Biochimica et Biophysica Acta, 1067 (1991) 1–8

ADONIS 000527369100288O

BBAMEM 75295

Localization of regions of the *Torpedo californica* nicotinic acetylcholine receptor labeled with an aryl azide derivative of phosphatidylserine

Michael P. Blanton and Howard H. Wang

Department of Biology, University of California, Santa Cruz, CA (U.S.A.)

(Received 17 January 1991)

Key words: Membrane topology; Probe; Nicotinic acetylcholine receptor; Acetylcholine receptor; Phosphatidylserine; Cyanogen bromide; (*T. californica*)

A photoactivatable analog of phosphatidylserine, ^{125}I-labeled 4-azidosalicylic acid-phosphatidylserine (^{125}I-ASA-PS) (Blanton, M. and Wang, H.H. (1990) Biochemistry 29, 1186–1194) was used to label the nicotinic acetylcholine receptor. The photoactivatable group of ^{125}I-ASA-PS is attached directly to the phospholipid head group making it an excellent probe of regions of the AchR structure in contact with the negatively-charged head group of phosphatidylserine. The 'binding domains' were localized by chemically cleaving the labeled receptor with cyanogen bromide (CNBr), separating the generated peptides by reverse-phase HPLC, and N-terminal sequence analysis of radiolabeled material. CNBr fragments containing flanking regions of the transmembrane spanning region M4 as well as within M3 were identified within HPLC separated radiolabeled material. The results suggest a topological arrangement of the transmembrane helices in which the hydrophobic faces of M3 and M4 form the boundary of the receptor complex in contact with the lipid bilayer.

The nicotinic acetylcholine receptor (AchR) is one of the best characterized membrane-bound allosteric proteins; it carries the cholinergic binding sites, contains the ion channel and mediates the conformational transitions responsible for the regulation of ion translocation [2–5]. The AchR is a heterologous pentamer made up of four different though homologous subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ [6]. Each of the subunits span the membrane [7] and are thought to contribute structurally to form the ion channel.

The complete primary structure of each of the receptor subunits has been established by cDNA cloning and sequencing in *Torpedo californica* [8–11]. Models for the transmembrane organization of the mature subunits have been proposed based on these sequences [10–14].

In line with its transmembrane organization the AchR has been shown to be quite sensitive to the lipid environment [15–18]. While it has been established that an appropriate membrane fluidity is necessary for receptor state transitions [18], the additional presence of both sterol and negatively charged phospholipids are required for both receptor state transitions and ion channel gating [15,16,18]. The specific requirement for negatively charged phospholipids such as phosphatidylserine indicates that interactions between the negatively charged phospholipid head group and regions of the AchR structure are important for receptor function.

Photoactivatable analogs of cholesterol [19] and phosphatidylserine [1] have been used to demonstrate a direct interaction with all four receptor subunits. Both analogs are probes of protein regions in contact with the lipid head group area with the photosensitive group attached directly to the phosphatidylserine head group and for the cholesterol analog to the β-hydroxyl group. Not surprisingly, the labeling stoichiometry of receptor subunits (α, β, γ, δ) is very similar, 2:1:1:1 for phosphatidylserine (obtained from densiometric scans of autoradiographs) and 2:1:1:2 for the cholesterol analog on a per mole basis. The binding site domains on the AchR subunits for the phosphatidylserine analog have been localized by *Staphylococcus aureus* V8 proteinase mapping. The majority of label

Abbreviations: AchR, nicotinic acetylcholine receptor; ^{125}I-ASA-PS, ^{125}I-labeled 4-azidosalicylic acid-phosphatidylserine; V8, *Staphylococcus aureus* V8 proteinase; CNBr, cyanogen bromide.

Correspondence: H.H. Wang, Department of Biology, University of California, Santa Cruz, CA 95064, U.S.A.

incorporated in the α-subunit is localized to a region of the polypeptide chain extending from Asn-339 to the carboxy-terminus of the subunit and which contains the putative transmembrane region M4 [9,10] as well as MA [13,14]. In addition, the region between Ser-173/Ser-162 and Glu-338 which contains the transmembrane regions M1, M2, and M3 also incorporated significant amounts of label.

An important first step in determining how the interaction of the negatively charged phospholipid head group with regions of the AchR structure affects receptor function is locating the helical regions of the subunit structure that interact with the phospholipid head group. It is interesting to us to determine whether ^{125}I-ASA-PS interact with membrane domains (M1–M4) of the receptor preferentially. In the following paper we have utilized chemical cleavage of the labeled AchR with cyanogen bromide and fragment separation by reverse-phase high performance liquid chromatography (HPLC) to further resolve the binding site domains on the AchR subunits for the photoactivatable analog of phosphatidylserine ^{125}I-ASA-PS. The results indicate that ^{125}I-ASA-PS is not only a useful probe of protein regions in contact with the negatively charged head group of phosphatidylserine, but also of the topology of the AchR transmembrane domains.

