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Pyridoxal Phosphate as a Probe of the Cytoplasmic Domains of Transmembrane Proteins: Application to the Nicotinic Acetylcholine Receptor†

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ABSTRACT: A novel procedure has been developed to specifically label the cytoplasmic domains of transmembrane proteins with the aldehyde pyridoxal 5-phosphate (PLP). *Torpedo californica* acetylcholine receptor (AcChR) vesicles were loaded with [^{3}H]pyridoxine 5-phosphate ([^{3}H]PNP) and pyridoxine-5-phosphate oxidase, followed by intravesicular enzymatic oxidation of [^{3}H]PNP at 37 °C in the presence of externally added cytochrome *c* as a scavenger of possible leaking PLP product. The resulting Schiff's bases between PLP and AcChR amino groups were reduced with $NaCNBH_3$, and the pyridoxylated proteins were analyzed by fluorography. The four receptor subunits were labeled whether the reaction was carried out on the internal surface or separately designed to mark the external one. On the other hand, the relative pyridoxylation of the subunits differed in both cases, reflecting differences in accessible lysyl residues in each side of the membrane. Proteinase K treatment of labeled AcChR vesicles generated a peptide of 13 kDa that could be detected with anti-PLP antibodies only when the pyridoxylation was carried out on the internal surface of the vesicles. Even though there are no large differences in the total lysine content among the subunits and there are two copies of the α-subunit, internal surface labeling by PLP was greatest for the highest molecular weight (δ) subunit, reinforcing the concept that the four receptor subunits are transmembranous and may protrude into the cytoplasmic face in a fashion [Strader, C. D., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5807-5811] that is proportional to their subunit molecular weight. Yet, the labeling data do not fit well to any of the models proposed for AcChR subunit folding. The method described can be used for selective labeling of the cytoplasmic domains of transmembrane proteins in sealed membrane vesicles.

Knowledge of the topology of the proteins associated with biological membranes is of basic importance to the understanding of structure–function relationships of membrane proteins. Several procedures for the identification and localization of surfaces of membrane proteins have been developed. Those methods include a number of membrane-impermeable agents, both anionic and cationic, that label proteins on the external surface of membrane vesicles (Rifkin et al., 1972; Steck & Dawson, 1974; Cabantchick & Rothstein, 1974; Thompson et al., 1987; Kyte et al., 1987; Dwyer, 1988). These probes have been designed to react with either nucleophilic or electrophilic groups of proteins on the membrane surface (Jackson, 1975; Berg, 1969). However, ligands that are inaccessible from the outside cannot be directly identified in the intact cell by such impermeant probes. Pyridoxal 5-phosphate (PLP)[1] is one of these probes used to label the outer surfaces

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of virus particles, both normal and transformed chick embryo fibroblasts (Edger & Rifkin, 1977), red blood cells (Cabantchick et al., 1975), (Na^+, K^+)-activated ATPase (Kyte et al., 1987), and the AcChR (Dwyer, 1988).

The use of PLP as a probe is based on the fact that it reacts with free amino groups with a high degree of specificity, forming a reversible Schiff's base that can be fixed as an irreversible covalent bond upon reduction. Formation of PLP protein derivatives can be followed by using ^3H-, ^{14}C-, or ^{32}P-radiolabeled PLP (Edger & Rifkin, 1977), electronic absorption or fluorescence spectroscopy (Matsushima & Martell, 1967; Churchich, 1965), or ^{31}P nuclear magnetic resonance spectroscopy (Martinez-Carrion, 1975).

Because of its poor membrane permeability, PLP (Cabantchick et al., 1975; Kyte et al., 1987; Dwyer, 1988) preferentially labels proteins on the external surface of the membrane. This poor permeability can be used advantageously if, instead, PLP could be trapped inside membrane vesicles and enzymatically generated by oxidation of intravesicularly trapped PNP and PNPoxidase. The resulting PLP could label lysyl residues encountered at the cytoplasmic domains of transmembranous proteins, which, in intact membranes, are protein regions difficult to access by other known protein marker procedures.

