 from Aquatic Research Consultants, San Pedro, CA) as decribed previously (Hertling-Jaweed et al., 1990). Only the purest fractions (as assessed by SDS−PAGE) recovered after sucrose density gradient centrifugation were used and resuspended in *Torpedo* physiological saline (TPS) composed of 250 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM $Na_xH_xPO_4$, pH 7.4. Aprotinin and leupeptin were added to a final concentration of 10 μg/mL, respectively. The protein concentration was determined according to Bradford (1976). The membranes contained 3.5−5.5 nmol of bungarotoxin binding sites/mg of protein as determined by [^{125}I]-α-bungarotoxin binding. Considering the stoichiometric presence of the 43 kDa peripheral membrane protein, also termed rapsyn, in preparations of receptor-rich membranes, the nAChR contributed at least 50−90% to the total amount of integral membrane protein.

Lipid Extraction of Receptor-Rich Membranes. Lipid extraction of receptor-rich membranes was performed according to Bligh and Dyer (1959). The extracted lipids were dried under N_2 and quantified by determination of phosphate content according to Rouser et al. (1966). Subsequently, the lipids were resuspended in TPS to a final concentration corresponding to that in native receptor-rich membranes.

Degradation of the Extramembrane Regions of the nAChR. In order to remove the extramembrane portions of the nAChR, receptor-rich membranes were digested by proteinase K (Boehringer, Mannheim) as described (Görne-Tschelnokow et al., 1994). The reaction was stopped by adding phenylmethanesulfonyl fluoride (PMSF) to a final concentration of 0.1 mM. Two milligrams of receptor-rich membranes was degraded in a volume of 1 mL in the presence of 1 mg/mL proteinase K. About 70−75% of the protein in the preparation was removed after degradation.

ESR Spectroscopy. We used the spin-labeled phospholipid analogues 1-palmitoyl-2-(4-doxylpentanoyl)-PC [(0,2)PC], 1-palmitoyl-2-(4-doxylpentanoyl)-PS [(0,2)PS], and 1-palmitoyl-2-(4-doxylpentanoyl)-PE [(0,2)PE] and also 1-palmitoyl-2-(4-doxylpentanoyl)-SM [(0,2)SM], synthesized according to Seigneuret et al. (1984). Due to the short fatty acid chain in the β-position, these analogues are partially water-soluble and can be easily incorporated into membranes. Furthermore, 3-doxyl-17β-hydroxy-5α-androstane was used as a cholesterol analogue, and the spin-labeled fatty acid I(1,-14), also termed 16-doxylstearic acid (both obtained from Sigma Chemie, Munich). The concentration of spin-labeled probes corresponded to about 1.5 mol % of the endogenous lipid in receptor-rich membranes, referring to data of total lipid analysis reported by Gonzales-Ros et al. (1982). Prior to labeling of the receptor-rich membranes, the desired amount of spin-labeled lipid was dried from chloroform/methanol (1:1 v/v) under N_2 and resuspended in 10 μL of TPS by vortexing and sonication. Then 50 μL of receptor-rich membranes (6 mg of protein/mL) or liposomes after lipid extraction of receptor-rich membranes were added and gently mixed with the suspension of spin-labels. The spin-labeled lipids were immediately incorporated into the membranes as deduced from the line shape. There was no progressive hydrolysis of spin-labeled probes during the time course of the measurements.

ESR spectra were recorded on a Bruker ECS 106 ESR spectrometer with 100 kHz magnetic field modulation. The capillary tube containing the sample was surrounded by a

single-jacket quartz Dewar flask through which dry nitrogen gas was passed at constant temperature. Temperature was measured by a wire thermocouple located at the bottom of the microwave cavity. Spectra were recorded immediately after labeling of the sample at a microwave power of 20 mW. To support spectral deconvolution, spectra were recorded at temperatures low enough to minimize exchange of spin-labeled probes between the bulk lipid phase and the protein boundary, but still high enough to allow visual differentiation between both spectral components. Measurements using spin-labeled phospholipids or sphingomyelin were performed at 4 °C with a modulation amplitude of 4 G. I(1,14) spin-labeled stearic acid was measured at 10 °C with a modulation amplitude of 2 G and spin-labeled androstane at 15 and 23 °C according to Ellena et al. (1983) with modulation amplitudes of 2 and 1 G, respectively. A total of 8 or 16 spectra per sample were accumulated to improve the signal-to-noise ratio. Spectral base line correction, integration, and subtraction of spectra were performed by the software provided with the spectrometer ECS 106.

Desensitization of the nAChR. Measurements of nAChR-rich membranes in the presence of carbamoylcholine were performed as follows: After ESR spectra of a spin-labeled lipid species in native receptor-rich membranes in the absence of the agonist were recorded, the sample (>50 μL) was recovered from the capillary tube. Five microliters of a carbamoylcholine stock solution was added and gently mixed with the sample. The final concentration of carbamoylcholine was 10 mM. The sample was immediately placed into a new capillary tube, and ESR spectra were recorded as described above.

