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Labeling of the nicotinic acetylcholine receptor by a photoactivatable steroid probe: effects of cholesterol and cholinergic ligands

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A photoactivatable steroid, *p*-azidophenacyl 3α-hydroxy-5β-cholan-24-ate (APL), has been synthesized and used instead of cholesterol to functionally reconstitute purified acetylcholine receptor (AcChR) into vesicles made of asolectin phospholipids. Upon irradiation, the extent of AcChR photolabeling by APL is directly proportional to the amount of APL incorporated into the reconstituted vesicles and the maximum stoichiometry observed corresponds to approx. 50 mol of APL bound per mol of AcChR. Furthermore, all four subunits of the AcChR become labeled by APL and the observed labeling pattern resembles the 2:1:1:1 stoichiometry characteristic of these subunits within the AcChR complex. The presence of either cholesterol or neutral lipids from asolectin in the reconstituted bilayer decreases both, the incorporation of APL into the vesicles and the covalent labeling of the AcChR upon irradiation, without altering the stoichiometry of labeling in AcChR subunits stated above. This suggests that the potential interaction sites for the photoactivatable probe in the reconstituted AcChR are mostly those normally occupied by the natural neutral lipids. Carbamylcholine, a cholinergic agonist, also reduces the extent of APL photolabeling of the AcChR in a dose-dependent manner but, in contrast to the effects of cholesterol, the presence of carbamylcholine alters the stoichiometry of labeling in the AcChR subunits. This, along with the observation that such a decrease in the extent of APL photolabeling caused by carbamylcholine can be blocked by preincubation with α-bungarotoxin, suggest that AcChR desensitization induced by prolongued exposure to cholinergic agonists encompasses a rearrangement of transmembrane portions of the AcChR protein, which can be sensed by the photoactivatable probe. Conversely, presence of (+)-tubocurarine, a competitive cholinergic antagonist, has no effects on altering either the extent of APL photolabeling of the AcChR or the distribution of the labeling among AcChR subunits.

Introduction

Cholesterol is among the most ubiquitous lipid components of eucaryote plasma membranes. The interaction of cholesterol with membrane phospholipids, as well as its role in regulating physical properties of the lipid bilayer such as membrane permeability or apparent fluidity, has been extensively documented [1–3]. Furthermore, the functional properties of several membrane proteins have been shown to be modulated by the presence of cholesterol within the bilayer (see for instance, Refs. 4–13). It is not known, however, whether such effects of cholesterol on the activity of those proteins are exerted indirectly, through modification of physical properties of the bilayer, or through a more direct interaction between cholesterol and the membrane proteins.

The nicotinic acetylcholine receptor (AcChR) from *Torpedo* is a transmembrane glycoprotein composed of four different polypeptide subunits (α, β, γ and δ) in a 2:1:1:1 stoichiometry [14–16]. Binding of cholinergic agonists to sites on extracellular domains of the Ac-

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Abbreviations: AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; APL, *p*-azidophenacyl 3α-hydroxy-5β-cholan-24-ate (*p*-azidophenacyl lithocholate); PC, phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DSC, differential scanning calorimetry; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TLC, thin-layer chromatography; CHAPS, 3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

ChR elicits the formation of a transient cation channel, responsible for the initiation of postsynaptic membrane depolarization. On continuous exposure to the agonist, however, the channel-opening response becomes blocked and the affinity for the agonists increases, a process known as desensitization.

Studies of reconstitution of the purified AcChR protein into artificial liposomes of defined composition have shown that the presence of cholesterol in the reconstituted samples, at concentrations similar to those found in the native AcChR membranes [17], preserves the ability of the reconstituted AcChR to undergo agonist-induced affinity transitions between sensitized and desensitized states or to exhibit an optimal cation-channel activity [4,7,8,10,13]. Based on the apparent correlation between presence of cholesterol, modification of membrane fluidity and alteration of AcChR function, an 'optimal fluidity' hypothesis was proposed to tentatively account for the effects of cholesterol on the AcChR [10]. On the other hand, the possibility of a direct interaction between cholesterol and the AcChR has received support from studies using spin labeled steroid probes [18,19], infrared spectroscopy [20] or calorimetry [21]. Moreover, use of a brominated steroid analogue in fluorescence quenching studies [22], has shown that the transmembrane portion of the AcChR protein, which is predictably formed by a bundle of membrane-spanning segments contributed by each AcChR subunit [15,16], contains at least two different populations of lipid binding sites, called 'annular' and 'non-annular', differing in their affinity for binding of either phospholipids or cholesterol. The 'non-annular' sites (5–10 sites per AcChR molecule) are occluded to phospholipids and would have an approx. 20-fold higher affinity for binding of cholesterol than that exhibited by the 'annular' sites (approx. 45 sites per AcChR), which should be occupied mostly by phospholipids [22]. It is conceivable, therefore, that the effects of cholesterol on AcChR activity are mediated by complex mechanisms involving more than simply membrane-fluidity alterations. In an attempt to explore the possibility that such effects include a direct interaction of cholesterol with transmembrane portions of the AcChR protein, we describe here the synthesis and properties of a photoactivatable cholesterol analogue and its use in experiments of AcChR photolabeling.