AcChR-enriched vesicles have been used as a model system for structural and functional studies of membrane proteins (Conti-Tronconi & Raftery, 1982; Wan & Lindstrom, 1984). This is a transmembrane glycoprotein, whose structural features are known in sufficient detail (Conti-Tronconi & Raftery, 1982; Barrantes, 1983). The AcChR isolated from *Torpedo californica* has an overall molecular weight (M_r) of 270000 (Martinez-Carrion et al., 1975). Composed of four membrane-spanning subunits of M_r 40000 (α), 50000 (β), 60000 (γ), and 65000 (δ) in a 2:1:1:1 stoichiometry (Reynolds & Karlin, 1978; Raftery et al., 1980). A variety of electron microscopy, chemical cross-linking, and X-ray scattering experiments (Cartaud et al., 1973; Hucho, 1981; Klymkowsky & Stroud, 1979; Kistler et al., 1982; Karlin et al., 1983; Brisson & Unwin, 1985) indicate a cylindrical arrangement of five subunits around a central pit. The bulk of the receptor protein extends from the extracellular site of the membrane, while a much smaller portion extends from the intracellular face of the membrane. These structural features have been complemented by the elucidation of the entire sequence of the four receptor subunits (Noda et al., 1983), which has led to extensive predictive models of secondary structure arrangement of each subunit (Caludio et al., 1983; Devillers et al., 1983; Noda et al., 1983; Finer-Moore & Stroud, 1984; Guy, 1984; Ratnam et al., 1986) and attempts at the identification of putative membrane-spanning segments using a variety of immunological and chemical probes (Ratnam & Lindstrom, 1984; Ratnam et al., 1986; Giraudat et al., 1985; Dwyer, 1988).

Particularly, monoclonal antibodies against certain peptide sequences have recently been used as probes to label those regions of AcChR that are not accessible by other membrane labeling reagents (Ratnam & Lindstrom, 1984; Ratnam et al., 1986; Tzartos et al., 1988). Those procedures are also preferentially targeted to the extracytoplasmic side surface regions (Tzartos et al., 1988). In the present study, a novel procedure was developed to label specifically the cytoplasmic domains of membranous proteins using PLP enzymatically generated inside sealed AcChR vesicles. The methodology developed in this paper is a general approach to study the topography of transmembranous proteins in sealed vesicles.

Materials and Methods

Excised electroplax tissue from *Torpedo californica* was purchased from Pacific Biomarine Supplies Co. (Venice, CA) and was stored at approximately −70 °C until needed. *Bungarus multicinctus* venom was obtained from Miami Serpentarium Laboratories (Salt Lake City, UT). α-Bungarotoxin was purified (Mebs et al., 1972) and radiolabeled with ^{14}C as previously described (Calvo-Fernandez & Martinez-Carrion, 1981). Frozen rabbit liver was purchased from Pel-Freez Biologicals (Rogers, AR) and stored frozen (−70 °C) until use. Sodium [^3H]borohydride was obtained from Amersham. Sodium cyanoborohydride was from Aldrich Chemical Co. Inc., and recrystallized before use (Jentoft & Dearborn, 1983).

Preparation of AcChR-Enriched Vesicles. AcChR-enriched vesicles from *Torpedo californica* electroplax were prepared as previously described (Soler et al., 1984; Garcia-Borron et al., 1987) and further purified by alkaline extraction (Neubig et al., 1979; Elliot et al., 1979). These AcChR-enriched vesicles were resuspended in HEPES buffer (10 mM HEPES, 100 mM KCl, and 5 mM $CaCl_2$, pH 8.3), to a final protein concentration of 10 mg/mL. Protein concentrations were determined by the method of Lowry et al. (1951). Specific α-Bgt binding activities were determined by the DEAE filter disk assay (Schmidt & Raftery, 1973) and typically ranged from 1.5 to 2 nmol of α-Bgt bound/mg of protein. The vesicles were 96–98% right-side-out (Hartig & Raftery, 1979).

Preparation of Pyridoxine-5-phosphate Oxidase. PNPoxidase was prepared from frozen rabbit liver following a published procedure (Bowers-Komro et al., 1986). Enzymatic activities were determined by the method of Wada (1970), if not otherwise indicated. Specific activities ranged from 16500 to 17500 units/mg of protein.

[^3H]Pyridoxine 5-Phosphate. [^3H]PNP was prepared by reduction of PLP with sodium [^3H]borohydride (25 mCi, 12 Ci/mmol). PLP (2 μmol) was dissolved in 1 mL of sodium [^3H]borohydride and the pH adjusted to 7.5 with HCl. The reaction was carried out overnight at 4 °C and protected from light. The products were separated in an Amberlite CG-50 column (Peterson & Sober, 1954). Isolated [^3H]PNP was lyophilized and quantitated by absorption spectra in 0.1 M sodium phosphate buffer, pH 7, using a Hewlett–Packard diode array spectrophotometer at 325 nm with an extinction coefficient of 7400 M^{-1} cm^{-1} (Peterson & Sober, 1954). The final specific activity was 10 Ci/mmol.