Phosphorylation and Dephosphorylation of nAChR-Rich Membranes. The nAChR-rich membranes were phosphorylated as described (Mei & Huganir, 1991) with the following modifications: The reaction was performed for 60 min, leading to a maximum ^{32}P incorporation into the receptor in our experiments. DTT was excluded from the reaction buffer because its presence (1 mM) led to a decrease of [^{125}I]-α-bungarotoxin binding in the samples even after resuspension of the receptor-rich membranes in TPS. In each experiment, an aliquot of receptor-rich membranes was phosphorylated in the presence of [γ-^{32}P]ATP (∼100 cpm/pmol of ATP) and subjected to the determination of the stoichiometry of the *in vitro* phosphorylation, to phosphoamino acid analysis, and to two-dimensional phosphopeptide mapping. The samples prepared for ESR measurements were phosphorylated under the same conditions but without radioactive ATP. In each case, the final ATP concentration was 0.5 mM. Control samples were incubated under the same conditions as the phosphorylated samples, but in the absence of ATP. Receptor dephosphorylation was carried out with 0.1 mg/mL (14 units) alkaline phosphatase as described (Hopfield et al., 1988), except for the addition of 1 mM $ZnCl_2$ and 20 mM $MgCl_2$ to the reaction buffer and in the presence of 1 μg/mL each of leupeptin, aprotinin, and phosphoramidon, 0.1 mM PMSF, and 0.5 mM EGTA to block proteolytic degradation. The membranes were briefly sonicated and incubated at 30 °C for 1 h. All reactions were performed immediately after preparation.

Western blots were performed with aliquots taken from the samples for ESR measurements.

The reactions with samples for analytical purposes were stopped by adding sample buffer according to Laemmli (1970). In the samples for spectroscopic measurements, the reactions were stopped by pelletting the receptor-rich membranes at 25000*g* and 4 °C in a Biofuge 28 RS (Heraeus). The membranes were resuspended in an appropriate volume of TPS and incubated for 1 h at 4 °C to allow the removal of substances of the reaction buffer and alkaline phosphatase. The membranes were centrifuged again and resuspended in TPS. The protein concentration was adjusted to 6 mg/mL. Aliquots were taken for SDS−PAGE to assess the absence of alkaline phosphatase in the samples of dephosphorylated membranes, to anti-phosphotyrosine Western blotting, and for the determination of [^{125}I]-α-bungarotoxin binding.

Phosphoamino Acid Analysis. After SDS−PAGE of receptor-rich membranes and transfer of the proteins onto a PVDF membrane by electroblotting, phosphorylated subunits of the nAChR were hydrolyzed with 1 N HCl and subjected to one-dimensional thin-layer electrophoresis essentially as described by Hirano et al. (1988).

Two-Dimensional Phosphopeptide Mapping. Tryptic digestion of phosphorylated receptor subunits after SDS−PAGE and transfer on PVDF membranes by electroblotting as well as separation of the peptides by thin-layer electrophoresis in the first and subsequent thin-layer chromatography in the second dimension were performed essentially as described by Boyle et al. (1991).

Assessment of Tyrosine Phosphorylation by Quantitative Western Blotting. Quantitative detection of tyrosine phosphorylation of the receptor subunits was performed based on the method described by Jahn et al. (1984) and modified by Hopfield et al. (1988). mAb anti-phosphotyrosine PY 20 (Sigma Chemie, Munich) was used as the primary antibody and [^{125}I]protein A (Amersham) as the detection system. [^{125}I]Protein A binding was quantified by counting labeled bands after autoradiography and excision from the blot membrane in a γ-counter (Kontron, Munich). A polyclonal rabbit anti-mouse antibody (Sigma) was used as secondary antibody to allow subsequent binding of [^{125}I]-protein A. Specificity and quantification of the signals were not affected by this modification of the procedure. The specific activity of [^{125}I]protein A labeling (cpm per mole of phosphotyrosine) was calculated by comparing the stoichiometry of ^{32}P incorporated in the samples phosphorylated in the presence of [γ-^{32}P]ATP with the increase of [^{125}I]-protein A labeling during the time course of phosphorylation using the formula [(cpm of [^{125}I]protein A labeling of subunit phosphorylated *in vitro*) − (cpm of [^{125}I]protein A labeling of nonphosphorylated subunit)/mol of ^{32}P incorporated *in vitro*].

RESULTS

A main concern of our study was the characterization of the nAChR−lipid interaction with respect to events associated with signal transduction. For this purpose, we regarded it as essential to monitor native nAChR-rich membranes.

A main aspect in using native membranes is the variability of protein−lipid interaction caused by biological variability as well as sample preparation. Whereas the specificity of receptor−lipid interaction has been well characterized for reconstituted membranes (e.g., Raines & Miller, 1993), there is some discrepancy to the rare data obtained for native

