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A topographical study of the electroplax sodium channel with site-directed antibodies

Hitoshi Nakayama [a], Ken-ichi Nakayama [a,1], Yoshiaki Nonomura [b], Mari Kobayashi [a], Kenji Kangawa [c], Hisayuki Matsuo [c,2] and Yuichi Kanaoka [a,3]

[a] *Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo (Japan),* [b] *Department of Pharmacology, Faculty of Medicine, University of Tokyo, Tokyo (Japan) and* [c] *Department of Biochemistry, Miyazaki Medical College, Kiyotake, Miyazaki (Japan)*

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Three peptides corresponding to residues (13–23C), (Y1419–1431), and (1809–1820) of the electric eel sodium channel have been synthesized and used to raise antisera in rabbits. All the antibodies produced specifically recognized the corresponding peptides in an ELISA assay. However, their avidities to the channel protein were different. Two antibodies, against the sequence (1809–1820) and (13–23C) which are directed to the C-terminus region and to the adjacent portion of N-terminus, respectively, recognized the 250 kDa channel protein in the immunoblot and an ELISA assay. Binding of the two antibodies to the sodium channel in oriented electroplax membrane vesicles was increased 4–5-fold after permeabilizing the vesicles with 0.01% saponin, implying cytoplasmic orientation for the two peptides. The cytoplasmic orientation of the N- and C-terminal regions were further confirmed by immunogold electron microscopy. By contrast, antibody raised against the sequence (Y1419–1431) which has been proposed to be the transmembrane segment S4 of internal repeat IV, did not react with the channel protein not only after the saponin treatment but also even under the immunoblot conditions following SDS-PAGE. The antibody could recognize fragments of the channel protein after digestion with lysyl endoproteinase, suggesting that the region may apparently form a strictly well-ordered conformation in the transmembrane part of the channel molecule.

Introduction

Voltage-sensitive sodium channels that govern the depolarizing currents of the action potential have been isolated from several sources including eel electroplax, rat brain, and rat and rabbit muscle (for review, see Ref. 1). Their complete amino acid sequences [2–7] as well as from rat and human heart [8,9] have been elucidated and several models [2,3,10–13] have also been proposed to describe the structure of the sodium channel molecules in membranes, i.e., to illustrate which regions of the polypeptide chain are transmembrane and which are exposed on the cell surface or in the cytoplasmic side. These model are mostly based on the analyses of hydrophobicity, probable secondary structure, and regional conservation of amino acid sequence. Several pieces of experimental evidence mainly in hydrophilic segments [14–17,29] have been reported, but it is still required to accumulate more information to draw a fine structure of the sodium channel. We have prepared three antibodies against synthetic peptides corresponding to three particular regions, N- and C-terminal portions as well as one of the proposed transmembrane segments S4, of eel electroplax sodium channel and used them to study structure and topography of the sodium channel. The results obtained permit us to refine its topography.

Experimental methods

Peptide synthesis and antibody generation. Synthetic peptides corresponding to particular regions of eel electroplax sodium channel sequence [2] were synthesized by the solid phase method [18]. Polyclonal antisera were raised in rabbits against peptides corre-

Correspondence to: H. Nakayama, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060 Japan.
[1] Present address: National Chemical Laboratory for Industry, Tsukuba 305 Japan.
[2] Present address: National Cardiovascular Center, Research Institute, Suita, 565 Japan.
[3] Present address: Toyama Women's College, Gankaiji 444, Toyama, 930-01 Japan.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

sponding to the following amino acid residues of the channel sequence: (13–23) plus C-terminal cysteine (13–23C), (1419–1431) plus N-terminal tyrosine (Y1419–1431]), and (1809–1820). Peptides were purified by reverse-phase HPLC and their structures were confirmed by amino acid sequence analysis and amino acid analysis. Peptide (13–23C) was coupled with porcine thyroglobulin using *N*-hydroxysuccinimidyl-*m*-maleimidobenzoate through cysteine, as described previously [27]. Two other peptides (7–9 mg) were dissolved in 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) and coupled with porcine thyroglobulin (10 mg) by addition of 120 μl of 5% glutaraldehyde. After incubation for 40 min on ice, the reaction was terminated by extensive dialysis against phosphate-buffered saline (PBS). Three conjugates were used for immunization in rabbits.