Preparation of Polyclonal Antibodies against PLP. Antisera containing polyclonal antibodies against PLP have been prepared by immunizing New Zealand rabbits with a PLP–hemocyanin complex formed by derivatization of amino groups in the carrier with PLP (Kittler et al., 1986). Every 4 days, 5 mg of PLP–hemocyanin, in a 1:1 ratio in Freund's complete adjuvant, was subcutaneously injected during 1 month. After 1 month, the rabbits were injected with 5 mg of PLP–poly(DL-lysine) complex in a 1:1 ratio in Freund's incomplete adjuvant (Ungar-Waron & Sela, 1966). After a week, the rabbits were bled, the serum was extracted, and 10 mL of serum was adsorbed overnight at 4 °C with a mixture of 10 mg of AcChR and 10 mg of BSA cross-linked with a final concentration of 2% glutaraldehyde. The precipitate was

[1] Abbreviations: AcChR, acetylcholine receptor; BSA, bovine serum albumin; α-Bgt, α-bungarotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; octyl glucoside, *n*-octyl β-glucopyranoside; PMSF, phenylmethanesulfonyl fluoride; PNP, pyridoxine 5-phosphate; PNPoxidase, pyridoxine-5-phosphate oxidase; PLP, pyridoxal 5-phosphate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

eliminated by centrifugation for 30 min at 40000 rpm in a 50.2 Beckman rotor.

AcChR Vesicle Loading. AcChR vesicles (0.5–0.8 mg) were loaded first with [^{3}H]PNP by rapidly freezing a sample in liquid nitrogen containing 10 μL of AcChR vesicles and dried [^{3}H]PNP (12 nmol) in HEPES buffer (10 mM HEPES, 100 mM KCl, and 5 mM $CaCl_2$, pH 8.3) during 5 min and slowly thawing the sample on an ice bath. After the thawing, 10 μL of PNPoxidase (3000 units) was added, and two more freezing and thawing cycles were practiced. Extravesicular enzyme and substrate were removed by centrifugation, first in 10 mL of ice cold HEPES buffer at 50000 rpm in a Beckman type 80 rotor for 1 min at 0 °C and then in HEPES buffer containing 0.5 mg/mL cytochrome *c*. The vesicles were resuspended in 100 μL of the HEPES buffer containing 0.5 mg/mL cytochrome *c* to scavenge any possible leaking products and incubated at 37 °C for 10 min. The enzymatic reaction was stopped by adding $NaCNBH_3$ (20 mM final concentration) freshly prepared in HEPES buffer and kept 10 min at 0 °C. The vesicles were washed by centrifugation at 50000 rpm in the type 80 rotor in 100 mM Tris-HCl, pH 7.4.

Trichloroacetic Acid Precipitation. Pyridoxylated AcChR vesicles were solubilized in 50 μL of 2% SDS. To precipitate the proteins, 10 μL was added to 1 mL of cold 10% TCA and incubated on an ice bath for 10 min. Precipitates were collected by filtration on Whatman GF/C filters and washed with 30 mL of cold 10% TCA and 5 mL of cold 5% TCA. ^{3}H was quantitated in 5 mL of scintillation cocktail (3a70B, Research Products, Inc.).

Blocking of Surface Amino Groups. Amino groups exposed at the external surface were blocked by incubating 1 mL of AcChR vesicles (1 mg/mL) with 65 mM unlabeled PLP for 15 min at 0 °C. Schiff's bases were reduced with 0.12 M $NaCNBH_3$ for 10 min at 0 °C. AcChR vesicles with blocked amino groups were washed once with 20 mL of HEPES buffer, pH 8.3, containing 1 mg/mL cytochrome *c* and twice with 20 mL of the latter buffer without cytochrome *c*. After each wash, the vesicles were collected by centrifugation for 30 min at 40000 rpm in a Beckman type 50.2 rotor.

Gel Electrophoresis and Protein Quantitation. Samples were electrophoresed on 10% SDS–polyacrylamide mini gels with a 3% stacking gel. The solubilization buffer, the remaining reagents, and the electrophoresis and staining procedures are those of Laemmli (1970). For fluorography, gels, after being soaked in Fluoro-Hance solution (Research Products Inc.), were dried under vacuum and exposed to Kodak XAR-5 X-OMAT film for 15–20 days at −70 °C. The intensities of the protein bands were measured by scanning densitometry in an ISCO Model 1213 gel scanner.

Radioactivity of Gel Slices. Radioactive bands were cut horizontally from the gel in 1-mm fragments, using a Bio-Rad gel slicer, and solubilized overnight at 37 °C with 0.3 mL of solution composed of 95 parts of 30% H_2O_2 and 5 parts of 15 N NH_4OH. The radioactivity was measured in 5 mL of scintillation cocktail. The quenching was corrected by using an internal standard of [^{3}H]toluene (Amersham).

