Photoaffinity Labeling of the *Torpedo californica* Nicotinic Acetylcholine Receptor with an Aryl Azide Derivative of Phosphatidylserine†

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ABSTRACT: A photoactivatable analogue of phosphatidylserine, ^{125}I-labeled 4-azidosalicylic acid–phosphatidylserine (^{125}I ASA–PS), was used to label both native acetylcholine receptor (AchR)-rich membranes from *Torpedo californica* and AchR membranes affinity purified from *Torpedo* reconstituted into asolectin (a crude soybean lipid extract) vesicles. The radioiodinated arylazido group attaches directly to the phospholipid head group and thus probes for regions of the AchR structure in contact with the negatively charged head group of phosphatidylserine. All four subunits of the AchR incorporated the label, with the α subunit incorporating approximately twice as much as each of the other subunits on a per mole basis. The regions of the AchR α subunit that incorporated ^{125}I ASA–PS were mapped by *Staphylococcus aureus* V8 protease digestion. The majority of label incorporated into fragments representing a more complete digestion of the α subunit was localized to 11.7- and 10.1-kDa V8 cleavage fragments, both beginning at Asn-339 and of sufficient length to contain the hydrophobic region M4. An 18.7-kDa fragment beginning at Ser-173 and of sufficient length to contain the hydrophobic regions M1, M2, and M3 was also significantly labeled. In contrast, V8 cleavage fragments representing roughly a third of the amino-terminal portion of the α subunit incorporated little or no detectable amount of probe.

The nicotinic acetylcholine receptor (AchR)[1] is an integral membrane protein with subunit stoichiometry $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980). One of the best characterized membrane-bound allosteric proteins, it carries the acetylcholine binding sites, contains the ion channel, and mediates the conformational transitions responsible for the regulation of ion translocation by acetylcholine [reviews in Lindstrom (1983), Popot and Changeux (1984), Hucho (1986), and McCarthy et al. (1986)]. The AchR is a heterologous pentamer made up of four different though homologous subunits, each of which is thought to contribute structurally to form the ion channel. The complete primary structure of the subunits has been established by cDNA cloning and sequencing in *Torpedo californica* (Noda et al., 1982, 1983a,b; Claudio et al., 1983). Models for the transmembrane organization of the mature subunits have been proposed on the basis of these sequences (Claudio et al., 1983; Noda et al., 1983b; Devillers-Thiery et al., 1983; Finer-Moore & Stroud, 1984; Guy, 1984).

The role of lipids in the structure and function of the AchR remains an important area of research. The AchR has been shown to be quite sensitive to the lipid environment (Ochoa et al., 1983; Criado et al., 1984; Fong & McNamee, 1986; McNamee et al., 1986). Delipidation of the AchR stabilizes the receptor in a state of very low affinity for the agonist (Chang & Bock, 1979). A minimum number of lipid molecules (approximately 45) are required to prevent irreversible loss of AchR function (Jones et al., 1988). Exposure of the AchR to the lipid bilayer has been studied by using photoactivated probes that partition predominantly into the hydrophobic phase of the lipid bilayer. Several of these probes have been used to demonstrate exposure of the AchR subunits to the membrane (Sator et al., 1979; Gonzales-Ros et al., 1979; Tarrab-Hazdae et al., 1980, 1982; Middlemas & Raftery, 1983; Clarke et al., 1987; White & Cohen, 1988).

While it has been established that AchR function requires an appropriate membrane fluidity (Fong & McNamee, 1986), the protein's state transitions and ion channel gating require the additional presence of both sterol and negatively charged phospholipids (Ochoa et al., 1983; Criado et al., 1982, 1984; Fong & McNamee, 1986). The lipid requirement of AchR function is consistent with the high abundance of both cholesterol and phosphatidylserine in native electroplax membranes (Gonzales-Ros et al., 1982). Electron spin resonance spectroscopy has been used to demonstrate that negatively charged phosphatidic acid, cholesterol, and fatty acids interact preferentially with the AchR (Marsh et al., 1981; Ellena et al., 1983; Devaux & Seigneuret, 1985). The specific requirement for negatively charged phospholipids indicates that interactions between the negatively charged phospholipid head group and regions of the AchR structure are important for AchR function. Negatively charged phospholipids have been shown to increase the apparent β sheet content of the AchR, which are considered to be crucial in transmitting conformational changes induced by AchR agonists (Fong & McNamee, 1987). A photoactivatable analogue of cholesterol has been used to demonstrate labeling of the AchR subunits (Middlemas & Raftery, 1987); however, no detailed analysis of the regions that incorporate label has followed.

An important first step in determining how the interaction of specific lipids affects receptor structure/function is locating the regions of the subunit primary structure that directly interact with specific lipids. In this paper we have used a photoactivatable analogue of phosphatidylserine, ^{125}I ASA–PS, to resolve the binding site domains on the AchR subunits. The

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[1] Abbreviations: AchR, nicotinic acetylcholine receptor; ^{125}I ASA–PS, ^{125}I-labeled azidosalicylic acid–phosphatidylserine; V8, *Staphylococcus aureus* V8 protease; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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photoactivatable arylazido group is attached directly to the phospholipid head group and thus probes for AchR regions in contact with the negatively charged head group. These experiments also test existing transmembrane organization models of the mature AchR subunits.

Experimental Procedures

Materials. $Na^{125}I$ was from Amersham. Electrophoresis reagents and affinity column matrix (Affi-Gel 10) were purchased from Bio-Rad. Molecular weight markers were obtained from Sigma and Diversified Biotech. *N*-Hydroxysuccinimidyl-4-azidosalicylic acid (NHS-ASA) was purchased from Pierce Chemical Co., L-α-phosphatidyl-L-serine from Sigma, and protease type XVII from *Staphylococcus aureus* (strain V8) from United States Biochemical Co. Thin-layer chromatography was performed by using Merck type 60 silica gel plates from Alltech Associates.

Preparation of AchR-Rich Membranes. Affinity Column Purification and Reconstitution of AchR Membranes. AchR membranes were isolated from dissected *T. californica* electric organ. AchR-rich membranes were purified on a sucrose gradient as previously described (Blanton et al., 1988). The final membrane preparation contained approximately 0.5–0.6 nM acetylcholine binding sites/mg of protein as determined by the binding of a spin-labeled local anesthetic using electron spin resonance spectroscopy (ESR) (Blanton, 1989).