Materials and Methods

Epicholesterol (5-cholesten-3α-ol) was from Steraloids. Lithocholic acid (3α-hydroxi-5β-cholan-24-oic acid), *p*-azidophenacyl bromide, cholesterol, 5β-cholanic acid-3-one and crude extracts of phosphatidylcholine from soybean (type 2-S, asolectin lipids) were purchased from Sigma. Neutral lipids in asolectin were eliminated by passing the lipid extracts, dissolved in chloroform, through a silica gel 60 (230–400 mesh, Merck) column, followed by elution with chloroform. Phospholipids in asolectin were then eluted from the column by using chloroform/methanol mixtures. Egg phosphatidylcholine (PC) and 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) were obtained from Avanti Polar Lipids. [^{125}I]α-Bungarotoxin (α-Bgt), NaB^3H_4 and radioactive calibration compounds ([^{3}H]-toluene and [^{3}H]H_2O) were purchased from New England Nuclear.

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were recorded in Varian CFT-80A or Bruker NP 200-SY instruments, in $CDCl_3$, using tetramethylsilane as internal standard. Infrared (IR) spectra were taken in KBr pellets using a Philips Pye-Unicam SP3–200 instrument.

Synthesis and purification of p-azidophenacyl 3α-hydroxy-5β-cholan-24-ate (APL)

APL was synthesized from *p*-azidophenacyl bromide and lithocholic acid. All operations were carried out under dim light. *p*-azidophenacyl bromide (28.8 mg, 0.12 mmol) in 5 ml of acetone was added to a suspension of lithocholic acid (37.6 mg, 0.1 mmol) in acetone (10 ml), in presence of anhydrous K_2CO_3 (2 gr). The mixture was refluxed under nitrogen and the reaction monitored by TLC on silicagel G plates, using n-hexane/diethyl ether/methanol/acetic acid (60 : 40 : 1 : 1 (v/v)) as the solvent. The formation of the reaction product (R_f approx. 0.1) was maximal in about 3 h, after which excess of K_2CO_3 was discarded and the solvent removed in a rotary evaporator. The residue was dissolved in 2–3 ml of chloroform, washed with 0.1 M $NaHCO_3$ and distilled water, then dried over anhydrous Na_2SO_4. The extract was finally applied onto preparative 0.5 mm-thick silicagel G plates and the R_f approx. 0.1 band was scraped off the plates and extracted with chloroform. The purified product, obtained in a 70–80% yield, was stored at −20°C, under nitrogen and in the dark. Spectral data of the purified APL were as follows: (i) IR (wavenumber, cm^{-1}): 3400 (hydroxyl group); 2125 (azide); 1735, 1300 and 1250 (ester group) and 1700 (ketone). (ii) ^{1}H-NMR (chemical shift, ppm): 3.68 (m, 1 H, 3-$\underline{H}$); 7.09 (d, $J = 8.8$ Hz, 2 H, aromatic $\underline{H}$), 7.91 (d, $J = 8.8$ Hz, 2 H, aromatic $\underline{H}$). (iii) ^{13}C-NMR (chemical shift, ppm): 190.9 (s, aromatic ring-$\underline{C}$O); 173.7 (s, $\underline{C}$OO); 145.7 (s, aromatic $\underline{C}$-N_3); 131.1 (s, aromatic $\underline{C}$-CO); 129.8 (s, aromatic $\underline{C}$-H), 119.3 (s, aromatic $\underline{C}$-H) and 65.6 (s, O-$\underline{C}H_2$-CO), in addition to the resonances expected from the lithocholic acid moiety of APL [23]. These data are consistent with the structure of the compound shown in Fig. 1.

[^{3}H]APL was synthesized similarly, except that tritiated lithocholic acid, produced by NaB^3H_4 reduction of cholanic acid-3-one, was used as a reactant. For the

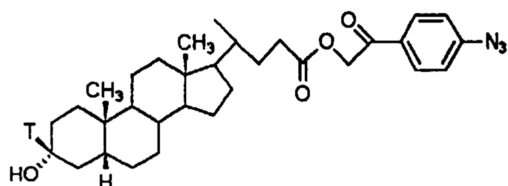

Fig. 1. Structural formula of APL. The location of the tritium label bound at the C-3 position of the steroid ring in [^{3}H]APL is also indicated.

reduction, 25 mCi of NaB^3H_4 (ranging 100–600 mCi/mmol, according to the manufacturer) dissolved at approx. 1 mg/ml in 0.01 M NaOH, was added dropwise to 0.13 mmol of cholanic acid-3-one dissolved in 10 ml of ethanol, under stirring at 4°C, and the reaction allowed to proceed for 5–10 min. Then, non-radioactive $NaBH_4$ (up to 0.2–0.25 mmol) was similarly added, until a complete reduction of cholanic acid-3-one into lithocholic acid was observed by TLC. One volume of distilled water was then added and the mixture was partly evaporated (to eliminate most of the ethanol present), acidified with 1 M HCl and the tritiated lithocholic acid was extracted with ether and dried over anhydrous Na_2SO_4. After evaporation of the solvent, the residue was dissolved in acetone and used for the synthesis of radioactive APL. ^{13}C and ^{1}H-NMR spectra of the product obtained by NaB^3H_4 reduction were identical to those obtained from commercial lithocholic acid, suggesting that the reduction product is also the 3α-OH isomer. The specific activity of the resulting [^{3}H]APL, determined from UV absorbance at 290 nm and scintillation counting, ranged 1–6 mCi/mmol.

Acetylcholine receptor purification and reconstitution

AcChR-enriched membranes were prepared from the electroplax of *Torpedo marmorata* [24]. The AcChR was purified from cholate extracts of those membranes by affinity chromatography in the presence of asolectin lipids [14]. The purified AcChR had specific activities of approx. 8 nmol of α-Bgt bound per mg of protein.