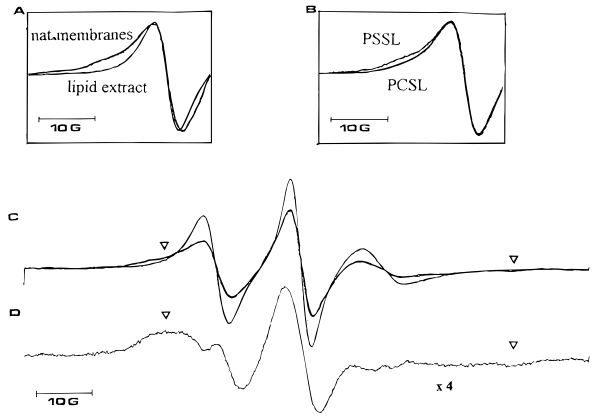

FIGURE 1: Interactions of spin-labeled phospholipid analogues with native nAChR-rich membranes. (A) PSSL in native membranes vs PSSL in lipid extract, normalized to the lower-field ESR line. (B) PSSL vs PCSL in native membranes, normalized as in (A). (C) PSSL in native membranes (broad ESR signal) vs PSSL in lipid extract (narrow signal). (D) Immobilized component spectrum derived from spectral subtraction of the spectra shown in (C). "×4" indicates that the immobilized component spectrum is shown enlarged in intensity by a factor of 4 as compared to the spectra in (C). PSSL, spin-labeled phosphatidylserine analogue; PCSL, phosphatidylcholine analogue.

membranes containing nAChR (e.g., Ellena et al., 1983). Therefore, as a first step, we investigated nAChR−lipid interactions in our preparation of native receptor-rich membranes.

Characteristics of Protein−Lipid Interactions in Native Receptor-Rich Membranes. The presence of nAChR gave rise to a two-component ESR spectrum: one corresponding to the motionally low restricted spin-labeled probes in the bulk lipid phase and the other, with the larger anisotropy (indicated by the outer wings of the spectra), corresponding to motionally restricted lipids in a protein-perturbed environment. The relative portions of motionally restricted lipids and lipids in the nonperturbed bulk lipid phase were obtained by spectral subtraction of the ESR signal of the corresponding spin-labeled probe in liposomes prepared from lipid extracts of native membranes. Applying the same procedure, the outer hyperfine splittings of the immobilized components were determined.

In all our experiments with spin-labeled phospholipids, the nAChR showed a preference for phosphatidylserine over phosphatidylcholine (Figure 1), consistent with data of others obtained from experiments with affinity-purified nAChR reconstituted in liposomes (Raines & Miller, 1993). About 38−57% of the phosphatidylserine present in the membrane was found to be perturbed, varying between different preparations, whereas in all preparations tested constantly 32% of total phosphatidylcholine was perturbed. The variance of the data obtained for phosphatidylserine was most likely due to the quality of the tissue from which the membranes were prepared. The extent of restriction in motion for all lipid species tested in native receptor-rich membranes is summarized in Table 1. The outer hyperfine splittings were the same for all phospholipid species and did not vary significantly between different experiments.

Because of the variations of the samples of receptor-rich membranes with respect to the relative content of nAChR and to the extent of tyrosine phosphorylation between different experiments, only spectra of samples from the same preparation were compared. Despite the variability of the preparations, native receptor-rich membranes, but not reconstituted systems, were chosen as the experimental system to be as close to *in vivo* conditions as possible.

Table 1 [a]

spin-labeled lipid analogue	fraction of immobilized lipid	outer hyperfine splitting (G)
PSSL	0.47 ± 0.02[b]	60.8 ± 0.2
PCSL	0.32 ± 0.01	60.5 ± 0.1
PESL	0.30 ± 0.04	60.8 ± 0.1
SMSL	0.28 ± 0.03	60.9 ± 0.3
ASL, 15 °C, 2 G	0.38 ± 0.01	−
ASL, 23 °C, 1 G	0.25 ± 0.01	−
FASL, 10 °C, 2 G	0.34 ± 0.04[b]	61.4 ± 0.3

[a] Fractions of immobilized spin-labeled lipid analogues in native nAChR-rich membranes. PCSL, phosphatidylcholine analogue; PSSL, phosphatidylserine analogue; PESL, phosphatidylethanolamine analogue; SMSL, sphingomyelin analogue; ASL, spin-labeled androstane; FASL, spin-labeled stearic acid. Spectra were recorded at 4 °C with a modulation amplitude of 4 G except for ASL and FASL as noted above. [b] For PSSL and FASL, the amount of motionally restricted spin-labels varied among different preparations of native membranes (0.38 up to 0.57 and 0.25 up to 0.45, respectively). We estimate the accuracy in the determination of the immobilized fractions to ±1−4% (given as standard error of estimate). The outer hyperfine splittings for ASL are not stated because the determination was not exact enough.

Protein−Lipid Interactions in Membranes after Digestion by Proteinase K. Jones and McNamee (1988) postulated two different classes of binding sites for lipids at the nAChR. The phospholipid binding sites were called the annular binding sites, whereas there exist additional binding sites for cholesterol termed non-annular binding sites. Free fatty acids were reported to bind to both classes of lipid binding sites.

After proteinase K digestion of the extramembrane domains of the nAChR, we observed a relative decrease of the portion of motionally restricted phospholipid analogues of all species as compared to the fraction of perturbed lipids as revealed by spectra obtained with native receptor-rich membranes. However, the preferential interaction of the receptor with phosphatidylserine over the other phospholipid species under investigation was maintained (Figure 2). We conclude from these results that the transmembrane segments provide the requirements for the selective binding of phospholipids to the nAChR.

