Characterization of Saccharide Moiety in the Electroplax Sodium Channel

Hitoshi Nakayama, a,b Shigeki Yamamoto, c Yasumaru Hatanaka, c Tetsuaki Hachis, d Susumu Tsunawasa, d Fumio Sakiyama, b and Yuichi Kano. a, c

Faculty of Pharmaceutical Sciences, Hokkaido University, a Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan and Institute for Protein Research, Osaka University, c 1-6, Yamadaoka, Suita 565, Japan. Received May 11, 1992

Carbohydrate chains on the large peptide of the voltage-sensitive sodium channel from Electrophorus electricus electroplax have been partially characterized by the lectin-blotting technique combined with digestion using three glucosidases: neuraminidase, endo-β-N-acetylgalactosaminidase H, and peptide-N-glycosidase F. The results show that both N-linked oligosaccharides and O-linked (mucin-type) oligosaccharides are present. In N-linked oligosaccharides, the results suggest the presence of complex- and hybrid-type oligosaccharides which contain bisecting N-acetylgalactosamine(s), as well as the complex-type oligosaccharides with the α-Fuc-GlcNAc-(Asn) residue(s). In O-linked oligosaccharides, they must carry Galβ1→3GalNAC-moieties which contain NeuAc residues in the terminal.

Keywords oligosaccharide chain; sodium channel; lectin-blot; glycosidase; N-glycoside; O-glycoside

Introduction

Voltage-sensitive sodium channels that are responsible for the depolarizing currents of the action potential have been isolated from several sources including eel electroplax, rat and rabbit muscle and rat brain (for review, see ref. 2). Successive efforts following the first elucidation of the primary structure of electroplax sodium channel 3) have made it possible to clone and sequence the sodium channels from some other origins. 2) The functionally important component of each channel species is a large polypeptide (M, 250—300 kDa). It is a striking characteristic that all of the large sodium channel peptides so far studied are heavily glycosylated. 2) This large peptide (250 kDa) is the sole component of the electroplax sodium channel to express its entire function, and contains approximately 29% carbohydrate by mass. 4) Its sugar composition, 4) the existence of α-2,8-linked polysialic acids, 5) and sensitivity to several glycosidases 3) have been reported. However, no further structural characterisation of the carbohydrate moiety has been performed. We therefore undertook to collect information about carbohydrate moieties as the first step in elucidating their structures. The carbohydrate moieties are examined by lectin-blotting technique 6) with five lectins of different specificities after successive digestion with three glycosidases of the electroplax sodium channel protein. In this paper, we present partial characterization of the carbohydrate chains of the electroplax sodium channel.

Results and Discussion

Carbohydrate Composition of the Eel Electroplax Sodium Channel The purified sodium channel protein shows a broad but single band at 250 kDa (Fig. 1A) owing to heavy glycosylation of the polypeptide portion which is homo-

![Fig. 1. Lectin Blotting of the Sodium Channel with HRP-Conjugated WGA before and after Glycosidase Treatments](image-url)

(A) The purified eel electroplax sodium channel was analyzed by SDS-PAGE on a 6% polyacrylamide gel and silver-stained. Only a single protein band was observed at ca. 250 kDa. The sample was used for the subsequent experiments. (B) The purified sodium channel on SDS-polyacrylamide gel was electrophoretically transferred to Durapore filter (DF) or nitrocellulose (NC) sheets. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated WGA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; Endo H, endo-β-N-acetylgalactosaminidase H; PNGase F, peptide; N-glycosidase F) on the sheets. Only the regions of the lectin-blotted sheets at molecular mass higher than 200 kDa are shown. Symbols + + +, + +, +, and − show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

© 1992 Pharmaceutical Society of Japan
Table 1. Carbohydrate Composition of the Purified Sodium Channel from E. electricus

<table>
<thead>
<tr>
<th>Carbohydrate/sodium channel protein (mol/mol)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>17^a</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>4^h</td>
</tr>
<tr>
<td>Mannose</td>
<td>29^f</td>
</tr>
<tr>
<td>Fucose</td>
<td>6^g</td>
</tr>
<tr>
<td>Galactose</td>
<td>24^g</td>
</tr>
<tr>
<td>Xylose</td>
<td>34^d</td>
</tr>
<tr>
<td>N-Acetylaneuraminic acid</td>
<td>92^d</td>
</tr>
</tbody>
</table>

^a) Moles of the purified sodium channel protein were determined by amino acid analysis and calculated as 95 mol for lysine residues based on the reported value from the total primary structure. ^b) Determined simultaneously by amino acid analysis. ^c) Determined by neutral carbohydrate analysis as described in Experimental. ^d) Determined by sialic acid analysis as described in Experimental.