Materials and Methods

^{125}I-4-Azidosalicylic acid-phosphatidylserine (^{125}I-ASA-PS; Fig. 1), (60 Ci/mmol) was prepared as described earlier [1]. Cyanogen bromide (CNBr) was purchased from Eastman Kodak. All solvents used were of HPLC grade.

Photolabeling of the AchR by ^{125}I-ASA-PS

Photolabeling of affinity purified and reconstituted AchR membranes was achieved as described earlier [1]. Briefly, an aliquot of the iodinated probe in a chloroform stock was removed, dried and resuspended by sonication into an aqueous buffer. The lipid probe suspension was added to AchR membranes (approx. 8 nmol of α-BgTx binding sites) to a final concentration of 3 μM probe and 2 mg/ml protein. The membrane suspension was stirred slowly, overnight at 4°C and any probe that had not partitioned into the membrane is then removed by centrifugation. The resuspended pellet was photolyzed for 10 min at 365 nm using a hand-held UVSL-25 lamp on the long-wave setting.

Photolyzed membranes were pelleted by centrifugation and the lipid extracted by adding 20 volumes of methanol/chloroform (2:1, v/v) to 1 volume of photolyzed membranes in a glass centrifuge tube. The mixture was shaken intermittently for 2 h, centrifuged at high speed in a clinical centrifuge and the supernatant removed. The pellet was extracted once more and the final pellet dried with a stream of N_2 gas.

Chemical cleavage of the AchR

The dried, lipid extracted AchR subunits were dissolved in 70% formic acid (2 mg/ml protein) and CNBr added to a final concentration of 0.07 M. The mixture in a glass stoppered vial was flushed with N_2 gas and incubated for 24 h at room temperature and in the dark. In subsequent runs CNBr was added to a concentration of 0.05 M incubated for 24 h, fresh CNBr added to a final concentration of 0.1 M and incubated for an additional 24 h. The digest was then diluted with 5–10 volumes of water and lyophilized.

Purification of CNBr peptides

The lyophilized CNBr pellets were resuspended in a small volume of formic acid and then diluted with 5 volumes of 0.1% trifluoroacetic acid/6.0% 2-propanol/3% acetonitrile. The suspension was centrifuged and the supernatant subjected to reverse-phase HPLC on a Bio-Rad RP-318 C18 column (10 × 250 mm) as detailed in the figure legends. Selected peaks of radiolabeled material were lyophilized and subjected to N-terminal gas-phase peptide sequencing (Protein Structure Lab, Davis, CA). To justify our methods and to ascertain that the sequenced peptides are indeed radiolabeled, the specific radioactivity of the purified fractions were examined in preliminary studies under identical conditions; the yields of sequenced peptides were consistent with the amount of radioactivity in the injected fraction.

Results

Affinity purified and reconstituted AchR membranes were photolabeled with ^{125}I-ASA-PS (Fig. 1) as described in an earlier paper [1]. Labeled AchR subunits were separated from lipid and unreacted probe by chloroform/methanol extraction as detailed in the Materials and Methods.

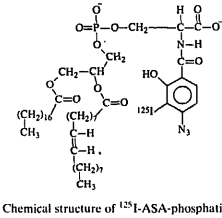

Fig. 1. Chemical structure of ^{125}I-ASA-phosphatidylserine.

Fractionation of CNBr digest

AchR subunits (approx. 1 mg) extracted from photolyzed membranes were chemically digested with CNBr. The lyophilized CNBr digest was completely solubilized in a small volume of 100% formic acid and then diluted with 5 volumes of HPLC starting solvent: 0.1% TFA/6% 2-propanol/3% acetonitrile/water for sample injection.

Analysis of the CNBr digest by reverse-phase HPLC yielded the chromatograph in Fig. 2. Approx. 7% of the injected radioactivity was associated with unbound material. Peaks of radioactivity eluting at 29.16% (IA) and 47.2% (IIA) solvent B were selected for further characterization.

Characterization of ^{125}I-ASA-PS labeled material in peak IA

As shown in Fig. 2, the radioactivity present in Peak IA comprises a sharp rising peak of ^{125}I-iodine (fractions 38–40). Sequence analysis of the pool of fractions 38–40 revealed three amino terminal sequences (Table I). The major sequence (Gly-Asn-Phe...) corresponds to a CNBr fragment of the δ-subunit beginning at Gly-476 (cleavage at Met-475) and on the basis that there are no further CNBr cleavage sites (Met), extends to the carboxy terminus of the subunit (Ala-501). Also present in the radiolabel-containing pool was a minor sequence (Lys-Leu...) corresponding to a CNBr fragment of the α-subunit beginning at Lys-145 (cleavage at Met-144) as well as a sequence which could not be unambiguously aligned with the known primary structures of *T. californica* subunits (Noda et al. (1983)).