Solid-phase ELISA. The binding of the antisera to the synthetic peptides and to sodium channel was analyzed by indirect solid phase ELISA [19]. The plates were coated with 50 μl of either the peptide (1 μg/ml) or the purified sodium channel (1 μg/ml) in 50 mM sodium carbonate buffer (pH 9.6). Alkaline phosphatase-conjugated goat anti-rabbit IgG was used for the second antibody followed by colorimetry with *p*-nitrophenyl phosphate as substrate.

Preparation of purified sodium channel from eel electroplax. Membrane fragments of electroplax from *Electrophorus electricus* were solubilized and purified by successive chromatography of DEAE-Sephadex and Sepharose 6B as described in the literature [20].

Proteolysis of the purified sodium channels. Concentrated Tris-HCl (4 M, pH 9.0) was added to the purified sodium channel (10–15 μg/50 μl) to make 40 mM Tris-HCl (pH 9.0) and 0.099% Lubrol-PX at a final concentration. The sample solution was then digested with lysyl endoproteinase (*Achromobacter* proteinase I) ($S/E = 100$, w/w) at 37°C for 12 h. The digested sample was subjected to the electrophoresis.

Electrophoresis and immunoblot. SDS-PAGE was carried out using 6% gel of polyacrylamide for the sodium channel or 10–30% gradient polyacrylamide gel for the proteinase-digested samples. Either of electrophoresed polypeptide bands or digested fragments on the gel were electrotransferred onto a nitrocellulose [21] or a diazophenylthioether paper (from Schleicher and Schuell, Ref. 22), respectively, and performed the immunoblot analysis [23]. Alkaline phosphatase-conjugated goat anti rabbit IgG (Cappel) was used as second antibody and visualized with β-naphthyl phosphate and Fast blue B.

Affinity-purification of antibodies by peptide-coupled Sepharose. Peptide (13–23C) (3 mg, 2 μmol) dissolved in 1.5 ml of water was coupled to AH-Sepharose 4B (1.5 ml, Pharmacia LKB) with ethyl diisopropylcarbodiimide hydrochloride (40 mg, 210 μmol) at pH 5.5 (titrated with 0.5 M NaOH) by gentle agitation for 24 h at room temperature. The affinity resin that contains 1.25 μmol of the peptide was obtained after extensive washing the gel with 0.1 M $NaHCO_3$/0.5 M NaCl (pH 8.3) followed by 0.1 M sodium acetate buffer (pH 4.0)/0.5 M NaCl and PBS. Onto the affinity column, antiserum (0.2 ml) against peptide (13–23C) was loaded. The column was washed with PBS until the absorbance of the washes at 280 nm reached the blank level, followed by washes with 50 mM phosphate buffer containing 1 M NaCl. The adsorbed protein was then eluted with 0.1 M glycine-HCl (pH 2.6) and the eluted materials was collected into tubes that contained 1M Na_2HPO_4 to adjust the pH to ~ 7. Antibody activity in fractions were monitored by the ELISA and active fractions were pooled, dialyzed against PBS, and stored at 4°C for further use. Affinity resin attached with peptide (Y1419–1431) was similarly prepared and used for purification of the anti-(Y1419–1431) antibody.

Preparation of 'right-side-out' vesicles and antibody binding to the vesicles. 'Right-side-out' vesicles were prepared by centrifugal separation of the membrane preparation in isotonic Nycodenz solution and their orientation was determined by [^{3}H]saxitoxin binding to the vesicles with or without 0.01% saponin treatment, as described in the literature [14]. Binding of the affinity-purified antibodies to the orientated vesicles were similarly determined as described [14].