Other Determinations. Turbidity was measured in an SLM 8000C Aminco spectrofluorometer interfaced to an IBM PC/XT computer. Measurements were taken at 460 nm in both excitation and emission monochromators in order to determine the relative changes in light at 90°. Proteolysis was carried out in 10 mM Tris-HCl buffer, pH 7.5, using proteinase K (IBI, International Biotechnologies, Inc., New Haven, CT). The reaction was carried out for 60 min on an ice bath using a ratio of protease to AcChR vesicles (protein)

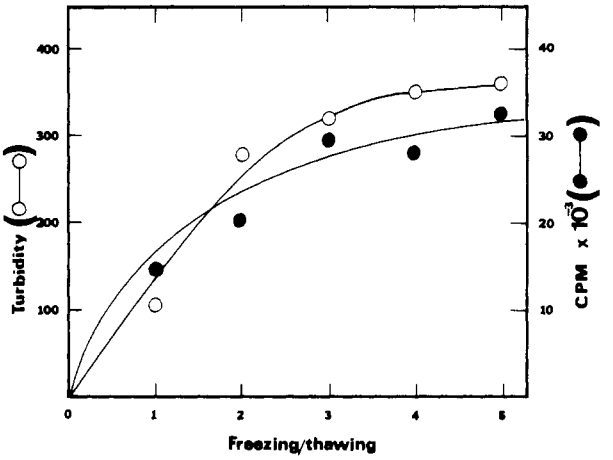

FIGURE 1: Effect of freezing/thawing cycles on the trapping capacity of AcChR vesicles. 0.4 mg of AcChR vesicles was loaded with 0.2 mM [^{3}H]PNP in a total volume of 15 μL. (●) Radioactivity trapped; (○) changes in turbidity of AcChR vesicles (0.5 mg/mL) after freezing/thawing cycles.

of 1:13 (w/w). The reaction was stopped by adding 2 mM final concentration of PMSF and keeping 15 min on an ice bath. Vesicles were collected by centrifugation in the Beckman Airfuge for 5 min at 30 psi and resuspended for electrophoresis. Proteins were separated in 15% SDS–polyacrylamide mini gels. For immunoblotting, the proteins were transferred to nitrocellulose sheets according to the method of Towbin et al. (1979). Rabbit anti-PLP was used as the first antibody. This primary antibody was detected with a biotinylated secondary antibody, horseradish peroxidase–avidin system (Vector Laboratories, Burlingame, CA).

RESULTS

Effect of Freezing and Thawing Cycles on the Trapping Capacity of AcChR Vesicles. It has been reported that cycles of rapid freezing in liquid nitrogen and slow thawing of liposomes result in disruption of the lipid phase organization (Morris, 1981) and also increase the size of the vesicles by a mechanism not entirely known but suspected to be vesicle fusion (Oku & McDonald, 1983; Pick, 1981). Rapid freezing and slow thawing of a mixture containing 0.5–0.8 mg of protein of AcChR vesicles and [^{3}H]PNP result in trapping of 0.3–0.6% of the total radioactivity added to the trapping media, reaching the maximal incorporation after three cycles of freezing and thawing (Figure 1). Moreover, turbidity measurement as a function of the freezing and thawing cycles as a means of visualizing the fusion process shows a maximal value after three freeze/thaw cycles (Figure 1).

PNPoxidase can be trapped by the same procedure, and the intravesicular localization of the enzyme is shown by the observation that addition of extravesicular substrate does not result in any sizable PLP production, unless the loaded vesicles are disrupted by addition of the detergent octyl glucoside. In that case, 6.9 nmol of PLP mL^{-1} h^{-1} is produced by AcChR vesicles containing 0.8 mg of membrane protein and loaded in 3000 units of PNPoxidase. This represents 1.4% of the total units added to the trapping media. The enzyme is active in the presence of octyl glucoside (data not shown). The difference in trapping efficiency for [^{3}H]PNP and the enzyme is most likely due to the loss of the trapped substrate during the large dilutions and rapid centrifugation steps (see Materials and Methods), where the vesicles might not be completely sealed at the low temperature.

Permeability of [^{3}H]PNP-Loaded AcChR Vesicles. It is generally admitted that temperature can cause changes in the