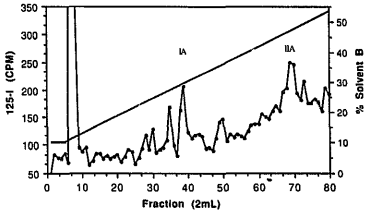

Fig. 2. Reverse-phase HPLC chromatograph of CNBr digests of the ^{125}I-ASA-phosphatidylserine-labeled AchR. The AchR subunits (approx. 1 mg) isolated from affinity purified and reconstituted AchR membranes photolabeled with ^{125}I-ASA-PS were cleaved with CNBr. The lyophilized digests were solubilized in a small volume (approx. 30 μl) of formic acid and then diluted with 5 volumes of HPLC equilibration buffer (0.1% trifluoroacetic acid/6% 2-propanol/3% acetonitrile) and the soluble material injected into a Bio-Rad RP-318 (C_{18}) reverse-phase HPLC column (10×250 mm). The column was equilibrated in 90% solvent A (0.1% trifluoroacetic acid)/10% solvent B (0.1% trifluoroacetic acid/60% 2-propanol/30% acetonitrile/10% H_2O) and material eluted at a flow rate of 1.0 ml/min with a gradient of solvent B indicated. 2-ml fractions were taken and directly counted in a gamma emission counter. Material contained in designated peaks IA (fractions 38–40) and IIA (fractions 68–71) were pooled, lyophilized and subjected to sequence analysis. An aliquot of the pool fractions was taken and analyzed by NaDodSO$_4$ polyacrylamide gel electrophoresis on an 18% polyacrylamide gel (see Fig. 3).

The α-subunit fragment beginning at Lys-145 presumably extends to the next available cleavage site at Met-171.

TABLE I

Sequence analysis of material in pools IA and IIA

Material in pools IA (fractions 38–40) and IIA (fractions 68–71) (Fig. 2) were lyophilized and subjected to N-terminal gas-phase peptide sequencing (UC Davis Protein Structure Lab). For each analysis PTH amino acids are designated by the conventional three-letter abbreviations and their yields in picomoles shown in parentheses. The amino terminal starting position in each AchR subunit is indicated near the bottom. n.q. denotes PTH amino acids identified but not quantified.

Cycle	HPLC peak IA Amino terminal sequence			HPLC peak IIA Amino terminal sequence		
	1 (yield)	2 (yield)	3 (yield)	1 (yield)	2 (yield)	3 (yield)
1	Gly (70)	Phe (65)	Lys (25)	Lys (440)	Pro (310)	Phe (210)
2	Asn (20)	Ile (30)	Leu (20)	Arg (180)	Asn (270)	Glu (80)
3	Phe (20)	Ala/Asp/Tyr (20/20/10)	–	(n.d.)	Ala (500)	Lys (60)
4	Ans (16)	Glu/Val (22/10)	–	Ser (240)	Ile (232)	Gln (72)
5	His (4)	Glu (28)	–	Lys (240)	Arg (40)	
6	Pro (n.q.)	Glu (8)	–	Glu (230)	Gln (50)	
7	Pro (n.q.)		Tyr (n.q.)	Lys (240)	Ile (120)	
8	Ala (13)			Gln (240)	Phe (120)	
9	Lys (16)			Glu (150)	Ile (80)	
10	Pro (n.q.)			Asn (120)		
11				Lys (140)		
Amino terminus	(δ-476)	(no fit)	(α-145)	(α-330)	(β-315)	(δ-383)

Characterization of ^{125}I-ASA-PS labeled material in peak IIA

It can be seen in Fig. 2 that in the midst of a rather broad peak of radiolabeled material is a sharp peak eluting at 47.2% solvent B (fractions 68–71) comprising peak IIA. Sequence analysis of the pool fractions revealed three amino-terminal sequences (Table I). A major sequence extending from α-Lys-330 (cleavage at Met-329), a sequence extending from β-Pro-315 (cleavage at Met-314) and a sequence extending from δ-Phe-383 (Met-382).