Colloidal gold immunolabeling. Electroplax was dissected out and cut into ~ 0.5 cm^3 blocks and fixed for 30 min with 1% (v/v) paraformaldehyde in PBS. The nonspecific binding sites were blocked by incubation with 1% bovine serum albumin (BSA) in PBS. The tissue was then incubated overnight with IgG fraction of either anti-(1809–1820) antibody or anti-(13–23C) antibody at 1:5 in PBS plus 1% BSA. After washing overnight, the tissue was incubated in colloidal gold (10 nm)-labeled protein A with 1% BSA in PBS. After an extensive wash in PBS, the tissue was fixed in 3% (v/v) glutaraldehyde in PBS. The fixed tissue was further treated in 1% (w/v) osmium tetroxide, dehydrated with series of ethanol, and processed for electron microscopy.

Results and Discussion

(1) Reactivity of the anti-peptide antibodies

In order to elicit antibodies, three synthetic peptides were conjugated to thyroglobulin and injected into rabbits. All the anti-peptide antibodies obtained exhibit high level of binding to their respective peptides in a solid phase ELISA (Fig. 1). However, they exhibited different reactivity with the sodium channel (Fig. 1). Two antibodies against the peptides corresponding sequences to (1809–1820) and (13–23C) show high reactivity but the antibody against the sequence

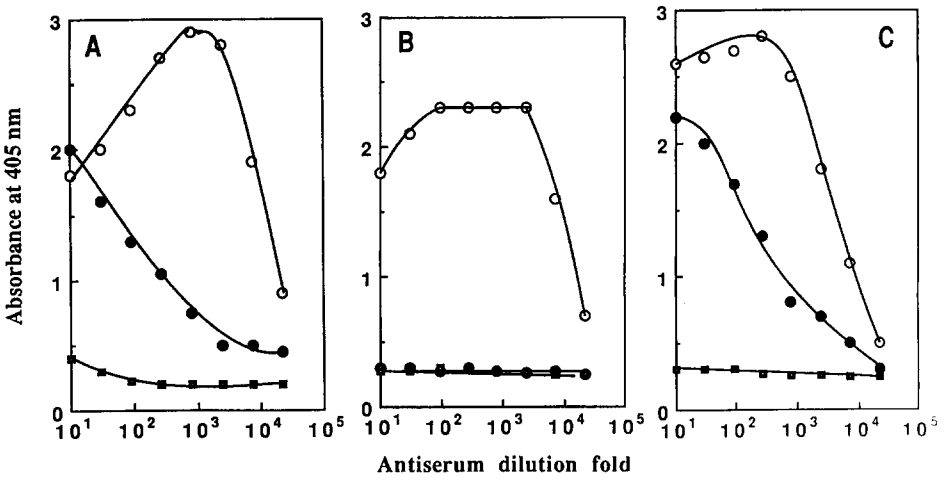

Fig. 1. Binding of anti-peptide antibodies to the corresponding peptides and the purified sodium channel from eel electroplax. The plates were coated with 50 μl of either the peptide (○, 1 μg/ml) or with the purified sodium channel (●, 2 μg/ml) in 50 mM sodium carbonate buffer (pH 9.6). Alkaline phosphatase-conjugated goat anti-rabbit IgG was used for the second antibody and color development for 10 min after the addition of *p*-nitrophenyl phosphate was determined by measuring absorbance at 405 nm. (A) Anti-(13–23C) antibody, (B) anti-(Y14199–1430) antibody, and (C) anti-(1809–1820) antibody. For control the binding of preimmune serum (■) is shown.

(Y1419–1431) does not. These results may reflect the exposure and/or conformation [24] of these sequences within the sodium channel molecule. In order to reduce this possibility, we carried out immunoblot analysis where the sodium channel protein was first electrophoresed on the polyacrylamide gel containing a detergent, SDS. Two antibodies against (1809–1820) and (13–23C) recognized the 250 kDa sodium channel protein, while the antibody against (Y1419–1431) still did not, as shown in Fig. 2. It is of note that both of anti-(1809–1820) antibody and anti-(13–23C) antibody did not crossreact with the sodium channel protein from rat brains. It has no homologous sequences to those of eel sequences' (1809–1820) and (13–23), suggesting that the recognition of the antibodies is in sequence-specific manners.