The AchR was isolated from detergent (cholate) extracts of crude AchR membranes by affinity chromatography. Affinity column purification was performed by using an acetylcholine affinity matrix according to the procedure of Ellena et al. (1983) with several modifications. Briefly, Affi-Gel 10 was used as the solid support matrix to which cystamine was linked. The cystamine was then reduced to provide the sulfhydryl linkage to attach bromoacetylcholine bromide. A crude soybean lipid extract (asolectin) was used in the column washes and in the final reconstitution of the AchR. The AchR was reconstituted at a lipid to protein ratio of 800:1 on a mole per mole basis and stored at −70 °C.

Synthesis of ASA-Phosphatidylserine. ASA-phosphatidylserine (ASA-PS) was synthesized by using the procedure of Shanahan et al. (1985) with the following modifications. Phosphatidylserine (100 mg, 0.127 mmol) was dissolved in 3 mL of chloroform, and 50 μL of triethylamine (0.36 mmol) was added to neutralize the solution. NHS-ASA (100 mg, 0.036 mmol) in 2 mL of chloroform was then added to the mixture. The reaction was allowed to proceed for 48 h at room temperature with constant stirring, after which time the solvent was removed by vacuum drying and the material dissolved in 1.5 mL of chloroform. The ASA-PS was purified by thin-layer (0.5 mm) chromatography on silica plates developed with chloroform:methanol:water (65:30:5). The developed TLC plate contained two UV-positive bands (R_f = 0.84 and 0.24) of which the major band (R_f = 0.24) also stained positive for the presence of phosphate. This UV-positive, phosphate-staining band (R_f = 0.24) was scraped from the plate and extracted with 20 mL of chloroform and then again with 20 mL of chloroform:methanol (2:1). The solvent from the combined extracts was removed on a rotary evaporator and the material dissolved in 15 mL of chloroform, flushed with N_2 gas, and stored at −20 °C in a vacuum desiccator. UV and IR spectroscopy were performed on the compound for analysis: UV (10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, pH 7.5; VDB buffer) λ_{max} 264 nm (ϵ 23 815) with a shoulder at 304 nM; IR (KBr) 2217 cm^{-1} (azide) and 3400 cm^{-1} (broad OH). GC analysis indicated that phosphatidylserine consisted of fatty acid chains of 18:0 and 18:1 in the approximate abundance of 60% and 40%, respectively.

Synthesis of ^{125}I ASA-Phosphatidylserine. Iodination of ASA-PS was performed by using the method of Hunter and Greenwood (1962). Five microliters of 0.5 M sodium phosphate (pH 7.5) was added to a vial containing 10 μL of $Na^{125}I$ (1 mCi) in 10 μL of 0.1 M NaOH, followed by addition of 10 μL (1 nmol) of ASA-PS solution (0.05 M sodium phosphate buffer, pH 7.4). To this solution was added 5 μL (2.5 nmol) of chloramine T (0.5 M sodium phosphate buffer, pH 7.4), and the reaction was allowed to proceed for 2 min; 500 μL of chloroform was then added to the mixture, followed by 500 μL of 10% NaCl. The chloroform layer was removed and washed by another 500 μL of 10% NaCl, and the chloroform layer was removed again. The specific activity of the radioiodinated product was determined from the initial amount of ASA-PS added and by γ emission counting. ^{125}I ASA-PS in chloroform was stored at −20 °C at a specific activity of 60 Ci/mmol.

Photoaffinity Labeling. To introduce the photoaffinity probe into AchR membrane preparations, typically 8–10 μCi (0.15 nM) of ^{125}I ASA-PS was added to a 5-mL pear-shaped flask and the solvent removed on a rotary evaporator; 500 μL of buffer (VDB) was added and the solution sonicated for 5 min under a stream of nitrogen. Five hundred microliters (750 μg) of reconstituted AchR preparation or native AchR-rich membranes was added and the mixture allowed to stir slowly overnight at 4 °C. All operations were carried out either in the dark or under a red safety light. At this point the material was centrifuged and the membrane pellet resuspended in 500 μL of buffer to remove most of the probe that had not partitioned into the membrane, thereby reducing nonspecific labeling. The preparation was divided into aliquots, and any chemical agents were added (e.g., carbamoylcholine) and allowed to incubate for 1 h. Samples were illuminated in a quartz cuvette for 10 min at 4 °C with a long-wave UV lamp 1 cm away (UVSL-25, Ultraviolet Products, on the long-wavelength setting 366 nm). The sample was diluted with approximately 20 volumes of buffer and centrifuged at high speed, and the pellet was resuspended for electrophoresis.

Electrophoresis. Proteins were separated by SDS-PAGE essentially by the method of Laemmli (1970) on 9% or 15% gels with a 5% stacking gel [2.6% bis(acrylamide)/acrylamide]. All gels were fixed in 50% methanol/10% acetic acid for 30 min prior to staining with Coomassie Brilliant Blue-G (in 45% methanol/10% acetic acid).

Proteolytic mapping of the AchR subunits with *S. aureus* V8 protease was performed according to the procedure of Cleveland et al. (1977) and as described by Pederson et al. (1986). Receptor bands (typically 35 μg for reconstituted AchR) were resolved on a 9% slab gel and visualized by staining overnight and destaining over several hours. The bands were excised and soaked overnight in overlay buffer (125 mM Tris, 5% sucrose, 0.1% SDS, pH 6.8) plus 1 mM DTT. The excised bands were trimmed to 7 mm and transferred to the wells of a mapping gel (15% acrylamide). Between 15 and 20 μL of *S. aureus* V8 protease (3–4 μg) was added to each well followed by 15–20 μL of overlay buffer without DTT. Electrophoresis was carried out at 70-V constant voltage, until the sample reached the end of the stacking gel (typically 3–3.5 h). Electrophoresis was then carried out at 120–140 V until the end of the run.

^{125}I ASA-PS labeled proteins and peptides in stained, dried gels were visualized by autoradiography on preflashed Kodak XAR-5 film. The amount of radioactivity incorporated into particular bands was measured directly by γ counting of

manually sliced gels and indirectly by scanning densitometric analysis of the developed autoradiographic films.

Electroblotting and Sequencing of V8 Protease Cleavage Fragments. AchR subunit fragments from V8 protease digests were resolved on 15% acrylamide gels as described above. Following the electrophoretic run, gels were rinsed in distilled H_2O for 5 min to reduce the amount of SDS, soaked in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3), and electrophoretically transferred to an immobilon P membrane (24 V for 2 h). Membranes were then rinsed in distilled H_2O for 10 min to remove as much glycine as possible. Immobilon P membranes were stained briefly and destained, and selected bands were excised and subjected to N-terminal gas-phase peptide sequencing (UCSF Biomolecular Resource Center).