Plain lipid vesicles used for reconstitution were prepared by a CHAPS dialysis procedure [25] from asolectin phospholipids at approx. 30 mg/ml, with or without added APL and/or cholesterol. Aliquots of the above components in chloroform were mixed and the solvent removed by vacuum drying. The residue was hydrated for 1 h in 10 mM Tris buffer (pH 7.4) containing 100 mM NaCl and 2% CHAPS, vortexed and sonicated in a probe-type Soniprep 150 apparatus during 3 periods of 3 min. Samples appeared transparent at the end of sonication. Lipid vesicles were formed by elimination of the detergent by dialysis. The dialyzed samples were resolubilized in 2% sodium cholate and used immediately for reconstitution.

Reconstituted AcChR samples were prepared by mixing aliquots of purified AcChR with the solubilized lipid vesicles from above. Final concentrations in the reconstitution mixtures were: AcChR, approx. 1 mg/ml; asolectin phospholipids, approx. 15 mg/ml; sodium cholate, 1.5% and APL and/or cholesterol at molar percentages ranging from 0 to 40%, with respect to the phospholipids. Reconstitution was accomplished by dialysis at 4°C for about 50 h (8 × 1-litre changes in 10 mM Tris (pH 7.4) containing 100 mM NaCl, 0.1 mM EDTA, 0.5 mM PMSF and 5 mM iodoacetamide). Protein determination in these vesicles by most colorimetric procedures is hindered by the interference of asolectin at high concentration. For this reason, aliquots of reconstituted samples were solubilized by 1% SDS and the protein precipitated by adding a 10-fold excess (v/v) of acetone/triethylamine/acetic acid (95:5:5 (v/v)) [26], resolubilized in 1% SDS and the protein concentration determined as in Ref. 27. Phospholipids (lipid phosphorus) and cholesterol were determined as described [28].

The ability of the reconstituted AcChR to undergo agonist-induced affinity state transitions was determined by measuring differences in the rate of α-Bgt binding upon preincubation with carbamylcholine to produce AcChR desensitization [29]. Agonist-mediated cation translocation through the AcChR was monitored by using a 'stopped-flow fluorescence quenching' assay of Tl^+ influx [30].

Photolysis of APL and labeling of AcChR

All samples were photolyzed in 1 × 1 cm quartz cuvettes, using a UVGL-25 lamp (Ultraviolet Products) on the long-wavelength setting and at room temperature. The distance between the lamp and the cuvette was 1 cm. AcChR photolabeling was determined by radioactive counting of the protein-bound photoproducts remaining upon solubilization of the labeled vesicles by SDS and protein precipitation as described above. No significant contribution of labeled lipids to the radioactivity attributed to photolabeling of the AcChR protein was observed by processing similar reconstituted vesicles prepared in the absence of protein. Photolabeled AcChR was also subjected to analysis by SDS-PAGE and the extent of labeling at the different AcChR subunits was determined by autoradiography. For this, the gels were soaked for 10 min into Amplify solution (Amersham), dried under vacuum and exposed to Kodak XAR-5 X-OMAT films during 30–40 days, at −80°C.

Differential scanning calorimetry

Large multilamellar DPPC vesicles for DSC measurements were prepared as described previously [31], in the absence or in the presence of cholesterol, epicholesterol or APL. Differences in the heat capacity

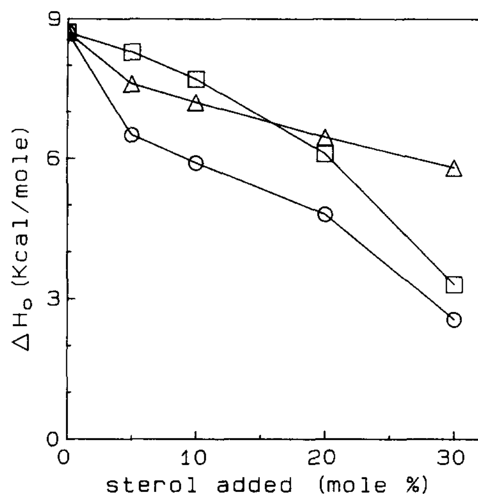

Fig. 2. Effects of cholesterol (○), epicholesterol (□) and APL (△) on the enthalpy change corresponding to the DPPC gel to fluid phase transition observed by differential scanning calorimetry. The DPPC concentration was 2 mM (in terms of lipid phosphorus) in all samples.

between the sample and the reference cell were recorded in a Microcal MC-2 calorimeter, by raising the temperature at a constant rate of 45 C°/h. Transition temperatures and enthalpies were calculated by fitting the observed transitions to a single van 't Hoff component.

Results

The effects of APL, cholesterol and epicholesterol (the 3α-OH isomer of cholesterol) on the thermotropic phase transition of model DPPC bilayers were determined by DSC. Fig. 2 shows that APL at moderate concentrations (below 15% by mol), exhibits a behaviour intermediate between those exhibited by cholesterol and epicholesterol in decreasing the enthalpy of the DPPC main phase transition. At higher molar percentages, however, it is observed that APL becomes less effective than either one of the cholesterol 3-OH isomers in affecting the DPPC transition.