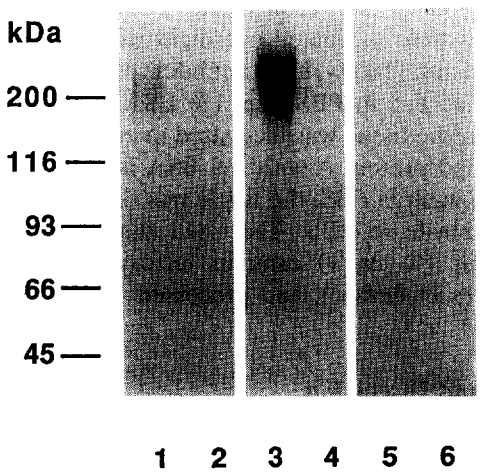

Fig. 2. Immunoblots of anti-peptide antibodies. SDS-PAGE of either partially purified eel electroplax sodium channel by DEAE-Sephadex and lysed P_3 fraction from rat brains was carried out using the 6% gel of polyacrylamide. Electrophoresed polypeptide bands were electrotransferred onto nitrocellulose and the immunoblot analysis was performed as described in Experimental methods. Electroplax sodium channels are immunoblotted with either anti-(13–23C) antibody (lane 1), anti-(1809–1820) antibody (lane 3), or anti-(Y1419–1431) antibody (lane 5). Rat brain sodium channels that are immunoblotted with each of three antibodies are shown in even numbers.

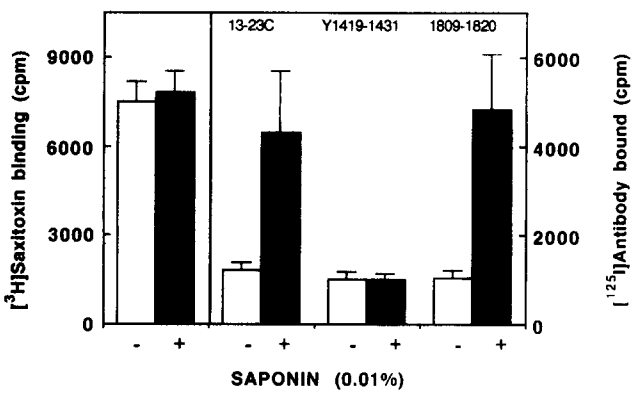

Fig. 3. Binding of [^{3}H]saxitoxin and the anti-peptide antibodies as identified by ^{125}I-labeled protein A binding. Sites labeled with [^{3}H]saxitoxin that binds on the external surface of the sodium channel, are all accessible in the intact 'right-side-out' vesicles, with little increase in binding detectable after permeabilization with 0.01% saponin (left pannel). Right pannel: The epitopes recognized by anti-(13–23C) antibody and anti-(1809–1820) antibody are not accessible to the external surface of the intact vesicles. Permeabilization increases bound antibodies against peptides (1809–1820) and (13–23C) 5-fold and 4-fold, respectively, implying cytoplasmic location for the two peptides. In contrast, the epitope recognized by anti-(Y1419–1431) antibody is accessible neither on the external surface of the intact vesicles nor after permealization with saponin, suggesting a possible transmembrane location.

(2) Antibody binding to the oriented vesicles

The topographic orientation of the peptide sequences recognized by two directed antibodies, anti-(1809–1820) antibody and anti-(13–23C) antibody, was evaluated by quantitating antibody bindings to oriented membrane vesicles from eel elctroplax. The specific bindings of the affinity-purified antibodies to these vesicles were assayed before and after permeabilization by treatment with saponin. The specific binding that is defined as binding displaceable by the presence of 10 μM corresponding synthetic peptide, was low for anti-(1809–1820) antibody to the intact vesicles. But 5-fold increase in the antibody binding was observed after treatment of the vesicles with saponin (Fig. 3). This is consistant with the previous observation by Gordon et al. [14] using antibody against the sequence 1783–1794 that is adjacent to C-terminal region. Similarly, the specific binding of anti-(13–23C) antibody increased by 4-fold after the treatment with saponin (Fig. 3). The sodium channels in these vesicles are assumed to be in a right-side-out orientation as indicated by the [^{3}H]saxitoxin binding data (Fig. 3). Therefore, the results of antibody bindings imply that not only the oligopeptide corresponding to the C-terminal but also the region adjacent to N-terminus of the sodium channel are accessible to their antibodies only on the cytoplasmic surface of the membrane.