Three rather high molecular weight bands are apparent on an 18% $NaDodSO_4$ polyacrylamide gel of radiolabeled material contained within peak IIA (Fig. 3, lane 3). Based on an examination of the primary structure of each of the subunits extending from the amino terminus of each of the CNBr fragments and the molecular weight of the bands on an 18% gel, it is evident that these are partial digestion fragments of the AchR which contain internal CNBr cleavage sites (Met). From a comparison of the sequencing yield of each of the fragments and the relative presence of each of the bands on an 18% gel, the bands were tentatively assigned as originating from the β-subunit (18 kDa), α-subunit (14 kDa) and δ-subunit (12 kDa). This assignment is consistent with the fact that only the fragment generated by cleavage at Met-315 of the β-subunit and extending to the carboxy terminus of the subunit is of sufficient mass to generate an 18 kDa band on the 18% polyacrylamide gel. In addition, any fragment generated by internal cleavage of the α-subunit between Lys-330 and the carboxy terminus is of insufficient mass to generate any of the bands on the 18% polyacrylamide gel regardless of the assignment. It is then evident that the CNBr fragments originating from the α- and β-subunits each extend to the carboxy terminus of the subunit, while the fragment originating from the δ-subunit presumably extends to Met-475 with the fragment present in peak IA beginning at Gly-476. The calculated molecular weights for each of these fragments is consistent with the apparent molecular weights of the three bands present on an 18% polyacrylamide gel.

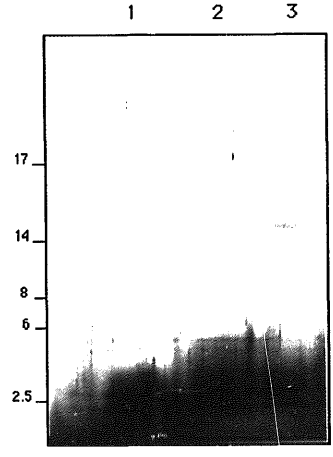

Fig. 3. Analysis of the CNBr digest of ^{125}I-ASA-PS labeled AchR resolved by reverse-phase HPLC and analyzed by SDS-PAGE. An aliquot of pool fractions from the reverse-phase HPLC separation of the CNBr digest of ^{125}I-ASA-PS-labeled AchR subunits was analyzed by electrophoresis in a $NaDodSO_4$ polyacrylamide gel (18% acrylamide), followed by silver staining. Material from HPLC peak IA, fraction 50 and peak IIA are present in lanes 1–3, respectively. The molecular mass standards are myoglobin (17000) and myoglobin fragments (Diversified Biotech) of molecular weight 14400, 8160, 6210, and 2510 daltons.

In subsequent runs labeled receptor subunits were digested with CNBr for a longer period of time in order to ensure more complete cleavage (see Materials and Methods). Examination of the chromatograph of the reverse-phase HPLC run (Fig. 4) reveals it to be extremely similar to the HPLC run shown in Fig. 2. Indeed, peak IVB in Fig. 4 and peak IA in Fig. 2 both contain radiolabeled material eluting at 29.16% solvent B, respectively. Peaks of radiolabeled material eluting at 16.4% (IB), 21.6% (IIB) and 26.2% (IIIB) were selected for further characterization.

Characterization of ^{125}I-ASA-PS material in peak IB

Sequence analysis of the peak radiolabel-containing fraction (fraction 61) in peak IB revealed the presence of two amino terminal sequences. Due to the rapid drop-off in the sequencing yield present in subsequent sequencing cycles the released amino acids could not be unambiguously assigned to either amino terminal sequences. Five possible permutations could be fit to the AchR primary structure (Table III). Four of the five possible fits are contained within the transmembrane spanning region M3 of α, β, γ, δ.

Characterization of ^{125}I-ASA-PS material in peaks IIB and IIIB

Sequence analysis of the peak radiolabel-containing fraction (fraction 86) in peak IIB revealed the presence of three amino terminal sequences (Table II). A major sequence extending from γ-Ile-359 (cleavage at Met-358), a sequence extending from α-Lys-387 (cleavage at Met-386) and a sequence which could not be aligned to the AchR primary structure. Assuming each of the fragments extends to the next CNBr cleavage site, the

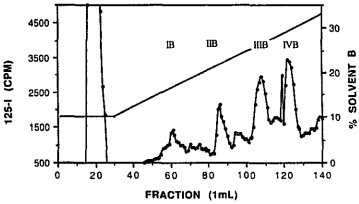

Fig. 4. Reverse-phase HPLC chromatograph of CNBr digest of the ^{125}I-ASA-phosphatidylserine-labeled AchR. The AchR subunits (approx. 1 mg) isolated from affinity purified and reconstituted AchR membranes photolabeled with ^{125}I-ASA-PS were chemically cleaved with CNBr. The lyophilized digests were solubilized in a small volume (approx. 30 μl) of formic acid and then diluted with 5 volumes of HPLC equilibration buffer (0.1% trifluoroacetic acid/6% 2-propanol/3% acetonitrile) and the soluble material injected into a Bio-Rad R-P 318 (C_{18}) reverse-phase HPLC column (10×250 mm). The column was equilibrated in 90% solvent A (0.1% trifluoroacetic), 10% solvent B (0.1% trifluoroacetic acid/60% 2-propanol/30% acetonitrile/10% H_2O). The column was eluted at a flow rate of 1.0 ml/min with an elution gradient of 0.2% solvent B/min. 1 ml fractions were taken and directly assayed by gamma emission counting. Material contained in designated peaks IB (fraction 61), IIB (fraction 86) and IIIB (fraction 107) were lyophilized and subjected to sequence analysis.