Results

Photodecomposition of ASA-PS. A photoactive analogue of phosphatidylserine suitable for photoaffinity labeling of the nicotinic acetylcholine receptor was provided by coupling a heterobifunctional photoactivatable compound (NHS-ASA) originally reported by Ji and Ji (1982) to phosphatidylserine to form ASA-PS. This compound was radioiodinated by using the method of Hunter and Greenwood (1962). The synthetic scheme and structures are presented in Figure 1. The UV absorbtion spectra following UV light activation of the aryl azide is presented in Figure 2. The compound exhibits considerable UV sensitivity as illustrated by the decrease in absorption spectra with increasing time of photolysis. Fifty percent of the aryl azide reacts within 72 s, and by 10 min the reaction is essentially complete (Figure 2, bottom).

Partitioning of ^{125}I ASA-PS into AchR Membranes. The phospholipid probe in a chloroform stock solution was dried, dispersed by sonication into an aqueous buffer, and added to either affinity-purified and reconstituted AchR membranes or native AchR-rich membranes, both at a final concentration of 0.15 μM ASA-PS and 0.75 mg/mL protein. After overnight incubation, the equilibrium distribution of the radioactive probe between the aqueous and membrane phase was measured by centrifugation. Typically 50–60% of the probe partitions into AchR membranes by simple incubation of sonicated probes.

^{125}I ASA-PS Labeling of AchR Proteins. To examine the incorporation of ^{125}I ASA-PS into *T. californica* AchR membranes, labeled polypeptides were resolved by electrophoresis on 9% SDS-polyacrylamide slab gels (see Experimental Procedures). In native AchR-rich membranes (Figure 3), all four subunits of the AchR incorporated ^{125}I ASA-PS. In addition to the AchR subunits, 95-kDa protein identified as the α subunit of Na^+/K^+-ATPase (White & Cohen, 1988) was also significantly labeled. The 43K (V1) protein was heavily labeled by the phosphatidylserine probe, consistent with this protein associating with the membrane (Porter & Froehner, 1985). Figure 4 shows the results of ^{125}I ASA-PS labeling of AchR reconstituted membranes. Inspection of the autoradiogram reveals that labeled lipid and free photolysis are visible and are migrating in the region of the tracking dye. All four subunits of the AchR reacted with the phosphatidylserine probe. Whereas the β, γ, and δ subunits each incorporated commensurable amounts of ^{125}I ASA-PS, the α subunit incorporated 4 times as much probe (Figure 4, bottom). Accounting for 2 mol equiv of the α subunit, the overall labeling stoichiometry per mole of subunit was $\alpha:\beta:\gamma:\delta$ = 2.36 (±0.20):1.07 (±0.03):1.25 (±0.04):1.0 (n = 4). A similar labeling stoichiometry has been reported for [^{3}H]cholesterol diazoacetate ($\alpha:\beta:\gamma:\delta$ = 2:1:1:2) in which the photogenerated carbene is situated near the lipid–water interface (Middlemas & Raftery, 1987).

Table I: V8 Protease Digestion Fragments of AchR Subunits

fragment mol wt ($\times10^{-3}$)			
α subunit	β subunit	γ subunit	δ subunit
19.1	21.6	23–24.2	21.8
17.0	20.9	14.6	14.6
12.0	19.1	12.6	12.1
10.0	17.6	9.4	11.1
6.7	16.4	7.4	10.0
	13.2		8.6
	12.1		7.0
	9.5		
	8.4		
	7.1		

[a] Principal proteolytic fragments of AchR subunits digested with *S. aureus* V8 protease on a 15% mapping gel (as described in Figure 5, top).

Table II: V8 Protease Digestion Fragments of AchR Subunits Labeled with ^{125}I ASA-PS[a]

α subunit		β subunit	γ subunit		δ subunit	
fragment mol wt ($\times10^{-3}$)	% label[b]	fragment mol wt ($\times10^{-3}$)	fragment mol wt ($\times10^{-3}$)	% label	fragment mol wt ($\times10^{-3}$)	% label
42		34	25.7	16.9	29.7	25.4
34.3			24.0	33.8	26.3	23.5
28.5			14.3	36.2	20.9	13.4
23.8					15.0	18.4
18.7	15				11.1	11.2
17.1	4					
11.7	81					
10.1						

[a] Proteolytic fragments of ^{125}I ASA-PS labeled AchR subunits digested with *S. aureus* V8 protease and run on a 15% mapping gel (as described in Figure 5, bottom). Densitometric analysis of the developed autoradiograph was used to qualitatively assess the relative incorporation into each of the peptides. [b] For the α subunit only those fragments that represent a more complete digestion of the polypeptide were used in determining the relative incorporation of label into each of the peptides.

Addition of AchR agonist carbamoylcholine (1 mM) resulted in no detectable change either in the amount of incorporation into AchR subunits or in the pattern of labeling for both native AchR-rich membranes (Figure 3, lanes 1 and 4) and reconstituted AchR membranes (Figure 4, lane 1).

Mapping of the ^{125}I ASA-PS Labeled Sites on AchR Subunits by V8 Protease. *S. aureus* V8 protease digestion of the AchR subunits provided a reproducible set of bands on a 15% SDS-polyacrylamide gel (Figure 5, top; Table I) with apparent molecular weights of 18 000–20 000 (V8–20), 17 000–19 000 (V8–18), 10 000–12 000 (V8–10, V8–12), and 4000–7000 (V8–7), consistent with previous reports (Gullick et al., 1981; Neumann et al., 1985; Pederson et al., 1986; White & Cohen, 1988). The V8 protease fragments are reproducibly found under varying amounts of enzyme to substrate levels (Pederson et al., 1986) and together represent the entire amino acid sequence of the α subunit. Fragment V8–20 contains the agonist/antagonist binding site in a peptide beginning at Ser-173. V18 contains the N-linked carbohydrate sites of the α subunit and begins at Thr-52. A variable fragment region exists between 10 000 and 12 000 daltons (V8–10, V8–12); the 10-kDa fragment begins at Asn-339. *S. aureus* V8 protease itself can be seen migrating in the 9% gel with an apparent molecular weight of 31 000.