In contrast, performing the experiment with the spin-labeled stearic acid I(1,14) and with spin-labeled androstane incorporated in digested receptor-rich membranes, the ESR spectra showed line shapes similar to those of these spin-labeled lipid analogues in lipid extracts (Figure 2). The spectra of the immobilized components revealed a dramatic decrease in the fraction of motionally restricted lipid analogues after removal of the extramembranal portions of the nAChR as shown for spin-labeled androstane in Figure 3. This indicates an extensive loss of binding sites, presumably non-annular sites, after proteinase K digestion.

It can be ruled out that the loss of these lipid binding sites is due to structural disordering upon proteinase K digestion because it was shown that the overwhelming portion of the transmembrane segments retains secondary structure after removal of the extramembrane portions of the receptor (Görne-Tschelnokow et al., 1994). Our data suggest that there are distinct binding sites for cholesterol and phospholipids on the receptor. Furthermore, we conclude that the binding of cholesterol and free fatty acids requires the

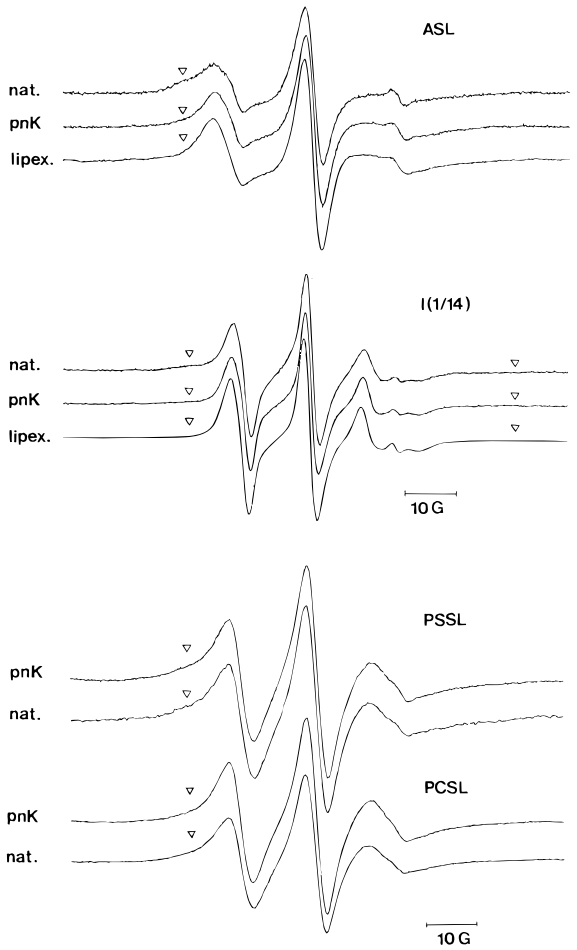

FIGURE 2: Removal of extramembrane portions of the nAChR does not severely affect the binding of phospholipids to the transmembrane regions of the receptor, but the binding sites for spin-labeled androstane and spin-labeled stearic acid get lost. For ASL and I(1/14), spectra are shown of the spin-labeled compound in native receptor-rich membranes (nat.), in proteinase K-digested native receptor-rich membranes (pnK), and in lipid extracts (lipex.). The spectra with ASL were recorded at 15 °C and 2 G modulation amplitude, those with I(1/14) FASL at 10 °C and 2 G modulation amplitude. Spectra with PSSL or PCSL in native receptor-rich membranes (nat.) or after proteinase K digestion (pnK) were recorded at 4 °C with 4 G modulation amplitude. (For comparison of spectra with PSSL in native receptor-rich membranes and in lipid extract, see Figure 1.)

interaction of these lipid species with regions of the nAChR protein adjacent to the transmembrane segments.

ESR Measurements of Native Membranes in the Presence of Carbamoylcholine. Spectra recorded with samples of native receptor-rich membranes were compared to those obtained after addition of carbamoylcholine to a final concentration of 10 mM, an agonist at the nAChR that transiently leads to the opening of the ion channel and then to desensitization. In the desensitized state, the affinity of the receptor toward the agonist is increased as compared to the resting state, but the ion channel remains closed [reviewed by Galzi and Changeux (1994)]. At the agonist concentration chosen (10 mM), the receptor is quantitatively in the desensitized state at the start of the ESR measurements. Under similar conditions with affinity-purified nAChR reconstituted in lipid vesicles, Narayanaswami and McNamee (1993) came to the following conclusion: They demonstrated that conformational changes in the nAChR accompanying desensitization alter the accessibility of transmembranously