fragments extend from γ-Ile-359 to Met-374 and α-Lys-387 to Met-404.

Sequence analysis of the peak radiolabel-containing fraction in peak IIIB (fraction 107) revealed a major sequence beginning at α-Lys-387 (cleavage at Met-386) present in a three-fold greater abundance than two minor sequences. One of the minor sequences was identified as a CNBr cleavage fragment of the δ-subunit beginning at Gly-476 (cleavage at Met-475) while the other minor sequence could not be aligned within the known primary structures of the *T. californica* AchR subunits (Table II). The CNBr fragment of the α-subunit beginning at Lys-387 interestingly is also present in peak IIB and in both cases the fragment presumably extends to the next available cleavage site at Met-404. Similarly, the minor sequence corresponding to a CNBr fragment of the δ-subunit beginning at Gly-475 is also present in peak IA and for reasons described earlier the fragment extends to the carboxy terminus of the subunit.

TABLE III

Sequence and location of material in peak IB

Material in peak IB (Fig. 4) was subjected to N-terminal gas-phase peptide sequencing and two amino terminal sequences were identified. However, the PTH amino acids released in subsequent cycles could not be unambiguously placed to either amino terminal sequences (see text and Table II). In this table possible permutations which can be aligned to the primary structure of AchR subunits are listed. Identified PTH amino acids are underscored for identification and the CNBR cleavage sites indicated by an arrow. The location of each potential CNBR fragment is indicated on the right.

Amino terminal residue	Sequence	Location
	(cleavage site) ↓ ↓	
α279	Met Leu-Phe-Thr Met	M3
β286	Met Phe Ile Met	M3
γ296	Met Leu Ile Val Met	M3
δ294	Met Phe Ile Met	M3
α325	Met Phe Phe Ser Thr Met	between M3 and M4

TABLE II

Sequence analysis of material in pools IB, IIB and IIIB

Material in pools IB (fraction 61), IIB (fraction 86) and IIIB (fraction 107) (Fig. 4) were lyophilized and subjected to N-terminal gas-phase peptide sequencing (UC Davis Protein Structure Lab). For each analysis PTH amino acids are designated by the conventional three-letter abbreviations and their yields in picomoles shown in parentheses. The amino terminal starting position in each AchR subunit is indicated near the bottom (for Peak IB see Table III).

Cycle	HPLC peak IB Amino terminal sequence		HPLC peak IIB Amino terminal sequence			HPLC peak IIIB Amino terminal sequence		
	1 (yield)	2 (yield)	1 (yield)	2 (yield)	3 (yield)	1 (yield)	2 (yield)	3 (yield)
1	Leu (94)	Phe (80)	Ile (115)	Lys (55)	Pro (32)	Lys (95)	–	Phe/Tyr (24/22)
2	Phe (75)/Ile (26)		Lys (110)	Ser (30)	Asp (20)	Ser (80)	Asn (20)	Gly (8)
3	Ser (20)/Thr (11)/Val (8)		Ala (88)	Asp (25)	Gly (16)	Asp (60)	Phe (12)	Ala (n.q.)
4	Thr (25)		Gly (79) *	Glu (79) *	Phe (5)	Glu (40)	Asn (8)	Asp (55)
5	–		Glu (92) *	Glu (92) *	Thr (5)	Glu (62) Ser (25)	His (10)	Asp (7)
Amino terminal	See Table III		(γ-359)	(α-387)	(no fit)	(α-387)	(δ-476)	(no fit)

* The glutamic acid residues in cycles 4 and 5 (peak IIB) are present in two of the sequences hence the yield represents the sum of the two sequences.