Fig. 3 illustrates the incorporation of APL or cholesterol, at different concentrations, into reconstituted AcChR vesicles. The incorporation of APL is more than 90% efficient at APL to phospholipids molar ratios up to 0.2, but becomes somewhat less efficient at higher molar fractions, which is almost identical to the pattern of incorporation of cholesterol into similar reconstituted vesicles obtained by the detergent dialysis procedure. Similarly to cholesterol, APL also allows the reconstituted AcChR protein to undergo agonist-induced sensitization-desensitization transitions and optimizes its cation channel function. Fig. 4 illustrates that the AcChR reconstituted into vesicles containing incorporated APL, responds to carbamylcholine by allowing a rapid flow of Tl^+ into the vesicles and that

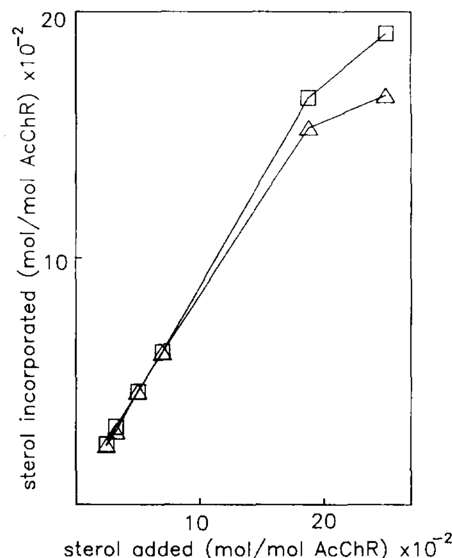

Fig. 3. Incorporation of APL (△) and cholesterol (□) into reconstituted AcChR vesicles formed by detergent dialysis. Since APL and cholesterol do not incorporate quantitatively in the reconstituted vesicles at all the concentrations tested, the final concentration of these components incorporated into the vesicles was determined after passing the reconstituted samples throught a Sephadex G-25 (coarse) column (1.5×30 cm). Error bars are comparable to the size of the symbols used in the representation.

such response to the cholinergic agonist is similar to that exhibited by reconstituted vesicles containing comparable concentrations of cholesterol.

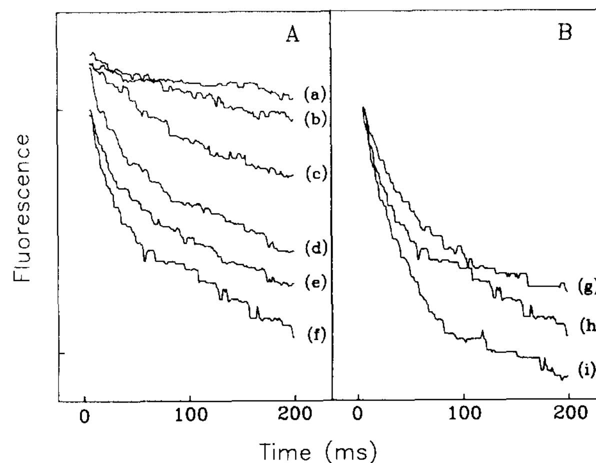

Fig. 4. Representative stopped-flow traces corresponding to the rapid collisional quenching of the fluorescence of 1,3,6,8-pyrene tetrasulfonate entrapped into reconstituted AcChR vesicles, by externally added Tl^+ (see Ref. 30). Panel A shows Tl^+ influx exhibited by reconstituted AcChR vesicles prepared from asolectin phospholipids and 20 mol% APL, in response to carbamylcholine at 0 (a), 25 (b), 37.5 (c), 50 (d), 100 (e) and 150 μM (f). Samples irradiated under similar conditions to those used in the photolysis of APL did not modified their ion-flux response to carbamylcholine. Panel B compares the Tl^+ influx responses to 150 μM carbamylcholine exhibited by reconstituted AcChR vesicles prepared from whole asolectin extracts, containing the endogenous neutral lipid components (i), or from asolectin phospholipids and 20 mol% of either APL (h) or cholesterol (g).

Unphotolyzed APL in chloroform solution exhibits an absorbance maximum at 290 nm and a molar extinction coefficient at this wavelength of 17100 $M^{-1} cm^{-1}$ (Fig. 5A). Upon irradiation with long-wavelength UV light, there is a shift in the absorbance maximum to shorter wavelengths and a decrease in the absorbance of the resulting compounds. Isosbestic points are also observed at 270 and 315 nm. Similar observations were made in small unilamellar egg PC vesicles containing incorporated APL (Fig. 5B). Aliquots of the labeled egg PC vesicles taken during the course of the photolysis exhibited an altered gas-liquid chromatographic pattern of the acyl components of egg PC (data not shown), thus suggesting that APL photoproducts are able to bind to the acyl chains of membrane phospholipids. The rate of photolysis of APL in either chloroform solution or egg PC vesicles can be estimated from the changes in the absorbance at 290 nm (Fig. 5C). Under either condition, the conversion of APL into photoproducts has a half-life ranging 130–150 s and reaches a maximum within 20–25 minutes of irradiation. Moreover, the photoproducts seem to be stable over longer periods of time, as indicated by the lack of large absorbance changes upon further irradiation of the sample. The optimum irradiation time of APL incorporated into reconstituted AcChR vesicles could not be determined from absorbance changes because of excessive light scattering of these samples. For this reason, we used AcChR membrane suspensions in front of a sample containing APL in chloroform solution, to filter light in a manner similar to when APL is incorporated into the vesicles. Under these conditions, the rate of photolysis decreases only slightly with respect to that observed in chloroform solution (Fig. 5C), and therefore, we chose irradiation times anywhere from 25 to 40 min to assure the complete photodecomposition of the incorporated APL.