S. aureus V8 protease digestion was used to map the sites of ^{125}I ASA-PS labeling on each of the AchR subunits. Inspection of the autoradiogram of the V8 protease digest (Figure 5, bottom; Table II) reveals a number of labeled fragments for each of the receptor subunits. In the α subunit

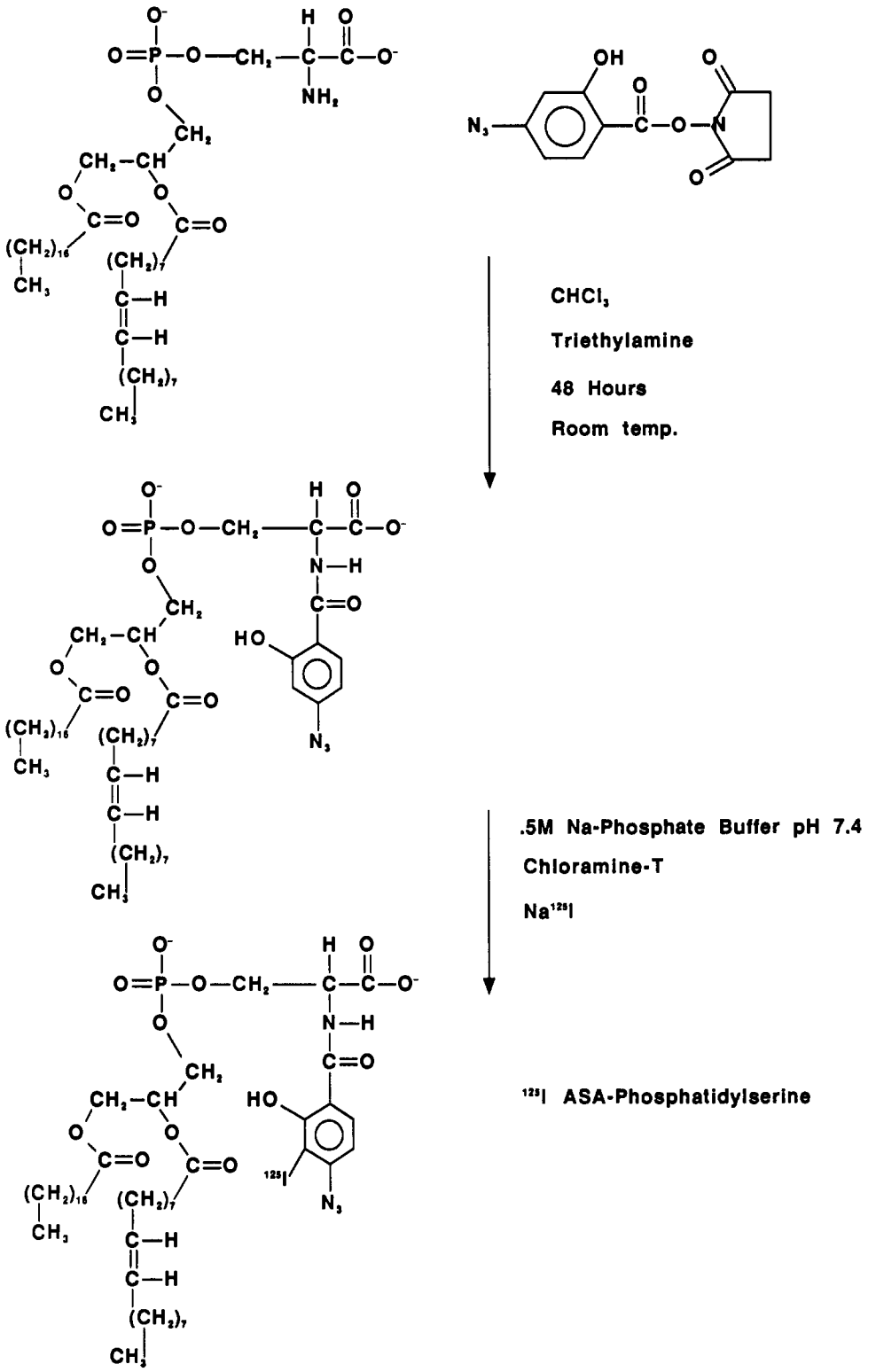

FIGURE 1: Schematic representation of the synthesis of ASA-PS and ^{125}I ASA-PS. $CHCl_3$, chloroform.

(Figure 5, bottom, lane 4), a number of bands incorporating ^{125}I ASA-PS are evident. The uppermost bands represent partial digestion products of the α subunit. Of the fragments representing a more complete digestion of the α subunit are peptides of 18.7, 17.1, 11.7, and 10.1 kDa. Densitometric analysis of the developed autoradiogram was used to qualitatively assess the incorporation of label into each of the labeled peptides which represent a more complete digestion of the α subunit. The majority of label (18%) is contained in the 11.7-(V8-12) and 10.1-kDa, (V8-10) fragments. The 18.7-kDa (V8-20) fragment accounts for 15% of the incorporated label. Interestingly, the 17.1-kDa (V8-18) fragment incorporated only a small amount of label (4%), while no detectable amount of label is contained within a 6.7-kDa (V8-7) fragment. The labeling pattern for each of the four AchR subunits is summarized in Table II.

Amino-Terminal Sequencing of the α Subunit 11.7-kDa V8 Protease Cleavage Fragment. To identify the position of the heavily labeled 11.7-kDa V8 cleavage fragment in the primary structure of the α subunit, the unlabeled peptide was isolated as described under Experimental Procedures and subjected to N-terminal gas-phase peptide sequencing (UCSF Biomolecular