Discussion

While an appropriate membrane fluidity is necessary for the AchR to undergo conformational changes associated with receptor state transitions [17], the additional presence of both sterol and negatively charged phospholipids are required for both state transitions and ion channel gating [15–17]. A specific requirement for negatively-charged phospholipids such as phosphatidylserine suggests that interactions between the negatively charged lipid head group and regions of the AchR structure are important for receptor function. One of our principal interests in labeling the AchR with the phosphatidylserine analog ^{125}I-ASA-PS was to localize regions of the AchR primary structure which specifically interact with the negatively-charged head group of phosphatidylserine. With the photoactive group attached directly to the lipid head group of phosphatidylserine, it provides an excellent probe for regions of the polypeptide in contact with the negatively-charged head group of the phospholipid. In an earlier paper [1] the 'binding domains' on the α-subunit were localized by *Staphylococcus aureus* V8 proteinase mapping. The majority of label was localized to V8 cleavage fragments of 11.7 and 10.1 kDa contained within the stretch of the polypeptide chain extending from Asn-339 to the carboxy terminus of the subunit. The proposed membrane spanning region M4 [9,10] as well as the amphipathic helix region MA [13,14] are both contained within this stretch of the polypeptide. An 18.5 (20) kDa V8 cleavage fragment of the α-subunit also incorporated significant amounts of label. This fragment extends from Ser-173/Ser-162 through the membrane spanning regions M1, M2 and M3 and terminates at or near Glu-338 [23].

In the present study we have further localized the 'binding domains' of ^{125}I-ASA-PS by chemically cleaving the labeled AchR with cyanogen bromide and purifying the generated fragments by reverse-phase HPLC. While interpretation of the sequence data is hampered by the presence of multiple sequences in any given radiolabel containing HPLC peak, previous work using *Staphylococcus aureus* V8 proteinase mapping to define the regions of the α-subunit which incorporated the phosphatidylserine photolabel [1] allowed us to constrain the labeled CNBr fragment(s) to those contained within the stretch of the polypeptide chain identified by V8 proteinase mapping. Central to this analysis is the consensus view that due to the high degree of homology between each of the receptor subunits, they fold in a similar manner to give rise to either four [10,12] or five [13,14] membrane-spanning regions. This conservative assumption allowed us to use information gathered for one subunit and apply it to each of the subunits as a whole. Finally, the pattern of locations of the identified peptides has allowed us to draw some valuable conclusions as to the regions of the AchR subunits which interact the phosphatidylserine analog employed in this study.

The results of the sequence data are summarized in Fig. 5 along with results previously obtained from V8 protease mapping. The three fragments identified in peak IIA (α-Lys-330, β-pro-315, and δ-Phe-383) each originate in a region of the polypeptide chain which is proposed to be located on the cytoplasmic side of the membrane [10,11,13,14] providing additional support for the view that each of the subunits fold in a similar manner. Incorporation of the phosphatidylserine analog into one or more of these fragments is entirely consistent with previous results demonstrating incorporation of label into V8 proteinase fragments of the a-subunit beginning at Asn-339 and extending to the carboxy terminus of the subunit [1].

Since the photoreactive portion of ^{125}I-ASA-PS is attached to the phospholipid head group, regions of the polypeptide chain in contact or in close proximity with the lipid bilayer are likely to be labeled. The proposed membrane-spanning region M4 as well as the amphipathic region MA are both contained within the stretch of the polypeptide chain found in the CNBr fragments identified within HPLC peak IIA as well as the V8 proteinase fragments of the α-subunit previously identified. Evidence from the substitution of hydrophobic domains [20] support a conclusion that the

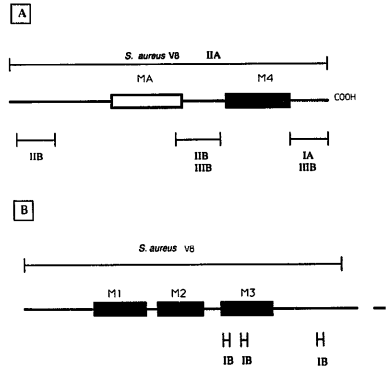

Fig. 5. Locations of sequenced peptides within AchR primary structure. Schematic drawing of the aligned primary sequences of the *T. californica* AchR subunits. Black boxes indicate the hydrophobic transmembrane segments [10], and the open box the amphipathic helix MA [13,14]. Identified CNBr and *Staphylococcus aureus* V8 proteinase peptides are localized along the sequence as indicated by fraction numbers which contained peptides labeled by ^{125}I-ASA-PS.

M4 region either spans the membrane or is at least integrally associated with it, while the region contained within MA is cytoplasmically located [21,22].

In our study, peptide fragments contained within the major radioactive HPLC peaks were characterized by sequence analysis. Because of the presence of both labeled and unlabeled peptides in each fraction, it is not possible to assign unambiguously an observed sequence to the radioactive peptide based on results from a single fraction. However, we have chosen to use independent sequencing results from the major radioactive fractions to arrive at a conclusion consistent with all the results. Based on the identified sequences from the fractions examined, we are able to obtain an overall pattern of peptides present in the radioactive fractions. The results show that the sequenced peptides present in the radioactive fractions are not randomly distributed, but include primarily membrane-spanning domains as would be expected from a phospholipid probe. This conclusion is also consistent with constraints based on our previous results using V8 proteinase digestion [1].