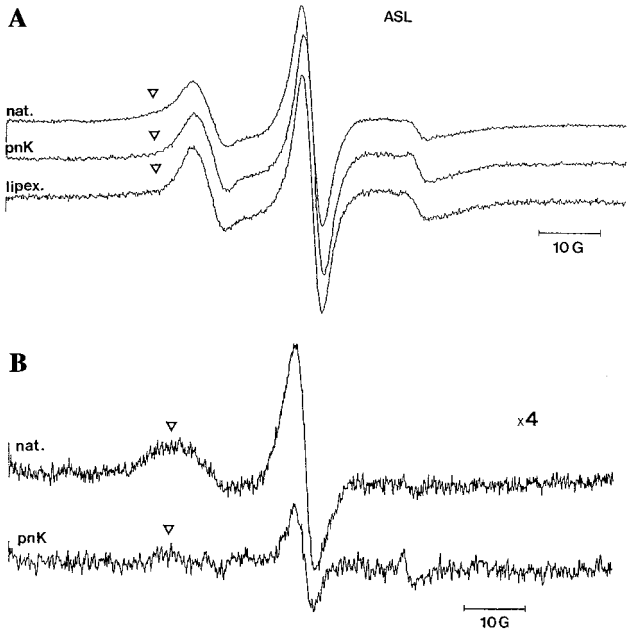

FIGURE 3: Loss of binding sites for spin-labeled androstane (ASL) upon removal of the extramembrane portions of the nAChR. (a) ASL in receptor-rich native membranes (nat.), in proteinase K-digested membranes (pnK), and in lipid extracts (lipex.) recorded at 23 °C with 1 G modulation amplitude. (b) Immobilized component spectra after subtraction of the spectrum of ASL in lipid extract. "×4" indicates that these spectra are shown enlarged in intensity by a factor of 4 as compared to the spectra in (A).

located fluorophores toward quenching by a cholesterol analogue (dibromocholesterol hemisuccinate) present at the non-annular binding sites. Comparison of spectra obtained with the spin-labeled phospholipids or spin-labeled androstane incorporated in the receptor-rich membranes revealed no changes upon addition of the agonist. In the case of I(1,-14), we observed a small and reproducible increase in the fraction of immobilized lipid. However, the extent of this effect (in the range of 3% increase) is too close to the detection limit of our experimental system to interpret this in terms of conformational changes in the nAChR (data not shown).

Measurements with Membrane-Bound AChR Tyrosine-Phosphorylated to Different Extents. The nAChR in the receptor-rich membranes contained 0.5−1.1 mol of phosphotyrosine/mol of receptor, varying among different preparations. In the native state, the β-subunit contained the relatively highest amount of phosphotyrosine compared to the other subunits. During *in vitro* phosphorylation by the endogenous tyrosine kinase, an additional 0.5−1 mol of phosphotyrosine/mol of receptor was incorporated, varying among different experiments. The nAChR was the major tyrosine-phosphorylated protein in the receptor-rich membranes (Figure 4A). As observed after immunoblotting using anti-phosphotyrosine mAb and quantification of ^{32}P incorporation, the β- and δ-subunits were almost quantitatively phosphorylated on their tyrosine phosphorylation sites, whereas the γ-subunit was phosphorylated to a lesser extent. *In vitro* phosphorylation occurred exclusively on tyrosine residues as assessed by phosphoamino acid analysis (Figure 4B). Two-dimensional phosphopetide mapping of tryptic peptides revealed that phosphorylation affected a single residue on the β-, γ-, and δ-subunit, respectively (Figure 4C), consistent with the notion that β(Y 355), γ(Y 364), and

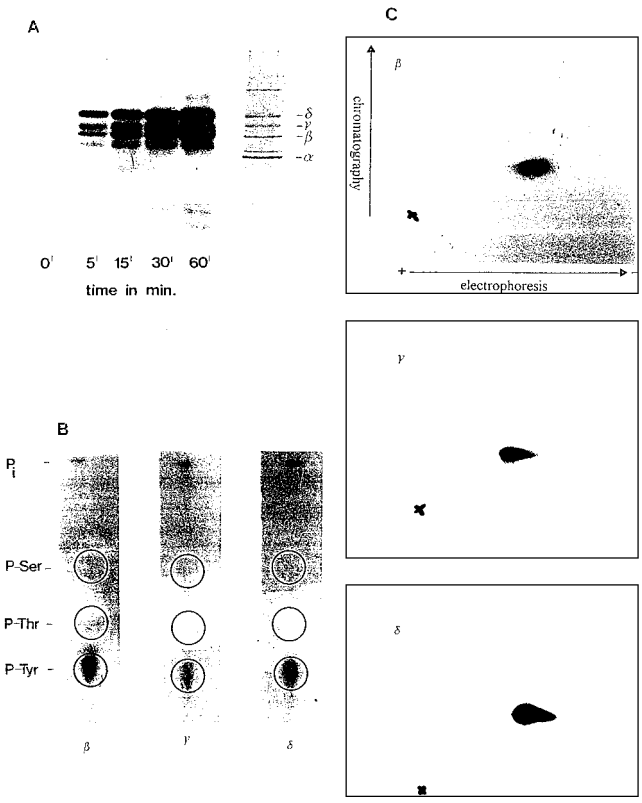

FIGURE 4: Analysis of tyrosine phosphorylation of the nAChR. (A) Time course of the *in vitro* phosphorylation of receptor-rich native membranes (in minutes). (B) Phosphoamino acid analysis as revealed by autoradiography after limited hydrolysis of the phosphorylated subunits and thin-layer electrophoresis. (C) Two-dimensional phosphopeptide map of the tyrosine-phosphorylated receptor subunits after tryptic digestion and separation of the peptides by thin-layer electrophoresis/thin-layer chromatography. α, *β*, *γ*, *δ*, subunits of the nAChR.