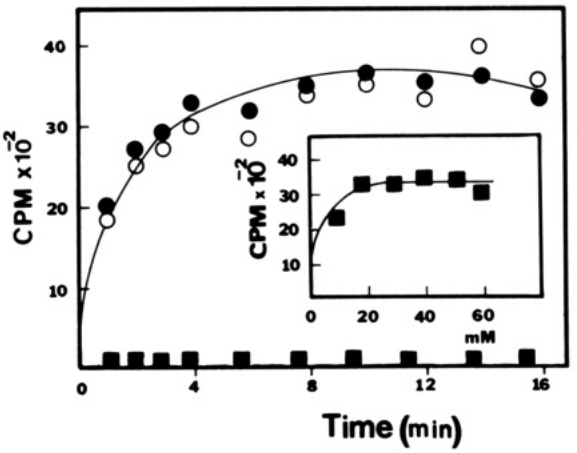

FIGURE 2: Time course of pyridoxylation of AcChR vesicles. 0.5 mg of AcChR vesicles, preblocked on the external surface, was loaded with 0.6 mM [^{3}H]PNP and 3000 units of PNPoxidase as described under Materials and Methods. Loaded vesicles were supplemented (●) or not (○) with 0.5 mg/mL cytochrome *c* and incubated at 37 °C. 10-μL aliquots were taken at selected times and the reactions stopped with $NaCNBH_3$ and assayed for radioactivity. (■) Vesicles lacking PNPoxidase and loaded only with [^{3}H]PNP. Insert: Effect of $NaCNBH_3$ concentration on Schiff's base reduction.

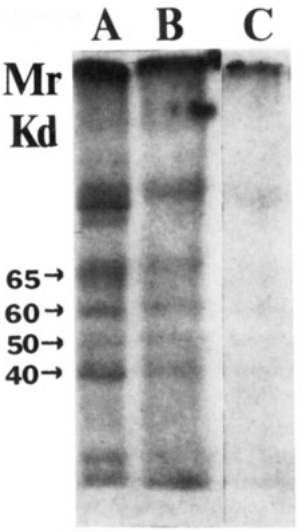

FIGURE 3: Fluorograms of electrophoresed proteins from pyridoxylated AcChR vesicles. (A) Vesicles labeled on the external face; (B) vesicles with the external face preblocked and labeled in the internal face; (C) external face preblocked and labeled on the external face. 80 μg of protein in each gel well.

physical properties of AcChR membrane vesicles (Farach & Martinez-Carrion, 1983; Soler et al., 1984). An obvious consequence of such perturbations might be alterations in membrane permeability. Therefore, we investigated the rate of leaking of internally trapped [^{3}H]PNP from AcChR vesicles at both 25 and 37 °C. These vesicles show a low permeability with 90% of the material remaining trapped upon incubation at 25 °C for 10 min, and a slight increase in the amount of [^{3}H]PNP leaking out (20% leaking) if the vesicles were incubated at 37 °C for 10 min (data not shown). Similar results were obtained with different preparations of AcChR vesicles.

Pyridoxylation of AcChR Vesicles. Labeling of the cytoplasmic face of the sealed membrane vesicles was achieved by carrying out an enzymatic oxidation of intramembranous trapped [^{3}H]PNP by PNPoxidase. ε-Amino groups of lysyl residues in proteins react with the enzymatically produced PLP to form Schiff's bases. Subsequent reduction of the imines with $NaCNBH_3$ results in formation of stable covalent pyridoxyl lysyl residues. The substrate and the enzyme were loaded sequentially inside AcChR vesicles preblocked on the external face as described under Materials and Methods. The data in Figure 2 show that enzymatically generated PLP is covalently attached to the internal face of the vesicles. A time-dependent incorporation of [^{3}H]PLP into the TCA-precipitable material was obtained. Under these conditions, the incorporation of [^{3}H]PLP was completed after 10 min of incubation. It is important to point out that addition of 0.5 mg/mL cytochrome *c* to the incubation mixture to scavenge any products that could leak from the vesicles did not affect the extent of the pyridoxylation reaction, indicating that [^{3}H]PLP leaks were insignificant in the interpretation of the results. Moreover, vesicle-entrapped pyridoxylation mixtures lacking PNPoxidase produced no detectable incorporation of [^{3}H]PLP into AcChR vesicle TCA-precipitable material, indicating that the oxidation of [^{3}H]PNP to [^{3}H]PLP must be enzymatic (Figure 2). Further characterization of this reaction showed that 20 mM $NaCNBH_3$ was the optimum for the reduction of Schiff's bases formed (Figure 2, insert).

Distribution of the radiolabel on the AcChR subunits examined after separation in 10% SDS–polyacrylamide gels (Figure 3) shows the comparison of labeling with enzymatically generated [^{3}H]PLP on both the external and internal surface of the vesicles after both were carried out under identical conditions in separate experiments. Pyridoxylated vesicles showed labeling of the four receptor subunits. The δ-subunit incorporated the highest amount of radioactivity whether the pyridoxylation reaction was carried out on the internal or external surface, and this was accompanied by a minor incorporation of [^{3}H]PLP at the α- and β-subunits (internal and external, respectively). The ratio of labeling among the receptor subunits is different under both sets of conditions, i.e., δ/γ α_2/γ ratios being larger after external labeling than after internal labeling (Figure 3). The extent of pyridoxylation was 2–3 times higher for reactions directed for a preferential labeling of the external surface of the vesicles compared to identical conditions used for cytoplasmic side labeling. The lack of any appreciable external labeling when the enzymatic generation of PLP was carried out after preblocking of the external face amino groups shows that the external amino groups have been effectively blocked with "cold" excess of PLP (Figure 3, lane C). In addition to AcChR, another protein component of our AcChR vesicle preparation was also labeled. A 95-kDa band, supposed to correspond to the catalytic subunit of the (Na^+/K^+)-ATPase (Perrone et al., 1975; Lindstrom et al., 1979), was labeled when the PLP was generated either inside or outside the vesicles. The label in this protein was also 2.5-fold higher when the pyridoxylation was carried out on the external surface compared with the internal one.