The extent of covalent photolabeling of the reconstituted AcChR protein at the different concentrations of APL (Fig. 6) is proportional to the extent of APL

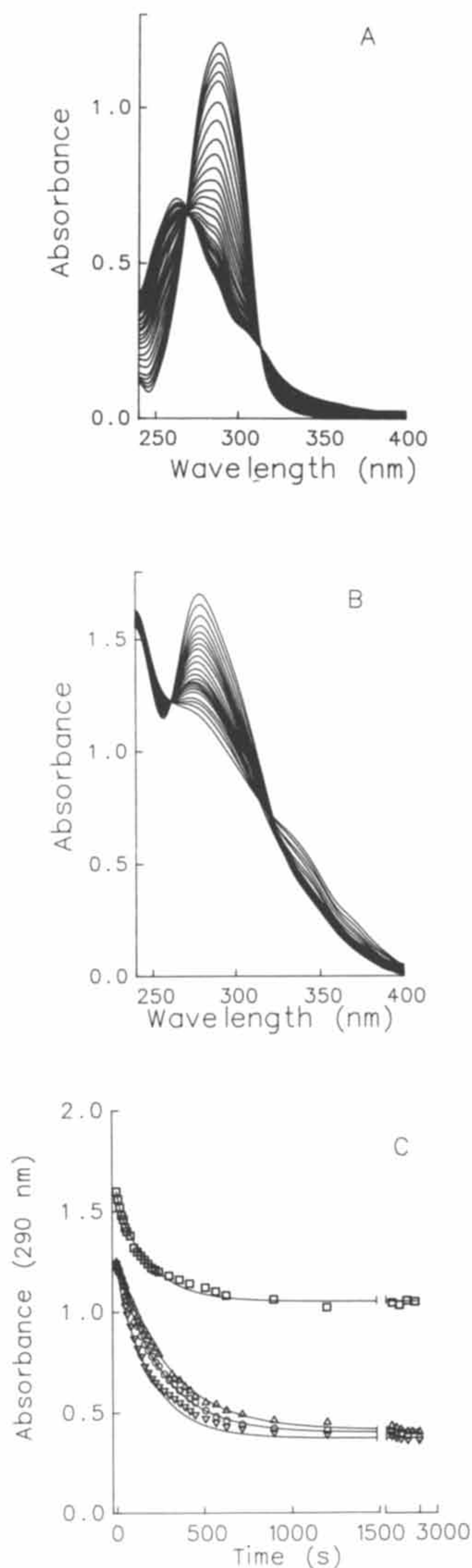

Fig. 5. Changes in the absorbance spectra of APL as a function of the irradiation time. Panel A shows the photolysis of APL (0.66 μM) in chloroform solution. The spectrum of the unphotolyzed compound corresponds to that exhibiting the highest absorbance at 290 nm, while the rest of spectra correspond to samples irradiated for increasingly longer periods of time (from 10 s up to 50 min). Panel B shows the photolysis of APL incorporated into small unilamellar vesicles made from egg PC. To prepare these vesicles, dried mixtures of egg PC and APL at a 0.4 molar ratio were suspended in 10 mM Hepes buffer (pH 7.4) containing 100 mM NaCl, to a final concentration of approx. 1 mM (in terms of lipid phosphorus), extensively sonicated and diluted in buffer to an adequate absorbance value. An apparent shift in the absorbance maximum of APL to 280 nm is observed when the unphotolyzed probe incorporates into lipid vesicles. Panel C shows plots of the absorbance changes at 290 nm vs. irradiation time, for the photolysis of APL (0.66 μM) in chloroform solution (▽) and in the same solution irradiated through a 1 cm-path length quartz cuvette containing a suspension of AcChR membranes at 1 (○) and 2 (△) mg of protein per ml. The photolysis of APL incorporated into the small unilamellar vesicles used in panel B (□) is also shown.

incorporation into the vesicles and in fact, plotting the amount of APL incorporated versus the amount of APL photoproducts covalently bound to the AcChR upon irradiation, results in a fairly straight line with a correlation coefficient of approx. 0.97 (not shown). The efficiency of covalent photolabeling of the AcChR was similar within the concentration range of APL assayed, amounting to approx. 3% of the total incorporated photoactivatable probe. Such low efficiency was expected since the photogenerated nitrenes are able to cross-link not only to proteins, but also to membrane lipids or to other APL molecules.

The distribution of radioactive APL photoproducts on the AcChR subunits was determined by autoradiography after their separation by SDS-PAGE. All four AcChR subunits become radioactively labeled upon irradiation (Fig. 7) and despite the limitations imposed by the low specific radioactivity of the tritium label, the observed labeling pattern resemble closely the 2:1:1:1 stoichiometry characteristic of these subunits within the AcChR complex (Table I), thus suggesting that all subunits are similarly accessible to labeling by the photoactivatable probe. Neither irradiation by itself, nor APL photolabeling of the AcChR under the conditions used for these experiments, result in a noticeable alteration of the electrophoretic pattern or the subunits' electrophoretic mobility.