δ(Y 372) are the only tyrosine phosphorylation sites present in the nAChR (Wagner et al., 1991).

Dephosphorylation by alkaline phosphatase decreased the amount of phosphotyrosine present in the receptor to 0.3 mol of phosphotyrosine/mol of receptor or less.

Therefore, the samples of AChR compared in these experiments covered a range of tyrosine phosphorylation from 0.3 mol of phosphotyrosine per mole of receptor up to 2.1 nmol of phosphotyrosine per mole of receptor.

Since the nAChR was the major phosphotyrosine-containing protein in the native membrane preparations (Figure 4), it could be ruled out that possible changes in the ESR spectra of differentially phosphorylated samples would have to be attributed to conformational changes in contaminating integral membrane proteins. As shown with phosphatidylserine as the spin-labeled probe in Figure 5, there were no detectable differences among spectra of differentially phosphorylated samples with any lipid species under investigation, neither with respect to the relative amount of perturbed lipid nor with respect to the outer hyperfine splittings revealed by the analysis of the immobilized component spectra after spectral subtraction. Thus, the selectivity in the receptor's interaction with the different lipid species under investigation was not affected by the tyrosine phosphorylation state of the nAChR.

Like in all other experiments, only spectra of samples from the same preparation were compared.

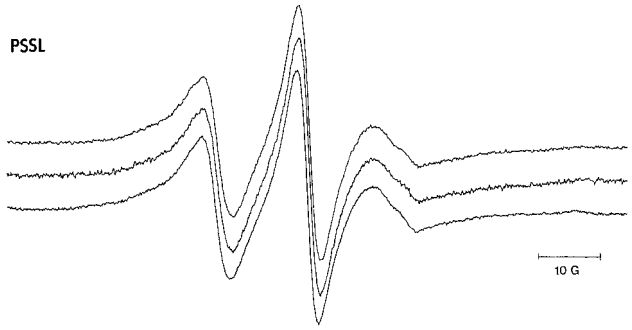

FIGURE 5: Tyrosine phosphorylation does not affect the protein−lipid interactions in receptor-rich native membranes. Top spectrum, PSSL in membranes after *in vitro* phosphorylation; middle, PSSL in membranes obtained from the preparation; bottom, PSSL in membranes after *in vitro* dephosphorylation. PSSL, spin-labeled phosphatidylserine analogue.

DISCUSSION

In the present work, we have investigated the interaction of the nAChR with spin-labeled lipid analogues. Previously, most of the investigations have been performed with reconstituted systems [reviewed by Barrantes (1993)]. Especially for measurements with samples of nAChR in different states of phosphorylation and desensitization, we regarded it essential for the relevance of results that the nAChR remained in its native lipid environment.

A major concern in using lipid analogues is a possible perturbation of membrane structure and function by the label (reporter) group. However, the concentration of spin-labeled lipid analogues was less than 1.5 mol % of endogenous phospholipid concentration. This rather low amount of label is typically used for studying membrane lipid dynamics (Devaux & Seigneuret, 1985), e.g., in reconstituted membranes containing nAChR (Ellena et al., 1983). We assessed with our native receptor-rich membranes that α-bungarotoxin binding is not affected by the labeled lipid analogues. If the nitroxide moiety of the label used would cause significant alterations of the membrane organization and, thus, would be a main determinant of the structure and dynamics of the labels' microenvironment, we would not expect to detect the observed lipid specificity of the nAChR and its specific dependence on the presence of the receptor ectodomain. Moreover, by using other labeled lipid analogues (including fluorescent lipids) as well as comparison to endogeneous lipids, we and others have shown for different biological membranes, that the lipid analogues used here are faithful reporters of endogenous lipids and do not affect membrane integrity and function (Zachowski, 1993; Müller et al., 1994; Pomorski et al., 1996).

Spectra recorded with native receptor-rich membranes indicated that the immobilized components are less pronounced as compared to reconstituted membranes composed of nAChR and one lipid species. Therefore, Ellena et al. (1983) proposed the reconstitution of nAChR into defined lipid bilayers for performing ESR experiments monitoring the protein−lipid interactions of the receptor. However, it cannot be excluded that functional characteristics of the nAChR are altered upon affinity purification of the receptor and reconstitution. *In vivo*, the receptor is associated with a protein network underlying the plasma membrane of the electric organ or, in analogy, the neuromuscular junction (Apel et al., 1995). Receptor function may well be influ-

enced by the interaction with accessory proteins. Furthermore, much of the published data concerning the protein−lipid interaction of the nAChR was performed in reconstituted systems with a higher lipid:protein ratio as compared to native membranes. A major problem using intact biological membranes is the efficient and quantitative insertion of appropriate probes, e.g., as those of spin-labeled phospholipids. To overcome these difficulties, we have used spin-labeled phospholipid analogues with a short fatty acid chain in the β-position. As has been shown for other membranes (Seigneuret et al., 1984) and confirmed here for nAChR-rich membranes, these labels incorporate in less than 1 min into the membrane.