For a more quantitative analysis of the radioactivity associated with each AcChR subunit, the gels, after being stained with Coomassie blue, were sliced into 1-mm fragments, and the radioactivity was determined after solubilization as described under Materials and Methods. Figure 4 shows the effect of preblocking the external face of the vesicles on the cytoplasmic pyridoxylation. Both the preblocked and unblocked vesicles showed a similar distribution of radioactivity. Comparison of the electrophoretic pattern after Coomassie staining indicated that, in both cases, the highest amount of label was associated with the δ-subunit while the α-subunit was less labeled. The radioactivity associated with the dye front was variable. Ultraviolet absorbance at 325 nm, typical of reduced Schiff's bases (Churchich, 1965), was not detected. On the other hand, pyridoxylated vesicles show the fluorescent emission (398 nm) characteristic of pyridoxyl derivatives

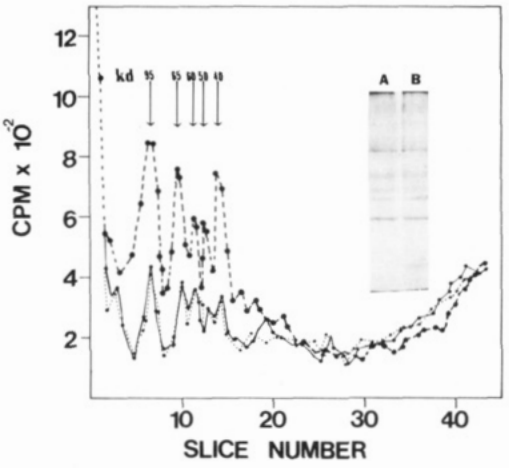

FIGURE 4: SDS–polyacrylamide gel profiles of labeled AcChR vesicles. (---) Vesicles labeled on the external face. (—) Labeled on the internal face with external face preblocked with unlabeled PLP. (···) Internal label without preblocking. (Right) Coomassie pattern of AcChR vesicles labeled in the internal face: (A) unblocked; (B) blocked on the external face. 80 μg of protein was loaded in the gel.

Table I: Pyridoxylation of AcChR Subunits in the Cytoplasmic Surface: Comparison with the Proposed Models

subunit	pmol of PLP/nmol of subunit	no. of cytoplasmic lysines			
		I[a]	II[b]	III[b]	IV[c]
α	8	11	6	11	12
β	10	8	4	8	8
γ	16	16	12	17	17
δ	18	11	8	12	12

[a] From the four-helix model (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983). [b] Five-helix model (Finer-Moore & Stroud, 1984; Guy, 1984). [c] From the model based on immunolocalization experiments (Ratnam et al., 1986).

(Churchich, 1965) (data not shown). From α-Bgt binding activities, we calculated the amount of receptor subunits loaded on the gel, and from the radiolabel associated with each subunit, we calculated the apparent stoichiometries for cytoplasmic pyridoxylation. These were 0.008, 0.010, 0.016, and 0.018 nmol of PLP (nmol of subunit)$^{-1}$ for α-, β-, γ-, and δ-subunits, respectively (Table I).

Proteinase K Digestion of Labeled AcChR Vesicles. After extensive digestion with proteinase K, the PLP-labeled AcChR vesicles yielded a major band of apparent molecular mass of 13 kDa and several of higher molecular mass (see Figure 5, lanes 2 and 4). However, after immunoblotting (Figure 5, lanes 6 and 8), only digested AcChR vesicles that were pyridoxylated on the cytoplasmic face could be recognized by anti-PLP antibodies.