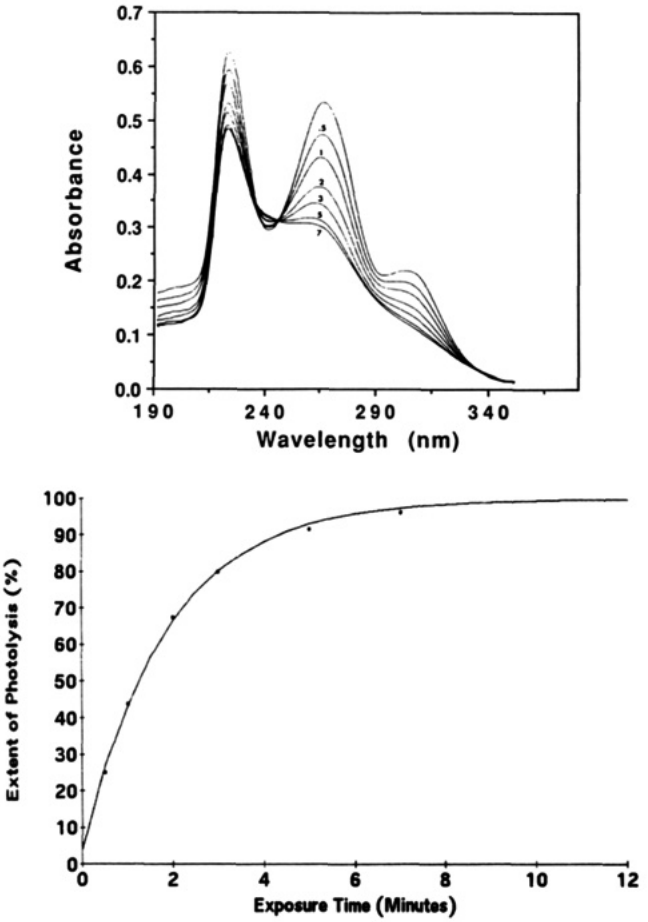

FIGURE 2: (Top) Photodecomposition of ASA–PS. A 22 μM aqueous solution of ASA–PS was irradiated by placing the solution in a quartz cuvette 1 cm from a mineralight UV-25 lamp on the long-wavelength setting (366 nm). The ultraviolet spectra were recorded following the indicated time periods (minutes). (Bottom) Time course of ASA–PS photolysis in aqueous solution.

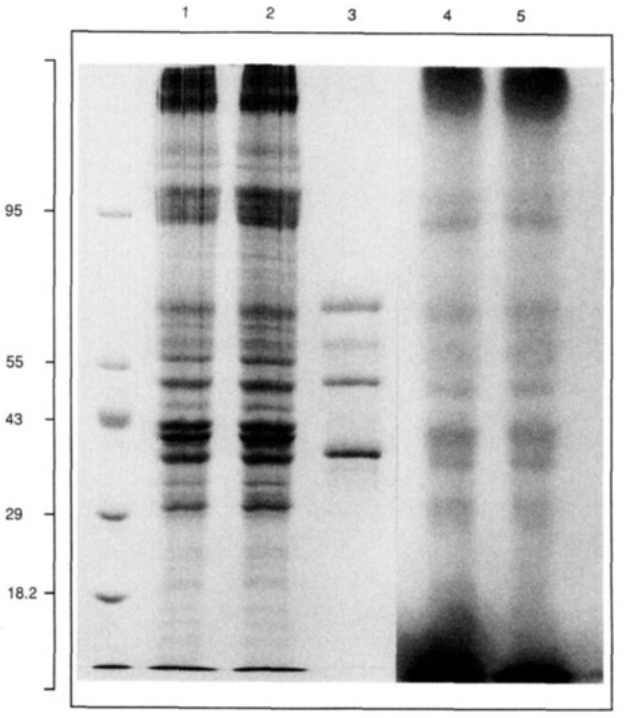

FIGURE 3: Photoincorporation of ^{125}I ASA–PS into native AchR-rich membranes in the presence and absence of cholinergic agonist. AchR-rich membranes were labeled with ^{125}I ASA–PS in the presence (lanes 1, 4) and in the absence (lanes 2, 5) of carbamoylcholine (1 mM). After photolysis, membranes were pelleted by centrifugation (see Experimental Procedures) and subjected to SDS–PAGE on 9% acrylamide gels. Lanes 1 and 2 are Coomassie Blue-G stained bands. Lanes 4 and 5 represent the autoradiogram of lanes 1 and 2. Affinity-purified AchR subunits are present in lane 3 to help identify native AchR-rich receptor subunits. Molecular weight markers are indicated on the left for phosphorylase *b* (95 000), glutamate dehydrogenase (55 000), ovalbumin (43 000), lactate dehydrogenase (36 000), carbonic anhydrase (29 000), and β-lactglobulin (18 400). Material migrating at the top of the gel represents protein not resolved in the separating gel and is likely a consequence of chemical cross-linking.

Resource Center). The first 14 cycles of the amino-terminal sequence yielded

Asn-Lys-Ile-Phe-Ala-Asp-Asp-Ile-Asp-Ile-(Thr, Ser)-Asp-X-Thr

Examination of the amino acid sequence of the *T. californica* α subunit indicates that the 11.7-kDa fragment begins at Asn-339. The molecular weight of the fragment indicates its carboxy terminus is close to the α subunit's carboxy terminus (Gly-437). This is the same start position as that reported for the 10.0-kDa V8 protease cleavage fragment (White & Cohen, 1988).

Discussion

In the present studies we have provided an initial characterization of the labeling of AchR membranes with a photoactivatable analogue of phosphatidylserine, ^{125}I ASA–PS. Upon photolysis ^{125}I ASA–PS reacts with both lipid and membrane proteins. All four subunits of the AchR are labeled in native AchR-rich membranes. In addition to AchR subunits, a protein with an apparent molecular weight of 95 000 was labeled by ^{125}I ASA–PS. This 95-kDa protein has been identified as the α subunit of the Na^+/K^+-ATPase. The significant incorporation of ^{125}I ASA–PS into the 43K protein is in contrast with only limited labeling of this protein observed with probes for the hydrophobic regions of the membrane (Giraudat et al., 1985; Cohen & White, 1988). The increased labeling of the 43K protein by ^{125}I ASA–PS can be explained by the particular properties of the ASA–PS probe. The photoactivatable group of ^{125}I ASA–PS is attached directly to the phospholipid head group and thus is an excellent probe of protein regions in contact with lipid head groups. Furthermore, the 43K protein has been shown to associate with lipid membranes in the absence of protein (Porter & Froehner, 1985). The difference in labeling affinity between ^{125}I ASA–PS and hydrophobic probes suggests that the 43K protein associates with membranes primarily through interactions with the polar head group. This is not to say that the 43K protein interacts solely with the lipid; it may also interact with cytoplasmic regions of at least the AchR (Toyoshima & Unwin, 1988).