Whereas in the case of the phospholipid, sphingomyelin, and androstane analogues the reporter moiety is located near the water−lipid interface after incorporation into the native membranes, the doxyl moiety of I(1,14) spin-labeled fatty acid was most likely located near the center of the lipid bilayer (Seigneuret et al., 1984; Chattopadhyay & London, 1987). However, spin-labeled fatty acids were demonstrated to "sense" changes in the environment of their polar moiety at the water−membrane interface rather than deeper in the lipid bilayer (Raines et al., 1995).

The most striking feature of protein−lipid interactions in native receptor-rich membranes was the high preference of the receptor for the spin-labeled phosphatidylserine analogue as compared to the other analogues of phospholipids and sphingomyelin. This selectivity revealed in our measurements was consistent with data of others obtained with affinity-purified and reconstituted receptor (Raines & Miller, 1993). However, tissue variability may be an important parameter: In the case of phosphatidylserine, the variance in the portion of motionally restricted lipid obtained in different experiments (ranging from 38 to 57%) was most certainly due to a qualitative variance of the tissue from which the receptor-rich membranes were prepared. Possibly the protein:lipid ratio or the lipid composition is different in tissue pieces from peripheral as compared to central regions of the electric organ.

Our data deviate from the results of Ellena et al. (1983) obtained from experiments with native receptor-rich membranes, especially with respect to phosphatidylserine and to androstane. In the publication cited, the spin-labeled analogue of phosphatidylserine was bound to the receptor with a slightly reduced selectivity as compared to the phosphatidylcholine analogue. The selective binding of spin-labeled androstane was demonstrated with affinity-purified and reconstituted nAChR, but less clearly with native receptor-rich membranes. We think, however, our data are more reliable because the samples used in our experiments contained at least twice as much nAChR compared to the total amount of membrane protein. Furthermore, we avoided alkaline extraction of peripheral membrane proteins which causes damage of the receptor-associated protein network (Krikorian & Bloch, 1992). One has to be aware that a possible interaction between the peripheral 43 kDa protein (rapsyn) and membrane lipids may contribute to the immobilized component present in native membranes although the specificity of lipid interaction was qualitatively similar to that of reconstituted systems.

To investigate whether protein−lipid interactions observed in our experiments with native receptor-rich membranes were exclusively due to the direct interaction with the transmembrane segments of the receptor, we monitored the protein−lipid interactions after removal of the extramembrane portions of the receptor protein. By this procedure, 70−80% of the nAChR protein was degraded, whereas 20−30% remained in the sample. This was roughly the amount of protein predicted to be located in the membrane (Noda et al., 1983; Finer-Moore & Stroud, 1984).

We observed that the proteolytic degradation of the extramembrane portions of the nAChR with the nonspecific protease proteinase K led to a breakdown in the receptor's interaction with androstane and to a large reduction in the interaction with a stearic acid. This destruction of the non-annular cholesterol binding sites was not due to a breakdown of the membrane structure after proteolysis: As revealed by FTIR, the overwhelming portion of the remaining protein representing the membrane-spanning part of the receptor (and of contaminating proteins including ATPase) still shows α-helical and β-sheet structure (Görne-Tschelnokow et al., 1994). Furthermore, linear dichroism measurements with polarized infrared light and oriented samples presented in the same work proved the intactness of the lipid bilayer structure. Thus, the loss of cholesterol binding cannot be ascribed to a loss of secondary structure of the transmembrane segments and to a dissociation of the lipid bilayer. The non-annular binding sites for cholesterol were proposed to be located at the lipid-exposed M4 helix close to the interfaces between the subunits (Narayanaswami & McNamee, 1993). We conclude from our data that the extramembrane portions of the nAChR are involved in the binding of cholesterol and stearic acid. Charged amino acids adjacent to the putative transmembrane regions and absent after proteinase K digestion may be a prerequisite for the interaction with fatty acids. Indeed, electrostatic forces have been suggested to be responsible for the interaction of the nAChR with fatty acids (Bhushan & McNamee, 1993; Raines & Miller, 1993).

In contrast, the interaction of the nAChR transmembrane domain with phospholipids remained almost undisturbed by proteolysis, with respect to the lipid selectivity. The structural base for this interaction is unknown, but the binding of the phospholipids is presumably not affected by electrostatic interactions as reported for the interaction of spin-labeled fatty acids with the Na^+/K^+-ATPase from shark rectal glands (Esmann & Marsh, 1985). A similar finding was also reported for the nAChR (Bhushan & McNamee, 1993; Raines & Miller, 1993). We note that upon proteinase K digestion the relative contribution of the immobilized component of spin-labeled phospholipid analogues slightly decreased. But it is obvious that the remaining immobilized component cannot be ascribed to nAChR molecules not enzymatically degraded, because no intact receptor could be detected after proteinase K treatment.

Thus, we surmise that the structural basis of the specific interaction with phospholipids lies in the transmembrane sequences of the nAChR. At the present stage of investigation, we cannot decide whether the different membrane-spanning sequences are equally involved in the interaction with phospholipids. Recently, it has been shown that a 26-residue peptide which contains a single putative transmembrane domain of a small protein associated with voltage-gated potassium channels causes a motional restriction of spin-labeled stearic acid and phosphatidylserine (Horvath et al., 1995). This peptide adopts a β-sheet conformation in

the lipid bilayer. Earlier, Fong and McNamee (1987) suggested a stabilization of β-structure in reconstituted nAChR on the basis of Fourier transform infrared (FTIR) spectroscopy investigations.