On the other hand, anti-(Y1419–1431) antibody was not bound to the vesicles before and after treatment with saponin (Fig. 3). This is a clear contrast to the results of two other antibodies raised against N- and C-terminal portions. The result suggests that the region (1419–1431) must be located quite differently from those at N- and C-terminus.

(3) Immunogold electron microscopy of the antibodies directed to N- and C-terminus

To localize the sites of antibody binding to the sodium channel in tissue rather than vesicles without recourse to the detergent treatment, we examine sections of *E. electricus* electroplax by immunoelectron microscopy. Sectioned electroplax tissue that was

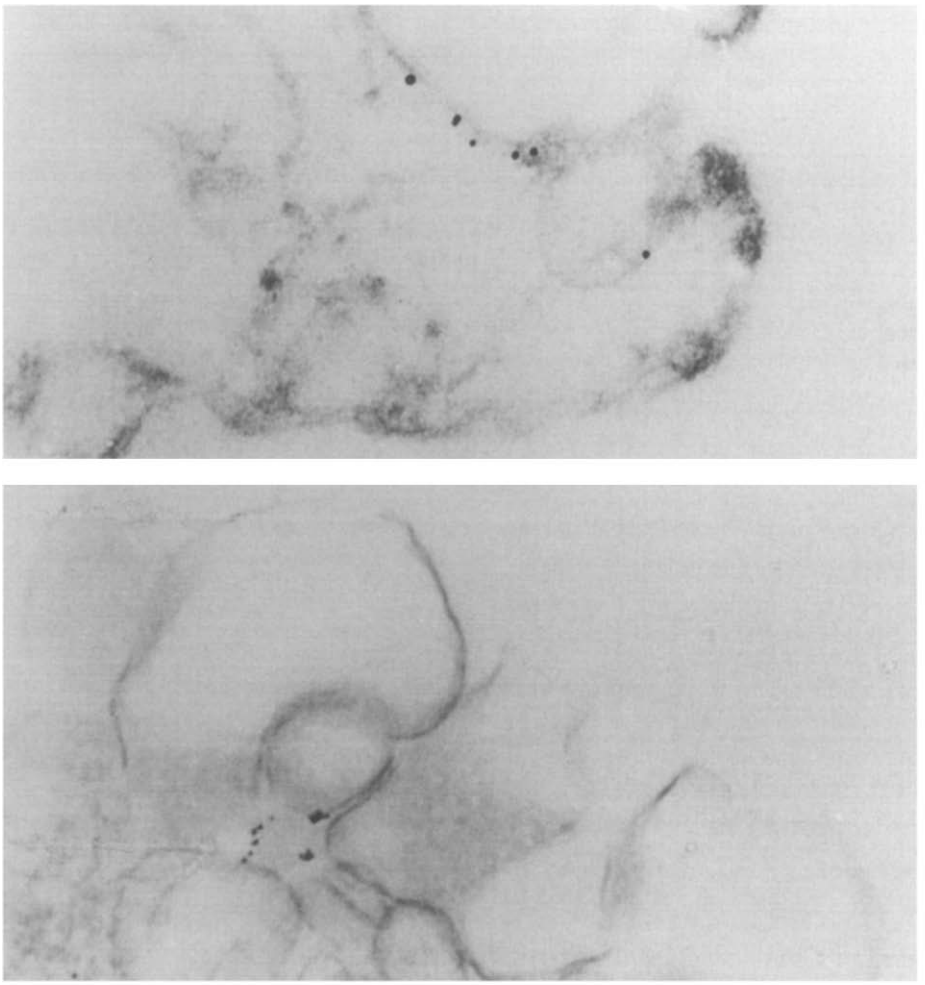

Fig. 4. Localization of anti-(1809–1820) antibody binding on the electroplax membrane. Gold particles were found on portion of the cytoplasmic surface of the membrane. (Original magnification: ×24000)