The incorporation of APL into reconstituted vesicles and the photolabeling of the AcChR protein referred above, can be partly prevented by the presence of cholesterol. Fig. 8 shows that APL and cholesterol can be simultaneously incorporated into the reconstituted vesicles, as long as the sum of the molar percentages of both compounds in the reconstitution mixtures does not exceed 25%, approximately, with respect to the phospholipids. Thus, in samples prepared at a molar percentage of APL of 10 (open circles, Fig. 8), it is observed that presence of cholesterol at similar molar ratios does not result in a large displacement of APL from the vesicles. However, increasing the molar percentage of cholesterol beyond 15–20% results in a larger decrease in the incorporation of APL. Similar experiments using vesicles prepared at a molar per-

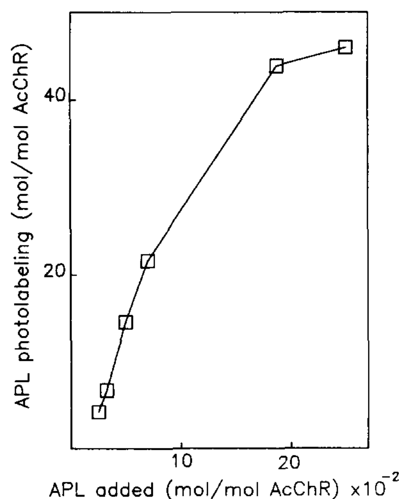

Fig. 6. APL photolabeling of reconstituted AcChR. The plot shows the amount of APL photoproducts covalently bound to the AcChR protein (see Materials and Methods), as a function of the amount of APL initially present in the reconstitution mixtures. Error bars are comparable to the size of the symbols used in the representation.

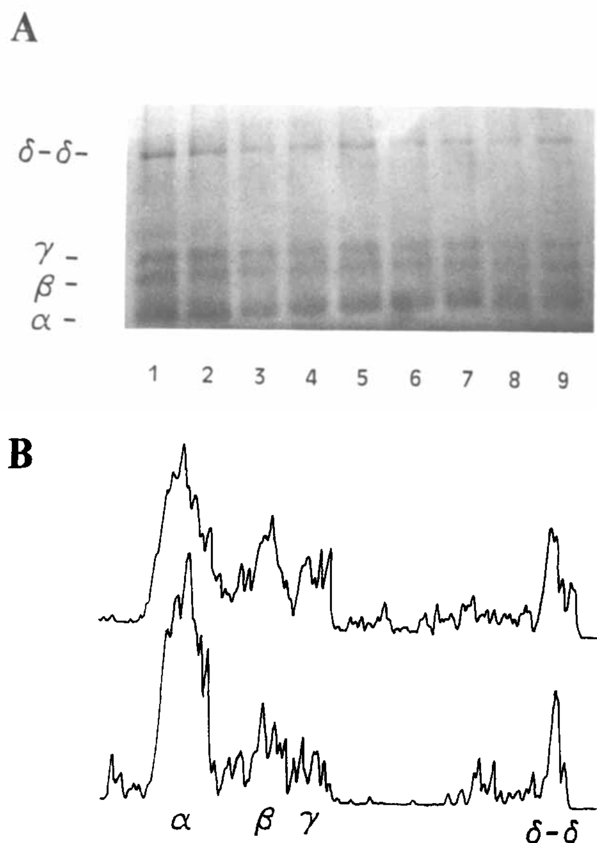

Fig. 7. APL photolabeling of AcChR subunits in the absence and in the presence of cholinergic ligands or cholesterol. Reconstituted AcChR vesicles prepared in the presence of APL at 10 mol%, were used in these studies. Prior to the electrophoretic separation of AcChR subunits, the photolabeled samples were either extracted with chloroform or the AcChR protein precipitated from SDS extracts (see Materials and Methods) to remove non-covalently bound photoproducts. For the electrophoresis, a 9% acrylamide slab gel and a 4% stacking gel were used, under non-reducing conditions [24]. Approx. 15 μg of AcChR protein were applied into each sample well. The different lanes in the autoradiogram of the gel (panel A) show the photolabeling obtained in the presence of 10^{-3} M (+)-tubocurarine (1); in the absence of either cholinergic ligands or cholesterol (2, control); in the presence of carbamylcholine at 10^{-5} M and 10^{-3} M, respectively (3 and 4) and in the presence of cholesterol incorporated into the reconstituted vesicles at 4, 7.8, 11.6, 15 and 22.3 mol%, respectively, with respect to the phospholipids (5 to 9). Estimates for the apparent molecular mass of AcChR subunits were 40 (α), 50 (β), 60 (γ) and 120 kDa (δ_2), regardless of whether unlabeled (control) or APL-photolabeled samples were used in the electrophoretic determinations. Panel B shows densitometric scans taken from lanes 2 (control, upper trace) and 4 (10^{-3} M carbamylcholine, lower trace).

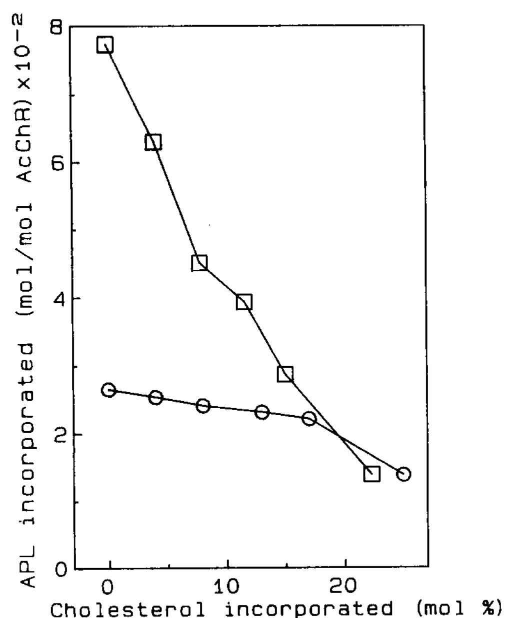

Fig. 8. Effects of cholesterol on the incorporation of APL into reconstituted AcChR vesicles. The results obtained using reconstituted vesicles prepared in the presence of 5, 10, 15, 20 and 30 mol% of cholesterol, in addition to either 10 (○) or 25 (□) mol% of APL, are shown.

centage of APL of 25 (open squares, Fig. 8) indicate that the addition of cholesterol, even at molar percentages as small as 5–10%, already results in a marked decrease in the amount of APL that can be incorporated. Reconstituting with whole asolectin (containing the endogenous neutral lipids) instead of using only the asolectin phospholipids, results also in a significant reduction of APL incorporation, which is comparable to that obtained at high molar percentages of cholesterol (data not shown).