Discussion

A method has been developed for the selective labeling of the cytoplasmic domains of transmembranous proteins using membrane-bound AcChR as a model system. The utilization of PLP as a covalent probe for labeling the internal surface of membrane proteins has so far been limited to analysis of disrupted membranes due to the fact that this is a membrane-impermeable aldehyde, and when added to membrane vesicles, it preferentially labels the external surface of membrane proteins (Rifkin et al., 1972; Cabantchick et al., 1975; Kyte et al., 1987). Few methods have been described to access the labeling of the internal surface of transmembrane proteins, and in all of those methods, the labeling of the cytoplasmic face has been possible only after prior blocking of the extravesicular surface followed by solubilization of the vesicles (Thompson et al., 1987; Kyte et al., 1987), or in some cases

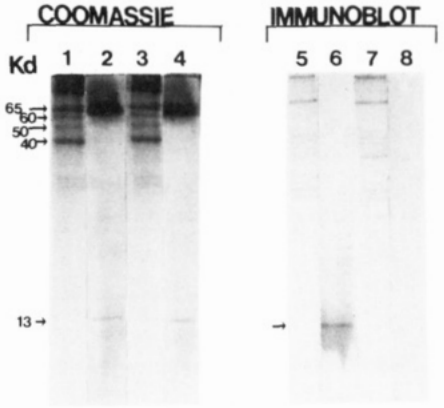

FIGURE 5: Coomassie staining and immunoblot of pyridoxylated AcChR vesicles digested with proteinase K as described under Materials and Methods. (Lanes 1 and 5) Labeled on the internal surface; (lanes 2 and 6) labeled on the internal surface and treated with proteinase K; (lanes 3 and 7) labeled on the external surface; (lanes 4 and 8) labeled on the external surface and treated with proteinase K.

after production of purified inside-out vesicles (Steck et al., 1970). The procedure presented here offers an alternative with minimal perturbation of the membrane protein/lipid interactions. This method takes advantage of the fact that PLP can be enzymatically produced by oxidation of PNP by PNPoxidase when both are trapped inside sealed right-side-out membrane vesicles; therefore, the labeling agent is retained for contact with the internal surface of transmembranous proteins. We also use AcChR because over 96% right-side-out membrane vesicles can be prepared from the electroplax of *Torpedo* fish (Hartig & Raftery, 1979).

Both [^{3}H]PNP and PNPoxidase can be loaded sequentially inside AcChR vesicles by cycles of freeze/thaw (Figures 1, 2, and 3). This method has been extensively used to load enzymes and organic molecules inside synthetic liposomes (Pick, 1981; Oku & McDonald, 1983) and has also been used in our laboratory to load fluorescent probes inside AcChR vesicles for further functional studies (Martinez-Carrion et al., 1984). The enzyme PNPoxidase can be successfully trapped inside the vesicles and retains its enzymatic activity upon freeze–thaw cycles and detergent solubilization of the vesicles. In addition, the PNPoxidase shows no measurable activity at low temperature (0–4 °C); it is also important to note that in intact enzyme loaded vesicles there is no detectable PLP formed if the substrate is added to the external medium, indicating that the enzyme remains preferentially sequestered in the vesicles. Furthermore, [^{3}H]PNP alone loaded inside the vesicles showed minimal leaking at both 25 and 37 °C. Thus, the 37 °C temperature was selected to carry out the initiation of the intravesicular enzymatic reaction when both [^{3}H]PNP and PNPoxidase were loaded inside the vesicles, inasmuch as significant amounts of product formed only at these high temperatures.

The cytoplasmic domains of the AcChR subunits could be labeled after blocking the external surface amino groups with excess unlabeled PLP and reduction with $NaCNBH_3$. This conclusion is supported by the fact that pyridoxylation of the external surface of vesicles that have previously been treated for blocking with unlabeled PLP does not show further incorporation of radioactivity when treated with our labeling procedure (Figure 3, lane C). However, when the preblocked vesicles were loaded with [^{3}H]PNP and PNPoxidase, a time-dependent incorporation of [^{3}H]PLP into TCA-precipitable material was obtained, indicating pyridoxylation on the cytoplasmic face of the vesicles. The fact that external addition

of cytochrome *c* to the incubation mixture containing enzyme and PNP-loaded AcChR vesicles does not alter the incorporation of radioactivity into AcChR, and that cytochrome *c* is not radiolabeled, suggests that potential leaks of PLP from the vesicles during the pyridoxylation sequence, including the $NaCNBH_3$ reduction, are an insignificant factor as a source for labeling external amino groups of the AcChR.

Fluorography and radiolabel counting of the PLP-labeled receptor subunits separated by SDS–polyacrylamide electrophoresis show distinctive differences when the pyridoxylation reaction was carried out on either the external or the internal surface of the vesicles (Figures 3 and 4). These data indicate a labeling of different regions of AcChR under each set of conditions, probably reflecting differences in the total amount, or exposure, of the lysyl residues on each side of the membrane. Analysis of the label per receptor subunit (Figures 3 and 4) shows that pyridoxylation of the internal surface of the receptor is higher for the larger molecular weight subunits, suggesting that more lysyl groups may be available or accessible for reaction with the aldehyde (see Table I). In addition to the four different AcChR subunits, a protein with apparent molecular mass, on SDS gels, of 95 kDa was labeled from both inside and outside the vesicles, which putatively correspond to the catalytic subunit of the (Na^+/K^+)-ATPase (Perrone et al., 1975), confirming the transmembranous nature of this protein. The transmembranous nature of all the receptor subunits is known, and it has been suspected that higher molecular weight subunits may be more exposed to the cytoplasmic face than the smaller ones (Strader & Raftery, 1980). Thus, pyridoxylation of the internal surface of AcChR reinforces previous data that the four receptor subunits span the membrane (Strader & Raftery, 1980).