Carbohydrate composition of the purified channel protein was determined in terms of molar ratio to the sodium channel protein, based on the molecular mass of 250 kDa (Table I). The molecular mass of the total carbohydrates was calculated as 45 kDa, which is in good accordance with the expected value obtained by subtraction of protein mass (20382 Da) calculated based on the primary structure^3) from the molecular mass of the intact sodium channel (250 kDa) estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Fig. 1A). N-Acetylaneuraminic acid (92 mol/mol of channel) is the most abundant component, as has been indicated in the literature.4,5) In addition to glucosamine, galactosamine was determined for the first time to be component in the electrophores sodium channel. Taken together with the determination of a considerable amount of galactose residues, the presence of O-linked saccharides (mucin-type) was indicated in the channel molecule.

Lectin-Blot Assay of Oligosaccharide Chains in the Electroplax Sodium Channel before and after Treatment with Glycosidases The electrophores sodium channel protein was purified by SDS-PAGE, electrotransferred to a Durapore filter sheet and assayed with horseradish peroxidase (HRP)-conjugated lectins. This procedure was used for the assay before and after treatment with glycosidases, neuraminidase (NA) and peptide: N-glycosidase F (PNGase F). However, when channel protein (eggwhite albumin as well) was treated with endo-β-N-acetylgalactosaminidase H (Endo H) on the Durapore filter, it did not react with any of five lectins (WGA, E-PHA, RCA120, PNA, and LCA) tested. Then, we substituted a nitrocellulose sheet for the Durapore sheet, on which the channel protein could be tested with the five lectins before and after Endo H treatment.

(i) Assay with Wheat Germ Agglutinin (WGA): WGA–HRP heavily stained the sodium channel blotted on the sheet, but the staining was not heavy after NA treatment (Fig. 1B). Treatment of the NA-digested channel protein with Endo H remarkably decreased the reactivity with WGA. Furthermore, upon PNGase F digestion, the NA-digested channel protein lost its WGA reactivity completely. These results suggest that the electrophores sodium channel protein bears NeuNAc and bisecting GlcNAc moieties in both hybrid-type N-glycans and complex-type N-glycans.

Fig. 2. Detection of Oligosaccharide Chains of the Sodium Channel with HRP-Conjugated E-PHA

The purified sodium channel on SDS-polyacrylamide gel was electrophoretically transferred to Durapore filter (DF) sheets. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated E-PHA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase, PNGase F, peptide: N-glycosidase F) on the sheets. Symbols ++ and + show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

Fig. 3. Detection of Oligosaccharide Chains of the Sodium Channel with HRP-Conjugated RCA120

The purified sodium channel was transferred to Durapore filter (DF) or nitrocellulose (NC) sheets by Western blotting. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated RCA120, before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase, Endo H, endo-β-N-acetylgalactosaminidase H, PNGase F, peptide: N-glycosidase F) on the sheets. Symbols ++, ++, +, and + show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

(ii) Assay with Erythroagglutinating Phytohemagglutinin (E-PHA): E-PHA–HRP stained the channel protein clearly on a Durapore filter (Fig. 2). NA treatment did not change the reactivity of the protein with E-PHA and the subsequent digestion with PNGase F abolished the reactivity to the lectin. These results support the suggestion described above, since E-PHA has specificity for biantennary and triantennary complex-type N-glycans. 7–9)

(iii) Assay with Ricinus communis Agglutinin120 (RCA120): RCA120 can specifically bind both galactose residue at the nonreductive terminal and Galβ1→4GlcNAc moiety in complex- and hybrid-type N-glycans strongly, and Galβ1→3GlcNAc moiety in mucin-type oligosaccharides moderately.10–12) RCA120–HRP stained the intact channel protein only slightly, but the protein was...
Fig. 4. Reaction of HRP-Conjugated PNA with the Sodium Channel before and after Treatment with Glycosidases