The efficiency of cholesterol in preventing protein photolabeling in vesicles prepared at 25 mol% of APL is similar to that exhibited in decreasing the incorporation of APL into the vesicles (see Table I and Fig. 8). However, similar experiments using reconstituted samples prepared at 10 mol% of APL suggests that, under these conditions, cholesterol is more efficient in protecting the protein against photolabeling (Table I) than in decreasing the incorporation of the photoactivatable probe into the reconstituted vesicles (Fig. 8). Thus, presence of cholesterol in these samples at an approx. 15 mol%, decreases only slightly (approx. 15%) the incorporation of APL into the reconstituted vesicle, but produces a considerable inhibition (approx. 40%)

TABLE I

APL photolabeling of the AcChR: effects of cholinergic ligands and cholesterol

Reconstituted AcChR vesicles prepared in the presence of 25 or 10 mol percent of [^{3}H]APL with respect to the phospholipids, with or without the indicated mol percentages of cholesterol, were incubated for 15 min, in the presence or in the absence of cholinergic ligands at the concentrations indicated and photolyzed during 25 min. Results are expressed as the percent of radioactivity (±S.D.) contained in the protein-bound photoproducts remaining upon SDS solubilization of the irradiated vesicles and protein precipitation, with respect to the controls (identical samples prepared without cholesterol and photolyzed in the absence of ligands). The labeling stoichiometries are the average of the densitometric scans of two autoradiograms, such as that shown in Fig. 7, using reconstituted membranes prepared at 10 mol percent of APL. The area of each subunit in the densitometric scans was related to the sum of the areas corresponding to the α, β, γ and δ subunits in each sample, which was made arbitrarily equal to 5 to determine the relative labeling stoichiometry.

	Relative labeling		Labeling stoichiometry in AcChR subunits ($\alpha:\beta:\gamma:\delta$)			
	25% APL	10% APL				
Control	100	100	2.1	0.7	0.8	1.1
(+)-Tubocurarine (10^{-3} M)	100±1	100±1	2.0	0.9	0.7	1.2
α-Bgt [a]	–	102±1	Not determined			
Carbamylcholine (10^{-5} M)	82±1	81±3	2.7	0.5	0.5	1.0
Carbamylcholine (10^{-3} M)	69±4	67±5	3.0	0.6	0.6	0.6
α-Bgt, then carbamylcholine (10^{-3} M)	–	102±1	Not determined			
Cholesterol						
5%	85±3	87±1	2.3	0.8	0.8	0.8
10%	80±3	75±1	2.2	0.8	0.8	0.9
15%	66±3	65±1	1.9	1.1	1.0	1.1
20%	54±2	53±3	2.1	0.9	0.8	1.0
30%	26±5	41±4	2.2	0.9	0.8	1.0

[a] Values represent duplicate determinations from a single batch of reconstituted AcChR vesicles containing a 10 mol percent of [^{3}H]APL with respect to the phospholipids. The vesicle samples were preincubated during 30 min and at room temperature, with a 2-fold molar excess of α-Bgt over the α-Bgt binding sites prior to irradiation and/or to the addition of carbamylcholine.

of AcChR photolabeling. Table I also shows that the inhibition in the photolabeling of the AcChR caused by the presence of increasing concentration of 'protecting' cholesterol in these samples, results in a progressive decrease in the radioactivity associated to each of the subunits, without a significant departure from the 2 : 1 : 1 : 1 labeling stoichiometry stated above.

Reconstituted vesicles prepared at a molar percentage of APL of either 10 or 25 were also used to determine the effects of cholinergic ligands on AcChR photolabeling. Incubation of the vesicles with such ligands does not produce alteration in the amount of APL incorporated in the vesicles. However, Table I shows that presence of the cholinergic agonist carbamylcholine, but not that of α-Bgt or the competitive antagonist (+)-tubocurarine, partly prevents the AcChR from photolabeling by APL. Such effect of the cholinergic agonist in protecting the AcChR against photolabeling (i) increases as the agonist concentration is increased up to 1 mM; (ii) can be blocked by preincubation of the receptor vesicles with an excess of α-Bgt and (iii) results in alteration of the distribution of the label among the AcChR subunits, which now shows a relatively greater photolabeling in the α-subunit (Table I). Conversely, comparable samples irradiated in presence of similar concentrations of the antagonist (+)-tubocurarine, which causes no alteration in the extent of APL photolabeling of the AcChR protein, exhibit a labeling distribution in the AcChR subunits indistinguishable from those obtained in the absence of cholinergic ligands.

Discussion

Photoactivatable cholesterol analogues that can be covalently bound to the putative interaction sites on the AcChR protein can provide an adequate tool to study the interaction between cholesterol and the AcChR. This approach has been already used by Middlemas and Raftery [32], which synthesized cholesteryl diazoacetate and found that it labeled all four AcChR subunits. Cholesteryl diazoacetate, however, lacks the cholesterol 3β-OH group, which is believed to mediate its interaction with phospholipids [33,34] and which might also be relevant in the interaction with membrane proteins. Also, labeling by cholesteryl diazoacetate is restricted to regions of the protein close to the lipid–water interface [32,35].