In reconstituted AchR membranes, all four subunits react with the phosphatidylserine probe. The α subunit incorporates approximately 4 times as much label as each of the other subunits. Accounting for the 2 mol equiv of the α subunit, the labeling stoichiometry per mole of subunit is $\alpha:\beta:\gamma:\delta$ = 2:1:1:1. The labeling pattern for ^{125}I ASA–PS was unaffected by the addition of cholinergic agonist carbamoylcholine (1 mM) for either reconstituted or native AchR-rich membranes. Similar results have been observed for photoactivatable analogues of cholesterol (Middlemas & Raftery, 1987) and phosphatidylcholine (Giraudat et al., 1985). A large-scale conformational change in the AchR protein upon binding of agonist in which the sites of protein–lipid interactions change

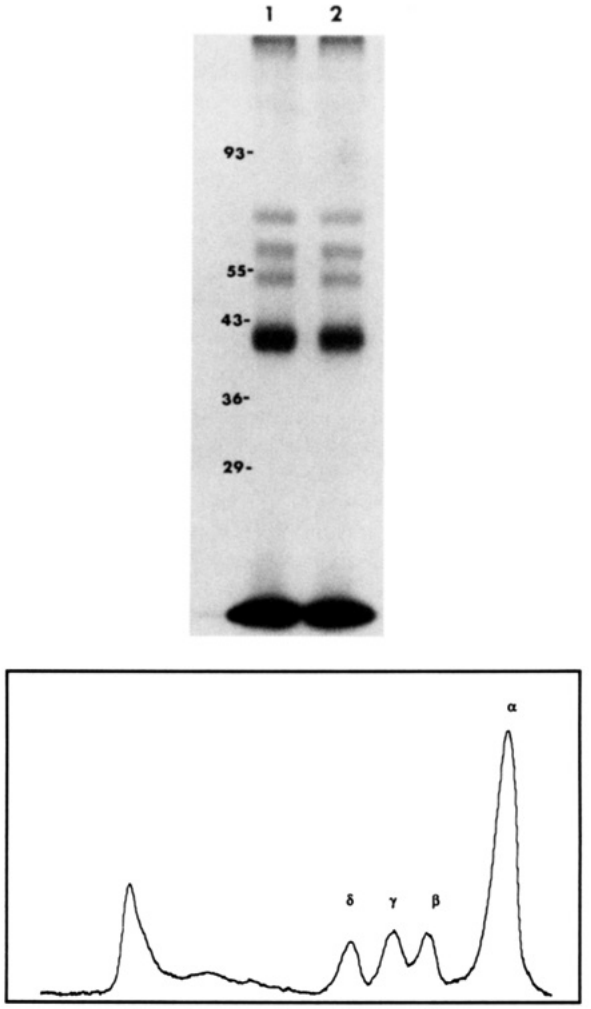

FIGURE 4: (Top) Photoincorporation of ^{125}I ASA–PS into reconstituted AchR membranes in the presence and absence of carbamoylcholine. Affinity-purified and reconstituted AchR membranes were labeled with ^{125}I ASA–PS in the presence (lane 1) or absence (lane 2) of 1 mM carbamoylcholine. After photolysis, membranes were pelleted by centrifugation (see Experimental Procedures) and subjected to SDS–PAGE on 9% polyacrylamide gels. The autoradiograph of the stained, dried gel is shown in lanes 1 and 2 (24-h exposure). The AchR subunits are identified on the right. Migration of molecular weight markers (Diversfied Biotech) is indicated on the left for phosphorylase *b* (95 000), glutamate dehydrogenase (55 000), ovalbumin (43 000), lactate dehydrogenase (36 000), and carbonic anhydrase (29 000). Material migrating at the top of the gel represents protein not resolved in the separating gel and is likely a consequence of chemical cross-linking. (Bottom) Densitometer recording of the autoradiograph in lane 2. The densitometer recording extends from the top of the separating gel to just past the α subunit. Integration of the subunit peak (data not shown) indicates that the percentage of the total area under each subunit peaks is (α) (60%, (β) 13%, (γ) 15%, and (δ) 12%, yielding a labeling stoichiometry of roughly α:β:γ:δ = 2:1:1:1 on a mole per mole basis.

dramatically does not appear evident on the basis of the present evidence. This observation is supported by collateral evidence. Fluorescence and chemical reactivity experiments (Witzemann & Raftery, 1979; Oswald & Changeux, 1981; Otero & Hamilton, 1984) as well as tritium-exchange experiments (McCarthy & Stroud, 1989) suggest that there is not a large conformational shift to a different conformation upon binding of agonist. In reconstructed electron microscopy images (18-Å resolution) both conformations have structures that appear very similar (Unwin et al., 1988). In view of the evidence that the agonist binding site and the binding site for noncompetitive inhibitors are between 20 and 40 Å apart (Herz et al., 1989), the binding of agonist may exert a global subtle conformational