Examination of the locations of sequences identified within HPLC radiolabel-containing peaks (Fig. 5A) indicate that either ^{125}I-ASA-PS is labeling regions of the polypeptide chain thought to be located in the cytoplasmic domain or the phosphatidylserine analog is labeling flanking segments of the membrane-spanning region M4. While the first case cannot be dismissed, ^{125}I-ASA-PS incorporation into flanking regions of M4 support the proposed models of the transmembrane organization of the AchR subunits in which the region M4 is thought to represent an α-helical membrane-spanning region [10–14]. This conclusion is also consistent with the phosphatidylserine analog labeling 'flanking' regions of M4 as a consequence of the fact that the photoreactive group is attached to the phospholipid head group and would most likely label flanking regions of transmembrane helices.

Similar labeling patterns observed for ^{125}I-ASA-PS and the hydrophobic probe ^{125}I-TID [23] in this region of the polypeptide chain are consistent with the interpretation that each probe labels different regions of a transmembrane helix. Using a photoreactive analog of phosphatidylcholine, Giraudat et al. [24] concluded that Cys-424 within the M4 region of the α-subunit of *T. mammorata* is labeled. The phosphatidylcholine analog employed in these studies was a superficial probe of the membrane bilayer with the photoreactive group substituted for one of the fatty acid chains. Labeling of Cys-424 is then consistent with ^{125}I-ASA-PS labeling a region slightly distal and in the region of helix in contact with the phospholipid head group.

Significant incorporation of both ^{125}I-ASA-PS and ^{125}I-TID into the region of the α-subunit which contains M4 suggests that of the membrane spanning regions (M1–M4), M4 is in greatest contact with the lipid bilayer. This conclusion is supported by the observation that M4 is the most hydrophobic of the proposed transmembrane spanning regions, exhibiting a face which is essentially devoid of residues with hydrogen bonding groups [3]. The M4 region is also the least conserved of the transmembrane spanning regions, suggesting that it interacts with the solvent (i.e., lipid) rather than solely with other regions of the polypeptide.

Several lines of evidence suggest that the M4 helix lies at the periphery of the ion channel [3], not the least compelling of which is that the M4 region can be replaced by foreign hydrophobic transmembrane sequences without loss of ion channel activity [20]. A peripheral location of the M4 helix with respect to the ion channel is then entirely consistent with it interacting with the lipid bilayer to a greater extent than that of the other membrane spanning regions.

Sequences identified within HPLC peak IB (Tables II, III) suggest that ^{125}I-ASA-PS is also incorporated into the transmembrane spanning region M3. Labeling of M3 is consistent with a significant amount of the total label incorporated into the α-subunit (15%) localized within a V8 cleavage fragment of the subunit containing the membrane-spanning regions M1–M3 [1]. However, based on the lower level of incorporation of ^{125}I-ASA-PS within the V8 cleavage fragment containing M3 as compared to that incorporated within the fragment containing M4, M3 would appear to be in less contact with the lipid bilayer. Like M4, M3 differs from M1 and M2 in that it is less well-conserved, and like M4, it exhibits a certain lateral amphipathy, having a face with rather polar residues as well as hydrophobic face.

While further work is necessary to determine whether helices M1 and M2 interact with the lipid bilayer, the labeling pattern of ^{125}I-ASA-PS enables one to construct a working model of the topological arrangement of the four transmembrane spanning helices (M1–M4). Labeling of the M2 region by photoreactive analogs of non-competitive inhibitors (NCB) [24,25] suggests that the M2 region of each of the receptor subunits form the lining of the ion channel. The hydrophobic faces of M3 as well as M4 would then form the boundary face of the channel complex in contact with the lipid bilayer with M4 forming the periphery in greatest contact with the membrane lipids. The hydrogen bonding faces of M3 and M4 are also presumably in contact with the other transmembrane helices. The significant hydrogen bonding capacity of the M1 helix suggests that it is primarily in contact with the other helices. The conserved proline residue in M1 (position 240) of the aligned primary sequences [9,10] probably breaks the helix up into two segments [14]. The lower segment of M1 may form a hydrophobic

pocket explaining the labeling of α-Cys-222 by pyrenyl maleimide (PM) [26]. Since labeling of α-Cys-222 by PM inhibits AchR-mediated cation translocation while the mutation α-Cys-222 to Ser yielded a functional receptor [27], labeling of Cys-222 may block a conformational change needed for channel opening possibly involving an isomerization of α-Pro-221. Undoubtedly M1 is integrally associated with the ion channel which is consistent with its relatively high degree of regional homology [9,10].