minced and lightly fixed, was incubated with the anti-peptide antibodies followed by protein A conjugated to colloidal gold. Gold particles were located on the cytoplasmic face of the membrane; not only the anti-(1809–1820) antibody (Fig. 4) but also the antibody against (13–23C) (Fig. 5) bound to the cytoplasmic side. The cytoplasmic location of the C-terminal region (1809–1820) is consistent with the previous observation that (1783–1794), which is at 15 residue's upper-stream than the present (1809–1820) sequence, is also cytoplasmic oriented [14]. The present result strongly suggests that proteolytic splicing near C-terminal region as reported in skeletal muscle calcium channel [28] is unlikely. In addition, this is the first demonstration by electron microscopy that N-terminal region is also located in the cytoplasmic side. These morphological

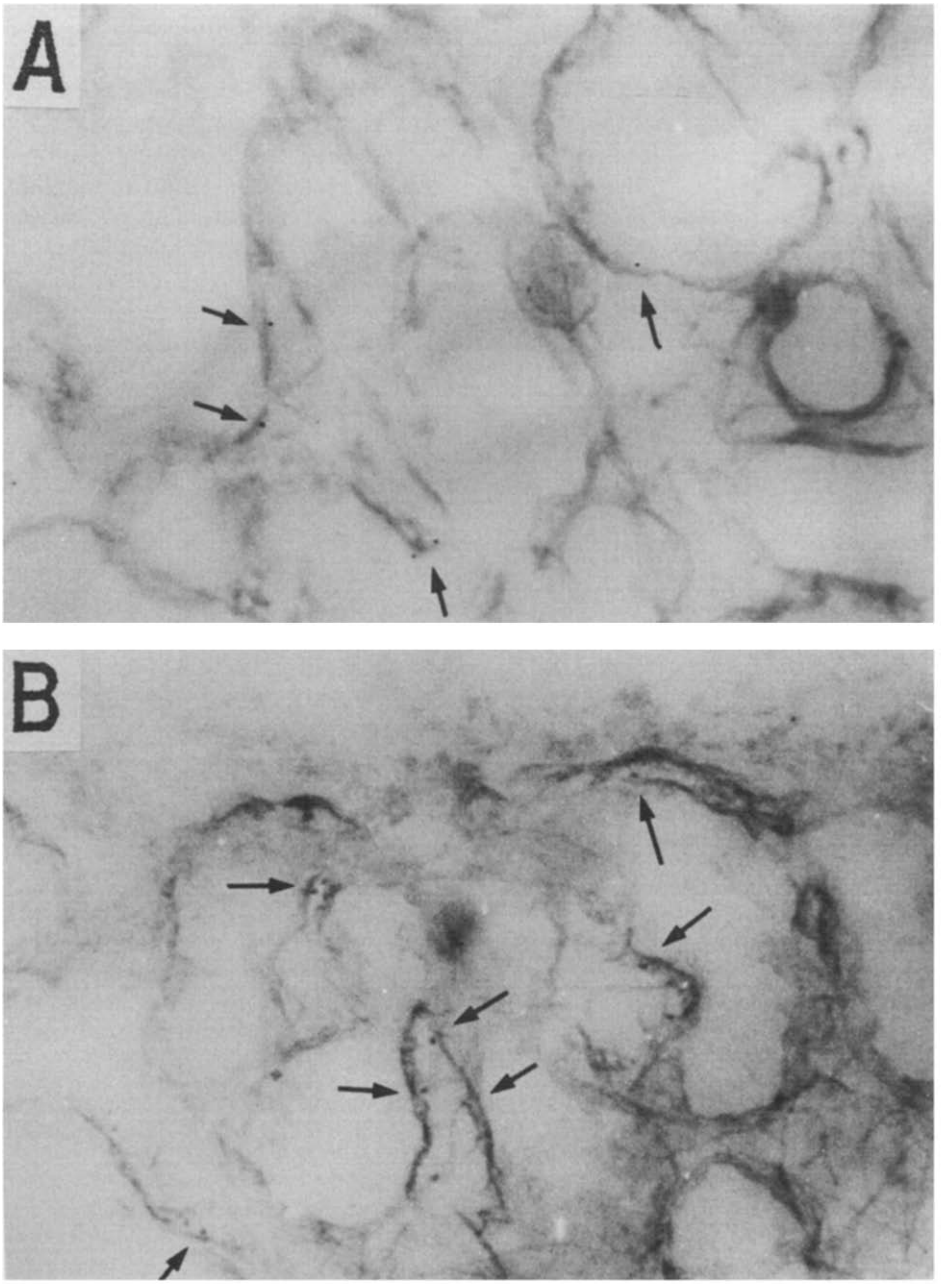

Fig. 5. Electron microscopic localization of anti-(13–23C) antibody on segments of electroplax membrane. (A) Colloidal gold particles identifying bound antibody are found on the cytoplasmic surface of the membrane (left portion) although an unfavorable location is observed (right portion). However, cytoplasmic location of colloidal golds are clearly observed in the membrane invaginations (B). Electroplax membrane without antibody treatment is also shown (C). (Original magnification: ×24000).