We have attempted a comparison of our results and the lysyl residue content of each subunit proposed to reside on the cytoplasmic face in four different AcChR transmembrane organization models (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983; Finer-Moore & Stroud, 1984; Guy, 1984; Ratnam et al., 1986) (Table I). This general comparison tends to favor the topography predicted by the three- or four-transmembrane helix models or the nonfunctional five-helix model. Less correlation was found with the functional five-helix model (including the amphipathic helix) of Finer-Moore and Stroud (1984). However, at the present time, we do not know if all the lysyl residues in the subunits are equally accessible to labeling aldehyde during the pyridoxylation reaction. From recent studies using PLP, a general lysyl residue modifying agent, in AcChR under native and denaturing or detergent-solubilized conditions, it was found that only a small number of lysyl residues were labeled by PLP (Dwyer, 1988; Kyte et al., 1987). This is probably due to low exposure of the lysyl residues or the presence of low amounts of Schiff's base accessible to the $NaBH_4$ reduction used by Dwyer (1988) and Kyte et al. (1987). A similar explanation can apply to our pyridoxylation reaction at even lower concentrations of PLP. The poor access to lysyl residues would also explain the relatively lower labeling of the α-subunit, since this subunit amino acid sequence does not show a significant lower lysyl content or probable cytoplasmic side lysyl residues than the other subunits in any of four models analyzed (Table I). Our analysis of the extent of pyridoxylation of the subunit's cytoplasmic domains is incapable of a higher degree of discrimination among the proposed AcChR peptide folding models. However, since detailed topology of the subunits as provided by X-ray analysis is not forthcoming, other alternatives of analysis must be devised. Guy and Hucho (1987) have recently proposed attacking this problem through localization of the phosphorylation and glycosylation sites or those in contact with lipids. We believe the mild labeling procedure with PLP and the future localization of the phosphopyridoxylated residues can provide valuable novel information regarding location of exposed lysyl residues at the cytoplasmic side of the membrane within the sequence of each subunit.

The fact that pyridoxylation of lysyl residues of membrane proteins seems to be a low-efficiency process has some drawbacks for analytical procedures, but it also has the advantage of inducing low protein structural perturbations, particularly when it introduces few molecules of PLP which change the electrostatic environment of the surface and add bulk to the lysyl ϵ-amino groups. However, in this case, as in other recent examples (Dwyer, 1988; Kyte et al., 1987), low amounts of PLP attach to protein-exposed lysyl residues in a seemingly random process. After external labeling, peptides with many pyridoxylation sites can be detected (Dwyer, 1988; Kyte et al., 1987). Similarly, for our cytoplasmic labeling conditions, we have no reason to suspect (Figure 5) anything but random pyridoxylation, also, and have the potential for producing membrane-bound AcChR molecules with little perturbation in their structure, which would be unlikely if the probe occupied all possible sites of attachment in each molecule.

Extensive digestion of the external surface of pyridoxylated AcChR vesicles using proteinase K produced, in addition to several larger peptides, a polypeptide of 13 kDa upon separation by SDS gels. The fact that these peptides were (Figure 5) immunodetected with monospecific antibodies against PLP only when the pyridoxylation reaction was carried out on the cytoplasmic face of the vesicles indicates heterogeneity in the peptides spanning the lipid bilayer and exposure of the internal surface as a "target" for the intravesicular pyridoxylation reaction. Of particular interest is the 13-kDa peptide, since it has been reported that protease digestion, such as trypsinolysis of α-subunit labeled in membrane vesicles with the hydrophobic probe 5-[^{125}I]iodonaphthyl 1-azide, gives a labeled, lipid-embedded domain of 13 kDa (Tarrab-Hazdai et al., 1980).

In conclusion, we have devised a procedure for specific covalent labeling of the internal surface of AcChR with PLP. The method we describe should be a convenient means for introducing a versatile probe to study the physical properties of the internal surfaces of membrane proteins.

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Registry No. PLP, 54-47-7; [^{3}H]PNP, 120577-90-4; PNP oxidase, 9055-72-5; sodium [^{3}H]borohydride, 61113-34-6.

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