The purified sodium channel was transferred to Durapore filter (DF) sheets by Western blotting. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated PNA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; PNGase F, peptidase N-glycosidase F) on the sheets. Symbols + + + + + show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

Fig. 5. SDS-PAGE Analysis of the Electroplax Sodium Channel before and after Treatment of Glycosidases

The purified sodium channel was treated in the absence (lane 1) and presence of neuraminidase (lane 2) or neuraminidase followed by PNGase F (lane 3). After treatment, the samples were boiled for 3 min in the 50 mM dithiothreitol, subjected to SDS-PAGE on a 6% polyacrylamide gel, and stained with silver.

Fig. 6. Reaction of HRP-Conjugated LCA with the Sodium Channel before and after Treatment with Glycosidases

The purified sodium channel protein was transferred to Durapore filter (DF) sheets by Western blotting. Oligosaccharide chains of the sodium channel were detected with HRP-conjugated LCA before (no treatment; N.T.) or after treatment of either glycosidase (NA, neuraminidase; PNGase F, peptidase N-glycosidase F) on the sheets. Symbols + + + + + show qualitative degree of HRP-catalyzed color development which reflects the extent of the blotted lectin.

heavily stained after NA treatment (Fig. 3). This is due to the exposure of terminal galactoside residue caused by removal of sialic acid residues. After Endo H digestion, the NA-treated protein was weakly stained by RCA<sub>120</sub>–HRP stain on nitrocellulose sheets, the presence of hybrid-type N-glycans containing Galβ1→4GlcNAc moieties being indicated. PNGase F digestion significantly reduced the reactivity of the NA-treated protein to RCA<sub>120</sub>–HRP stain indicating the presence of complex-type N-glycans. The fact that NA and PNGase F-treated protein retains the reactivity to RCA<sub>120</sub> suggests strongly that the Galβ1→3GlcNAc moiety exists in the portion of mucin-type oligosaccharides which is resistant to the PNGase F digestion of the electroplax sodium channel protein.

(iv) Assay with Peanut Agglutinin (PNA): PNA recognizes Galβ1→3GlcNAc moieties in mucin-type oligosaccharides strongly, and Galβ1→4GlcNAc moieties in complex- and hybrid-type N-glycans moderately.<sup>101</sup> This is rather the inverse of the specificity of RCA<sub>120</sub>. NA treatment markedly enhanced the reactivity of the channel protein to PNA as is well shown in (iii). However, the behavior of the protein at the PNGase F digestion was different: it retained the reactivity to the lectin (Fig. 4). These are complementary results obtained with RCA<sub>120</sub>. Since PNGase F removes all the N-glycosidic moieties in the NA-treated channel protein, as shown by the insensitivity to WGA, E-PHA, and LCA (see section v), it is deduced that the Galβ1→3GlcNAc moiety exists in the portion of mucin-type oligosaccharides.

The observation that molecular size lowered to ca. 200 kDa by NA- and PNGase F-treatments on SDS-PAGE (Fig. 5), which is in good accordance with the molecular mass of channel protein (208321 Da) estimated by the primary structure, also supports that PNGase F effectively cleaves N-linked oligosaccharides of the NA-treated sodium channel. Even after such extensive removal of N-glycans, some residual microheterogeneity observed as a rather broad band for the channel protein of ca. 200 kDa (lane 3 in Fig. 5), is another evidence for the existence of O-glycans. The primary amino acid sequence includes 10 potential N-glycosylation sites,<sup>39</sup> 9 of which may be extracellular and 7 of which are conserved in two brain polypeptides that have been cloned.<sup>13</sup> Remarkable reduction in the apparent molecular mass of the channel protein (from 250 kDa to ca. 200 kDa) by removing N-glycans suggests that N-linked oligosaccharides are major carbohydrate constituents in the electroplax sodium channel protein: grossly ca. 24 saccharide residues per one of the 10 potential N-glycosylation sites by assuming 210 as average molecular weight of saccharide unit. The number of O-linked saccharide units may be less than 4, the determined galactosamine residues (Table 1).