In view of the fact that no significant difference in the labeling pattern has been detected in the ^{125}I-ASA-PS labeling of the AchR in the presence or absence of receptor agonist [1], nor for any other photoactivatable lipid analog [19,24], it is probable that any conformational change induced by the binding of agonist is not sensed at the lipid/protein interface. A likely model which explains the requirement of negatively charged phospholipids on receptor function is one in which the binding of the negatively charged phospholipid head group with positively-charged residues located at the ends of at least the M3 and M4 helices, stabilizes the helices in the membrane and facilitates the formation of a stable ion channel capable of undergoing agonist induced activation.

Acknowledgments

This work is in part supported by research grants from the NIGMS, the Graduate Division and the Research Committee at the University of California, Santa Cruz. We thank Dr. Phil Limbacher, Jr. for reading the manuscript and Dotty Hollinger for typing the paper.

References

1 Blanton, M. and Wang, H.H. (1990) Biochemistry 29, 1186–1194.
2 Lindstrom, J. (1983) Neurosci. Comment. 1, 139–156.
3 Popot, J.L. and Changeux, J.P. (1984) Physiol. Rev. 64, 1162–1239.
4 Hucho, F. (1986) Eur. J. Biochem. 158, 211–226.
5 McCarthy, M.P., Earnest, J.P., Young, E.F., Choe, S. and Stroud, R.M. (1988) Annu. Rev. Neurosci. 9, 383–413.
6 Raftery, M.A., Hunkapiller, M.W., Stader, C.D. and Hood, L.E. (1980) Science (Washington, D.C.) 208, 1454–1457.
7 Strader, C.D. and Raftery, M.A. (1980) Proc. Natl. Acad. Sci. USA 77, 5807–5811.
8 Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T. and Numa, S. (1982) Nature (London) 229, 793–797.
9 Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983) Nature (London) 302, 528–532.
10 Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T. and Numa, S. (1983a) Nature (London) 301, 251–255.
11 Claudio, T., Ballivet, M, Patrick, J. and Heinemann, S. (1983) Proc. Natl. Acad. Sci. USA 80, 1111–1115.
12 Devillers-Thiery, A., Giraudat, J., Bentaboulet, M. and Changeux, J.P. (1983) Proc. Natl. Acad. Sci. USA 80, 2067–2071.
13 Finer-Moore, J. and Stroud, R.M. (1984) Proc. Natl. Acad. Sci. USA 81, 155–159.
14 Guy, H.R. (1984) Biophys. J. 45, 249–261.
15 Ochoa, E.L.M., Dalziel, A.W. and McNamee, M.G. (1983) Biochim. Biophys. Acta 727, 151–162.
16 Criado, M., Eibl, H. and Barrantes, F.J. (1984) J. Biol. Chem. 259, 9188–9198.
17 Fong, T.-M. and McNamee, M.G. (1986) Biochemistry 25, 830–840.
18 McNamee, M.G., Jones, O.T. and Fong, T.-M. (1986) in Ion Channel Reconstitution (Miller, C., ed.), pp. 231–273, Raven Press, New York.
19 Middlemas, D.S. and Raftery, M.A. (1987) Biochemistry 26, 1210–1223.
20 Tobinmatsu, T., Fujita, Y., Fukuda, K., Tanaka, K.-I., Mori, Y., Konno, T., Mishina, M. and Numa, S. (1987) FEBS Lett. 222, 56–62.
21 Ratnam, M., Sargent, P.B., Sarin, V., Fox, J.L., Nguyen, D.L., Rivier, J., Criado, M. and Lindstrom, J. (1986) Biochemistry 25, 2621–2632.
22 Dwyer, B.P. (1988) Biochemistry 27, 5586–5592.
23 White, B.H. and Cohen, J.B. (1988) Biochemistry 27, 8741–8751.
24 Giraudat, J., Montecucco, C., Bisson, R. and Changeux, J.P. (1985) Biochemistry 21, 3467–3474.
25 Oberthür, W., Muhn, P., Baumann, H., Lottspeich, F., Wittmann-Liebold, B. and Hucho, F. (1986) EMBO J. 5, 1815–1819.
26 Marquez, J., Iriarte, A. and Martinez-Carrion, M. (1989) Biochemistry 28, 7433–7439.
27 Mishina, M., Tobinmatsu, T., Imoto, K., Tanaka, K., Fujita, Y., Fukuda, K., Kurasaki, M., Takahashi, H., Morimoto, Y., Hirose, T., Inayama, S., Takahashi, T., Kuno, M., Numan S. (1985) Nature (London) 299, 793–797.