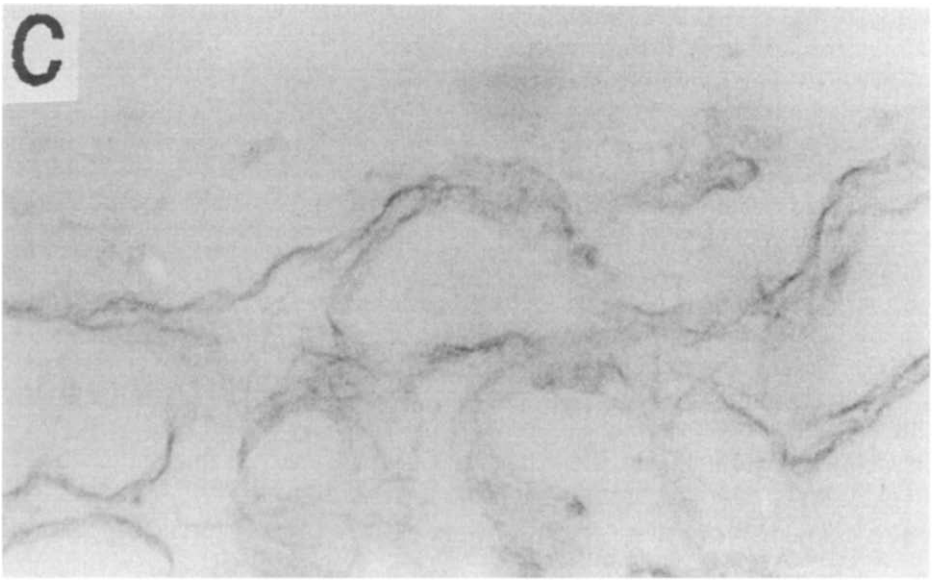

Fig. 5 (continued).

observations are in consistence with the antibody binding results in vesicles, although no saponin was used in the electron microscopic experiments.

(4) Conformational analysis of the region (1491–1431) with the site-directed antibody

In contrast to the cytoplasmic-oriented regions of sequence (13–23) and (1809–1820), (1419–1431) region of the sodium channel protein was not recognized by anti-(1419–1431) antibody before and after the saponin treatment as previously shown in Fig. 3. This suggests that the region may be located differently from those of (13–23) and (1809–1820), and possibly in transmembrane. In immunoblot analysis where lipid molecules were removed from the channel preparation by SDS-PAGE, the sodium channel protein did not react with the antibody, as shown in Fig. 2. Such insensitivity of the channel protein to the anti-peptide antibody recognition suggests that the region still has some ordered structures even after the temporal exposure with SDS. This interpretation is not surprising if we find reports on structural renaturation of proteins after transferring them to membrane sheets; e.g., enzyme activities lost during SDS-PAGE were recovered after transferring the enzyme proteins to membrane sheets [25,26].