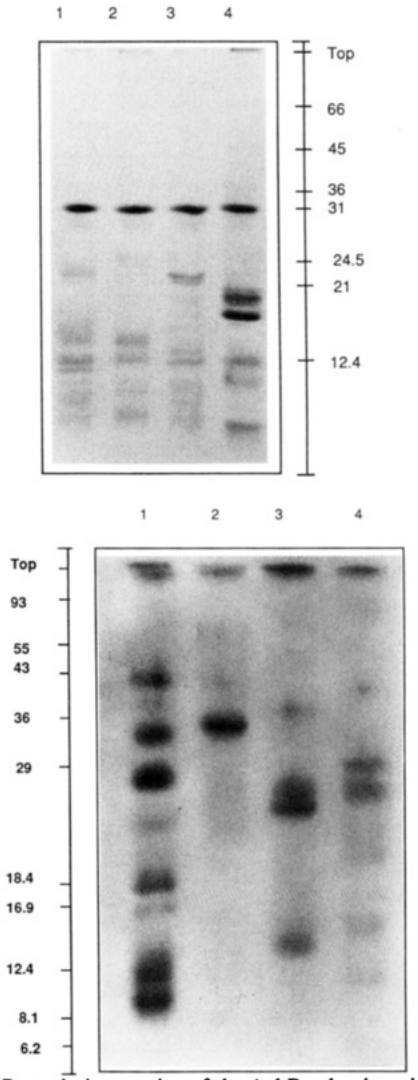

FIGURE 5: Proteolytic mapping of the AchR subunits using *S. aureus* V8 protease. Affinity-purified and reconstituted AchR membranes were subjected to SDS–PAGE on 9% polyacrylamide slab gels. The gel was then stained overnight in Coomassie Blue-G and destained over several hours, and the α, β, γ, and δ bands were excised. The bands were soaked overnight in overlay buffer with the addition of 1 mM DTT (see Experimental Procedures) and then transferred to the well of a 15% mapping gel and overlaid with 3–4 μg of *S. aureus* V8 protease. Following electrophoresis, the mapping gel was staining with Coomassie Blue-G, destained, and dried (lanes 1–4, δ, γ, β, α, respectively). The principal proteolytic fragments for each of the subunits are identified in Table I. *S.* Aureus V8 protease itself can be seen migrating in the 9% gel with an apparent molecular weight of 31 000. Migration of molecular weight standards (Sigma, Diversified Biotech) is indicated on the left for bovine serum albumin (66 000), ovalbumin (43 000), trypsinogen (24 000), trypsin inhibitor (21 000), and cytochrome *c* (12 400). (Bottom) Proteolytic mapping of the ^{125}I ASA–PS incorporation sites in the AchR subunits using *S. aureus* V8 protease. Affinity-purified and reconstituted AchR membranes were labeled with ^{125}I ASA–PS as described (see Experimental Procedures). Labeled membranes were subjected to SDS–PAGE on 9% polyacrylamide slab gels. The gels were stained and destained, and the α, β, γ, and δ subunits were excised. The excised bands were then transferred to the wells of a 15% mapping gel and overlaid with 3–4 μg of *S. aureus* V8 protease. Following electrophoresis, the gels were stained overnight, destained, and dried. An autoradiograph (3–5-day exposure typically) of the 15% *S. aureus* V8 protease mapping gel (lanes 1–4, α, β, γ, δ, respectively) is shown. The principal labeled fragments for each of the AchR subunits are described in Table II. Migration of molecular weight standards (Diversified Biotech) is indicated on the left for phosphorylase *b* (93 000), glutamate dehydrogenase (55 000), ovalbumin (43 000), LDH (36 000), carbonic anhydrase (29 000), and cytochrome *c* (12 400) as well as cleavage fragments of myoglobin (Sigma) (16 900, 8100, and 6200).

change in the AchR protein leading to channel activation. Channel activation may simply involve the concerted movement of electroattractive and/or electrorestrictive residues into or out of the mouth of the ion channel. It has been proposed that negatively charged amino acids located at the periphery of the M2 membrane spanning helix of each of the receptor subunits form "rings" of negatively charged residues which are major determinants of the rate of ion transport (Imoto et al., 1988). Adjacent to these negatively charged residues are conserved positively charged amino acids which are considered to be equally important. These positively charged residues are situated at each end of the channel mouth and may be important in forming a "charge gate". In the resting and desensitized state of the receptor, rings of positively charged amino acids would provide a barrier to the passage of cations and cationic anesthetics. The channel's cation selectivity would then be attributable to the presence of negatively charged residues and channel gating to the presence or absence of positively charged residues near the channel mouth.

Recently Cohen and White (1988) observed agonist-sensitive binding to the AchR with the hydrophobic probe 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazarine (^{125}I TID). The agonist-sensitive incorporation into the α subunit was restricted to a 20 kDa *S. aureus* V8 protease fragment which is of sufficient length to contain the transmembrane regions M1, M2, and M3. Three-fifths of the incorporation into this region was inhibited by the addition of 100 μM carbamoylcholine. However, 15% of the incorporation could not be abolished by increasing concentrations of agonist. One interpretation of these observations is that ^{125}I TID is labeling a hydrophobic pocket of the AchR that undergoes a substantial agonist-induced conformational change but which is not directly accessible to phospholipids. The labeling pattern of ^{125}I TID within the α subunit in the presence of agonist is consistent with the labeling pattern observed with ^{125}I ASA–PS, suggesting this incorporation is at regions in contact with the lipid membrane.

Negatively charged phospholipids and sterols have been shown to be required for both receptor conformational changes and ion channel gating (Ochoa et al., 1983; Schindler, 1983; Criado et al., 1984; Fong & McNamee, 1986; McNamee et al., 1986). For this reason, one of our principal interests in labeling the AchR with ^{125}I ASA–PS was to identify regions of the AchR subunits that specifically interact with negatively charged phospholipids. As an initial approach, we mapped the sites of ^{125}I ASA–PS incorporation into receptor subunits digested with *S. aureus* V8 protease. In the α subunit, the majority of the label was localized in the 11.7- and 10.7-kDa V8 cleavage fragments. N-Terminal sequencing of the 11.7-kDa fragment indicates it has the same amino-terminal start as previously determined for the 10-kDa fragment (White & Cohen, 1988). The two fragments therefore represent essentially the same stretch of polypeptide and are of sufficient length to contain the proposed transmembrane spanning regions M4 (Noda et al., 1982) and MA (or M5) (Finer-Moore & Stroud, 1984; Guy, 1984). Fifteen percent of the total label incorporated into the α subunit was localized to an 18.7-kDa V8 cleavage fragment previously demonstrated as beginning at Ser-173 (White & Cohen, 1988). The carboxy terminus of the labeled fragment is believed to be Glu-338, such that the peptide is of sufficient length to contain the transmembrane regions M1, M2, and M3. The 17.1- and 6.7-kDa V8 cleavage fragments incorporated little or no detectable amount of ^{125}I ASA–PS. Together, these two fragments represent roughly a third of the amino-terminal portion of the α subunit. This

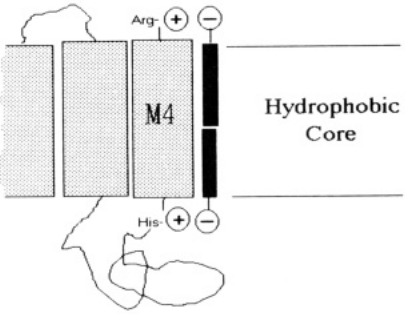

FIGURE 6: Model of phosphatidylserine binding to M4 transmembrane spanning region of the α subunit.

labeling pattern remains consistent with the hydrophobic regions proposed to span the membrane (Noda et al., 1982; Finer-Moore & Stroud, 1984; Guy, 1984).

Significant incorporation of ^{125}I ASA–PS into the V8 protease fragments of 11.7 and 10.1 kDa of the α subunit is consistent with the labeling patterns observed with the hydrophobic probe ^{125}I TID (White & Cohen, 1988) and with the selective labeling of a COOH-terminal tryptic of the α subunit of *T. mamarota* with a photoaffinity analogue of phosphatidylcholine (Giraudat et al., 1985). Similar labeling patterns observed with each of these three distinct probes suggest that a stretch of the α subunit polypeptide chain contained within Asn-339 and Gly-437 is in contact with both the hydrophobic core of the membrane and with the phospholipid head groups. One explanation is that each of these probes is interacting with a region of transmembrane spanning region. On the basis of the selective labeling of a COOH-terminal tryptic fragment of the α subunit of *T. mamarota* vs *T. californica*, and the presence of a single amino acid substitution in this region, Giraudat et al. (1988) proposed that a photoactivatable analogue of phosphatidylcholine labels Cys-424 of the M4 membrane spanning region in *T. mamarota*. A number of observations suggest that M4 is in contact with the lipid bilayer [see Popot and Changeux (1983)]. M4 is the least conserved of the proposed transmembrane spanning regions, suggesting it is in contact with solvent (lipid) rather than solely with other regions of the polypeptide chain. M4 is also the most hydrophobic of the membrane spanning regions, exhibiting a face that is virtually devoid of hydrogen-bonding groups. In addition, it has been determined that a large segment of the M4 region must be present for expression of functional AchR molecules on the cell surface (Tobinmatsu et al., 1987), a finding that suggests that M4 traverses the membrane or at least is integrally associated with it.