(v) Assay with Lens culinaris Agglutinin (LCA): LCA–HRP stained both intact and NA-treated sodium channel protein lightly; the extent of staining in the two being similar. The NA-treated protein lost the reactivity to LCA upon PNGase F digestion (Fig. 6), suggesting the presence of a complex-type oligosaccharide bearing the α-Fuc→GlcNAc-(Asn) residue.

Oligosaccharide Chains in the Electroplax Sodium Channel

In the present work, we have characterized the gross structures of several oligosaccharide chains in the electroplax sodium channel from Electrophorus electricus by the lectin-blotting technique combined with digestion using glycosidases of different substrate specificity. This technique was first developed by Kijimoto-Ochiai <i>et al.</i><sup>60</sup> and
proved feasible for detection of oligosaccharide moieties in situ using several glycoproteins of known oligosaccharide structure, although there are some limitations; for example, detection is qualitative rather than quantitative, and broad specificity and cross-reactivity of lectins sometimes hamper elucidation of the exact structure of oligosaccharide chains.\(^5\) However, the present work shows that the technique affords information reasonable and consistent with chemical compositional analysis and new information as well. The results strongly suggest that not only N-linked oligosaccharides but also O-linked (mucin-type) oligosaccharides are present in the sodium channel protein, although the former are more abundant.

As N-linked oligosaccharides, they suggest the presence of complex- and hybrid-type oligosaccharides which contain bisecting N-acetylglucosamine, as well as complex-type oligosaccharides with the -z-Fuc-GlcNAc(Asn) residue. Although the presence of hybrid-type N-glycans was suggested previously in the electroplax sodium channel,\(^5\) no further information on the N-linked oligosaccharide chain structures has been available.

This is the first demonstration that mucin-type oligosaccharides are actually present in the sodium channel. Galβ1→3GalNAc moieties might be included in the mucin-type oligosaccharide chains, as the results obtained using two different lectins, RCA\(_{120}\) and PNA, are complementary. This is also supported by the present data of carbohydrate composition analysis that galactosamine residues as well as a considerable amount of galactose residues were detected. The molecular mass of the channel protein (250 kDa) was reduced to ca. 210 kDa by NA digestion, while further PNGase F treatment caused decrease in its molecular mass to 200 kDa, only by 10 kDa, demonstrating that siadic acid is apparently the major component of the carbohydrate composition as observed previously.\(^4,5,14\) -z,2,8-Linked siadic acid homopolymers which are uncommon in vertebrate cells were characterized in the electrophil sodium channel.\(^5\) Siadic acids may contribute to surface charge effects on ion conductance and neurotoxin interactions.\(^17\) As siadic acid residues can be removed efficiently by the NA-treatment as shown here, it will be intriguing to assess their functional significance in the sodium channel.

**Experimental**

**Materials** Lectins (WGA, E-PHA (Phaseolus vulgaris agglutinin), RCA\(_{120}\), PNA, LCA) conjugated with HRP were purchased from Hohen Oil Company. NA from Azcomobacter ureafaciens was purchased from Nakarai Chemical Ltd. Enzymes from Streptomyces griseus and PNGase F from Flavobacterium meningosepticum were obtained from Seikagaku Kogyo Company Ltd. and Boehringer Mannheim, respectively. Durapore and nitrocellulose sheets were obtained from Millipore. Other chemicals (reagent grade) were obtained commercially.

**Isolation of the Sodium Channel Protein from Electrophil** Lubrol PX extracts from Electrophorus electricus electroplax were further purified by successive chromatography of DEAE-Sephadex, Sepharose 6B, Sepharose 4B, and TSK G4000SW, as described previously.\(^5\)

**Carbohydrate Composition Analysis** The purified sodium channel proteins (100 pmol) were dissolved in 100 μl of 0.1 M ammonium bicarbonate and subjected to composition analysis as follows:

1. Analysis of Neutral Carbohydrates: To ten microliters of the sample was added 1 N trifluoroacetic acid (TFA, 100 μl) and the resultant solution was sealed in a Pyrex tube in vacuo, followed by hydrolysis at 110 °C. After 6 h, the acid solvent was evaporated to dryness and residual materials were dissolved into water (50 μl) and subjected to analysis.

<table>
<thead>
<tr>
<th>References and Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Present address: Toyama Women’s College, Gionkai 444, Toyama 930-01, Japan.</td>
</tr>
</tbody>
</table>