In order to assess this possibility experimentally, we digested the channel protein with lysyl endoproteinase and analyzed the digest by immunoblot if any peptide fragment can react with the antibody. As shown in Fig. 6, anti-(1419–1431) antibody apparently stained four fragments of ~3, ~8, 16, 21 kDa. By inspection of the potential cleavage site with lysyl endoproteinase (lysine residues) outside the transmembrane segments, these fragments are possibly derived from cleavage at the following sites: Lys-1408 (N-terminus) and Lys-1435 (C-terminus; calculated molecular mass, 3.2 kDa), Lys-1408 (N-terminus) and Lys-1478 (C-terminus; calculated mass, 8.6 kDa), Lys-1351 (N-terminus) and Lys-1478 (C-terminus; calculated mass, 15.6 kDa), and Lys-1299 (N-terminus) and Lys-1478 (C-terminus; calculated mass, 21.6 kDa), respectively.

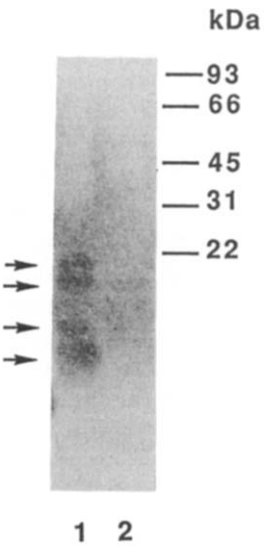

Fig. 6. Immunoblot of proteinase digested sodium channel with anti-(Y1419–1431) antibody. Purified sodium channel (10 μg/50 μl) in 40 mM Tris-HCl (pH 9.0) and 0.099% Lubrol-PX was digested with lysyl endoproteinase (*Achromobacter* proteinase I) ($S/E = 100$, w/w) at 37°C for 12 h. Samples before (lane 2) and after (lane 1) the digestion were subjected to the electrophoresis on a 10–30% gradient polyacrylamide gel followed by electrotransfer onto a diazophenylthioether paper. Immunoblot with anti-(Y1419–1431) antibody was carried out as described in Experimental methods.

Therefore, the region (1419–1431) in proteolytic fragments is very likely to have rather random conformation that the anti-(Y1419–1431) antibody can react with easily, whereas the region in intact sodium channel protein is in some ordered structures that the anti-peptide antibody can not recognize.

Sequence (1419–1431) is a major constituent of S4 in internal repeat IV of the sodium channel. S4 segments have been poroposed to exhibit strongly amphipathic and to form a possible α-helix or 3_{10}-helix structure [2]. It is demonstrated that amphipathic peptides such as melittin [30] and mastparan [31] have rather random structures in dilute aqueous solution but they form highly ordered α-helix structure in lipid bilayers. In analogy to the observations, our result that the sodium channel was recognized by the anti-(Y1419–1431) antibody only after it was digested by proteinase, is interpreted that the region of (1419–1431) in IVS4 is very likely to be transmembrane-oriented. The site-directed antibody can be only accessible to the region (1419–1431) after digesting to the peptide fragments of ~ 3, ~ 8, 16, 21 kDa, which have no such strict conformations of α-helix or 3_{10}-helix as that of native sodium channel in lipid bilayer.

It is known that antibodies raised against peptide antigens recognize not only their linear sequences but also their conformations [24]. Therefore, some anti-peptide antibodies can not react with parent protein molecules that apparently include the peptide sequences of antigens but have different conformations from the antigen peptides or their conjugates with carrier proteins [24, 32]. Anti-(Y1419–1431) antibody is regarded as such an antibody. Another result that the antibody did not react with intact sodium channels in immunoblot, suggests that the region possibly forms rather ordered conformations in intact sodium channels than random structures. The conformations may be different from highly ordered structures in native sodium channels, since the channel proteins were not surrounded by lipid molecules after SDS-PAGE.

This is an initial experimental result for topographical characterization that S4 segment region, in repeat IV at least, is transmembranous. Further studies using different experimental techniques are still required for refinement of the present observations.

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