The negatively charged head group of phosphatidylserine suggests a likely interaction with positively charged amino acids. Two positively charged amino acids, Arg-429 and His-408, are located at the periphery of the M4 transmembrane spanning region in the α subunit. These charged amino acids occupy positions located at the level of the phospholipid head groups in proposed transmembrane organization models of the mature AchR subunits (Noda et al., 1982). Positively charged residues provide a suitable interaction site for the negatively charged head group of phosphatidylserine (Figure 6). Interaction of the phosphatidylserine head group with Arg-429 is entirely consistent with the proposed labeling site of a photoactivatable analogue of phosphatidylcholine (Giraudat et al., 1985) to Cys-424 in *T. mamarota*. The phosphatidylcholine analogue employed in these studies contained an arylazido group substituted for one of the fatty acid chains and thus is a superficial probe of the membrane hydrophobic region. Labeling of Cys-424 is therefore consistent with the

zwitterionic phospholipid head group interacting with amino acids slightly removed and in the region of Gly-428 or Arg-429. Interaction of the negatively charged head group of phosphatidylserine with positively charged amino acids may serve to stabilize the transmembrane helix in the bilayer. This stabilization may facilitate agonist-induced conformational changes in the receptor, hence the requirement of negatively charged phospholipids for AchR function.

Incorporation of ^{125}I ASA-PS into the 18.7-kDa V8 cleavage fragment is likely to occur in an analogous fashion as in M4. Positively charged amino acids are present at the periphery of the transmembrane regions M1, M2, and M3 contained within this fragment.

In conclusion, our results demonstrate that ^{125}I ASA-PS can be used to identify regions of the AchR that interact with phosphatidylserine molecules. More detailed mapping of the regions of ^{125}I ASA-PS incorporation in the α subunit, as well as identification of the labeling sites on the other subunits, should provide important structural and functional information about the AchR.

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Registry No. ASA-PS, 124155-77-7; ^{125}I ASA-PS, 124155-78-8; PS, 124262-93-7; NHS-ASA, 96602-46-9.

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A Synthetic Peptide Corresponding to Human FSH β-Subunit 33-53 Binds to FSH Receptor, Stimulates Basal Estradiol Biosynthesis, and Is a Partial Antagonist of FSH†

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ABSTRACT: We have previously shown that hFSH-β 34-37 (KTCT) and 49-52 (TRDL) inhibit binding of ^{125}I-hFSH to FSH receptor in calf testis membranes and that hFSH-β 33-53, which encompasses these tetrapeptides, inhibits binding with increased potency. hFSH-β 33-53 rapidly dimerizes under conditions utilized in the receptor binding assay (pH 7.5) so that the binding inhibition reported earlier was due to the hFSH-β 33-53 dimer rather than the monomer. At pH 6.5, conversion to dimer does not occur, and binding inhibition could be unequivocally attributed to the monomer. Radioiodinated and alkylated hFSH-β 33-53 binds to the FSH receptor with a $K_d = (5.5 \pm 1.4) \times 10^{-5}$ M. The biological activity of hFSH-β 33-53 was assessed by its ability to affect the conversion of androstenedione to estradiol in rat Sertoli cells cultures. FSH-β 33-53 behaved as a partial antagonist of the FSH-induced estradiol synthesis. The required incubation medium, however, contains cystine as well as cysteine, which rapidly forms a hFSH-β Cys(51)-S-S-Cys derivative at the pH of the incubation, 7.4. When hFSH-β 33-53 was converted either to the hFSH-β Cys(51)-S-S-Cys or to a carboxymethylated derivative, inhibition of FSH-induced estradiol synthesis still was observed. This result demonstrates that the free R-SH group at Cys51 is not responsible for the inhibition. FSH-β 33-53 also significantly stimulated basal levels of estradiol synthesis, but not to maximal levels observed with FSH (partial agonist). Neither the carbohydrate content of hFSH-β nor the α subunit of FSH appears to be essential for signal transduction and expression of the hormone effect of FSH-β 33-53.

Follitropin (FSH), a pituitary glycoprotein hormone composed of two nonidentical subunits, plays an important role in ovulation in the female and spermatogenesis in the male. In an earlier study (Sluss et al., 1986), we reported inhibition of ^{125}I-hFSH binding to testicular FSH receptors by synthetic tetrapeptides corresponding to amino acids 34-37 (TRDL) and 49-52 (KTCT) of the β subunit of human follitropin (FSH). A subsequent report (Schneyer et al., 1988) concluded that the interaction of FSH with its receptor may involve multiple, discrete binding determinants, which included hFSH-β 34-37 (TRDL). In a preliminary report (Andersen et al., 1987), we noted that a synthetic peptide encompassing TRDL and KTCT, and representing hFSH-β 33-53 (H_2N-YTRDLVYKNPARPKIQKTCTF-$CONH_2$) (Pierce & Parsons, 1981), also inhibited ^{125}I-hFSH binding to receptor, at a potency significantly greater than that seen with either individual tetrapeptide. The presence of cystine at position 51 caused us to suspect the observed effect might be due, in part at least, to a dimer of the peptide. We report here the results of our studies on the receptor binding properties of monomer-dimer forms of hFSH-β 33-53, and on the ability of each form, as well as various cysteine-51 derivatives, to affect steroidogenesis in cultured rat Sertoli cells. Our results suggest hFSH-β 33-53 binds to the FSH receptor in a functional manner, affecting both basal and FSH-stimulated steroidogenesis. They also imply that neither carbohydrate content nor the FSH-α subunit is absolutely essential for FSH signal transduction.

Experimental Procedures

Materials. Highly purified human FSH (LER-1781-2, 4000 IU/mg) was radioiodinated and used as the radioligand in the radioreceptor assay. Purified ovine FSH (LER-1996-S, 8 NIH-FSH-S1 units/mg) was used in the Sertoli cell culture assay. Iodoacetamide, cysteine, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). All solvents and buffers utilized in the chromatographic procedures were HPLC-grade reagents.

Peptide Synthesis, Purification, and Characterization. The synthetic peptide corresponding to amino acids 33-53 of hFSH-β subunit was prepared by Dr. Jean Rivier, Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, San Diego, CA, under Contract No1-HD-7-2907 from the Contraceptive Development Branch, Center for Population

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