We describe here the synthesis of APL, a photoactivatable steroid that behaves similarly to cholesterol regarding (i) its interaction with phospholipids; (ii) its incorporation into reconstituted AcChR vesicles and (iii) the functional maintenance of the reconstituted AcChR. Furthermore, APL incorporation into the reconstituted membranes and photolabeling of the AcChR protein can be substantially inhibited by cholesterol or by the neutral lipids present in crude asolectin extracts, thus suggesting that many of the potential interaction sites for the photoactivatable probe within the reconstituted AcChR vesicles are shared by the natural lipids. Also, the dependence of the extent of photolabeling on the presence of cholesterol or other neutral lipids in the reconstituted bilayer, together with the observation that APL labels acyl chains of membrane phospholipids, provides evidence to support that the labeled protein sites are accessed from the membrane and deeply embedded in the bilayer.

Under conditions of maximal incorporation of APL into the reconstituted membranes and in the absence of competing neutral lipids, the stoichiometry of AcChR labeling amounts to approximately 50 mol of APL photoproducts bound per mol of AcChR. Such maximal stoichiometry exceeds that predicted for the occupation of the 'non-annular' AcChR binding sites [22], for which cholesterol should have the highest affinity, and approaches the summation of both, 'annular' and 'non-annular' lipid binding sites. A possible explanation for this observation is that the covalent binding of APL photoproducts to the 'annular' sites overcomes the expected low affinity of these sites for cholesterol-like molecules, thus, resulting in the effective displacement of the phospholipids normally involved in the interaction with the protein at these 'annular' sites.

Analysis of APL photolabeling on the different AcChR subunits reveals that all four subunits become labeled and that the labeling stoichiometry is similar to that exhibited by these subunits within the AcChR complex. Such similarity should in principle be expected (i) from the high degree of sequence homology reported previously for AcChR subunits and from the existence of similar clustering of hydrophilic and hydrophobic aminoacids along the chains (reviewed in Ref. 36), which should result in comparable tertiary structures, and (ii) from the low specificity of cross-linking of the highly reactive nitrenes.

The effects of cholesterol in decreasing the extent of AcChR photolabeling by APL, are translated into a comparable decrease in the extent of labeling at all four AcChR subunits, thus, maintaining the relative labeling stoichiometry. This further indicates that the four subunits contain homologous sites of interaction with APL and suggests that all the subunits interact with the lipid bilayer similarly. On the other hand, the presence during irradiation of the agonist carbamylcholine results also in a significant, concentration-dependent decrease in the extent of AcChR photolabeling, which can be prevented by preincubation with α-Bgt, but produces a change in the relative distribution of the label among the AcChR subunits. This implies that transmembrane regions of the AcChR protein, i.e., those containing the putative sites of interaction for the cholesterol-like probe, which would

normally be labeled in the absence of the agonist, become unaccessible to APL upon desensitization, when the agonist is bound at the ligand binding sites located 40–50 Å away from the membrane surface [37]. Comparable long-range structural perturbations transmitted through the AcChR protein were previously proposed from studies using a pyrene derivative covalently attached to the AcChR at the protein-lipid interface [38]. Likewise, cholesterol [21] or free fatty acids produced by hydrolysis of membrane phospholipids [39], were also shown to influence the stability of the α-Bgt binding sites on the AcChR, as well as the ability of the AcChR to undergo sensitization-desensitization transitions [7,8,10,13,40], which are known to affect mostly the conformation of the cholinergic binding region of the AcChR [41]. According to our data, the agonist-sensitive protein sites that become unaccessible to the probe, seem confined to the β, γ and δ subunits of the AcChR and, being lipid-exposed regions, they are likely to involve at least the M4 transmembrane segments of these subunits [42,43].

The observed sensitivity of APL photolabeling to the presence of cholinergic agonists is somewhat reminiscent of that found in the photolabeling of the AcChR with the hydrophobic probe 3-trifluoromethyl-3-(m-[^{125}I]iodophenyl)diazirine [44], which was found to be a non-competitive antagonist of the AcChR [45]. However, it seems unlikely that APL could also be a non-competitive antagonist, since unphotolyzed APL maintains AcChR ion-channel activity. Furthermore, based on the observation that other neutral lipids, including those in crude asolectin, cholestane, α-tocopherol or vitamin D-3, are also able to maintain AcChR ion-channel function [13], it seems more likely that the observed effects of cholesterol or cholesterol-like molecules on the AcChR are mediated by hydrophobic interactions involving sites on the hydrophobic surface of the AcChR protein.

The observed photolabeling of the AcChR protein by APL differs with that obtained by using other photoreactive derivatives of phospholipids [46,47] or cholesterol [32], which also labeled all AcChR subunits, but (i) at labeling stoichiometries which differed markedly [46,47] or only slightly [32] from the approximately 2:1:1:1 reported here and (ii) did not observed changes in the extent of labeling in the presence of agonists. The observed discrepancies could partly be due to a different accesibility of the photoreactive probes to the protein. As indicated above, there are restrictions in the access of cholesteryl diazoacetate to the transmembrane portions of AcChR [32,35]. Also, the photoreactive phospholipids [46,47] should be excluded from the 'non-annular' sites [22] and thus, may not have access to all the protein sites potentially available to APL. Since APL photolabeling could be useful in defining the transmembrane portions of the protein configuring the conformation of the AcChR in the resting and in the desensitized states, work is currently in progress in our laboratory to obtain APL of higher specific radioactivity and use selective proteolysis procedures in isolated AcChR subunits to identify the agonist-sensitive protein sites labeled by APL.

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