Taken together, our data extend the postulate of different binding sites for cholesterol and phospholipids on the nAChR (Jones & McNamee, 1988): We propose that extramembrane portions of the receptor close to the membrane surface contribute to the binding of cholesterol and of stearic acid, but not of phospholipids.

Another aspect of our experiments addressed the question whether the lipid phase "senses" conformational changes in the receptor protein triggered by signal transduction. Experiments with native receptor-rich membranes in the presence of carbamoylcholine showed that no extensive changes in protein−lipid interactions with the lipid species tested occurred. Since in the desensitized state the ion channel properties of the nAChR differ grossly from those in the resting state [reviewed by Galzi and Changeux (1994)], we conclude from our data that conformational changes in the transmembrane regions may not necessarily lead to significant changes in the protein−lipid interactions. The changes in channel properties associated with desensitization obviously are accompanied only by small structural changes, too small to affect the lipid environment.

The same seems to be true for the effect of tyrosine phosphorylation. Several ligand-gated ion channels have been found to be phosphorylated, and various data suggest that this may be a mechanism to modulate synaptic transmission [reviewed by Swope et al. (1992) and Raymond et al. (1993)]. Among this protein family, the nAChR, the NMDA-receptor, and the $GABA_A$-receptor are substrates of protein tyrosine kinases (Huganir et al., 1984; Moon et al., 1994; Moss et al., 1995). In the case of the nAChR of *Torpedo californica*, it was shown *in vitro* that tyrosine phosphorylation regulates the rate of receptor desensitization (Hopfield et al., 1988), but it remains to be shown that the desensitized state and its regulation by tyrosine phosphorylation occur under physiological conditions. For the neuromuscular junction, evidence was found that tyrosine phosphorylation induces the anchoring of the nAChR to the cytoskeleton (Wallace, 1995). Thus, rather than inducing overall conformational changes in the receptor as required for direct regulation of the ion channel and ligand binding properties, local conformational changes in the receptor's cytoplasmic portion leading to altered interactions with accessory proteins might occur. For example, the candidate protein tyrosine kinases being capable of phosphorylating the *Torpedo* nAChR were shown to bind preferentially to the receptor's tyrosine phosphorylation sites in the phosphorylated state (Swope & Huganir, 1994).

It has to be stressed that with the method we applied and the preparation of nAChR used we did not address the problem of membrane-achoring of the receptor depending on the degree of tyrosine phosphorylation as it occurs during development of neuromuscular junctions. Our aim was to test the hypothesis whether tyrosine phosphorylation in the cytoplasmic portion of the nAChR induced a "retrograde signal" leading to a conformational change in the transmembrane and extracellular region of the protein and possibly affecting the selectivity of the interactions of the transmembrane regions with the lipid environment. Such a retrograde signal would have to be postulated if tyrosine phosphorylation regulated the rate of desensitization of the nAChR in the presence of agonist as was reported by Hopfield et al. (1988).

By monitoring the protein−lipid interaction of spin-labeled lipids incorporated in native receptor-rich membranes with the receptor phosphorylated on its tyrosine phosphorylation sites to different degrees, we found no change in the selectivity of the interaction with any of the lipid species mentioned above. Whereas in the case of the spin-labeled stearic acid, the ESR spectra in the presence of carbamoylcholine were not exactly identical to those with samples of nAChR in the resting state, we did not observe a comparable change in the samples containing different amounts of phosphotyrosine. In addition, studies with these samples using FTIR revealed no detectable changes in the overall secondary structure of the receptor induced by tyrosine phosphorylation, but local changes affecting the cytoplasmic portions could not be ruled out (Dreger et al., 1995a,b). Therefore, we have no indication for a retrograde signal from the cytoplasmic domain of the nAChR to the transmembrane part and to the extracellular ligand binding site.

CONCLUSION

In summary, we demonstrated that distinct but different binding sites of cholesterol and phospholipids exist on the nAChR in its native lipid environment. In extension of previous data (Owen & McNamee, 1988), it was shown that domains of the nAChR adjacent to the transmembrane segments are required for the binding of cholesterol and stearic acid. Since the interaction with both negatively charged phospholipids and cholesterol is essential for the receptor's functional integrity (Fong & McNamee, 1986; Sunshine & McNamee, 1994), our data suggest that the maintenance of the receptor function can be dissected into different components requiring different lipid species.

Tyrosine phosphorylation of the cytoplasmic portion of the nAChR leaves the protein−lipid interaction unaffected. Including results from FTIR spectroscopy, it seems likely that effects of tyrosine phosphorylation on the nAChR conformation are restricted to the cytoplasmic region of the protein.

The data obtained in experiments with samples of nAChR in different conformational states induced by the agonist carbamoylcholine suggest that protein−lipid interactions near the membrane surface are unaltered and perhaps only slight changes might occur deeper in the membrane at the protein−lipid interface. From this, it may be concluded that the overall structure of the transmembrane domain is rigid and small changes are sufficient to define different functional states of the receptor.

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