Characterization of Rat Sodium Channels in Neuroendocrine Cells Using Region-specific Antibodies

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ABSTRACT

Using two anti-peptide antibodies, which recognized a conserved region within subtypes of α -subunit of rat sodium channel and a relatively divergent sequence specific to subtype II (R_{II}), rat sodium channels in neuroendocrine cells were examined. Rat adrenal medulla was stained by both antisera; RY 137, which was raised against peptide corresponding to well-conserved region of α -subunit between domain 4 segment 5 (D4S5) and D4S6, stained the nucleus of medulla cells, while RY 143, which was raised against R_{II} -specific region between D1S6 and D2S1, stained the cellular membrane. Antisera inhibited the cell burst response of mouse neuroblastoma cell line, Neuro-2a, against the combination treatment with ouabain, veratridine and brevetoxin PbTx-1. These results indicate clearly the region-specific antibodies produced in the present study were useful for detection and characterization of sodium channels in rats.

Voltage-dependent sodium channels are trans-membrane glycoprotein responsible for sodium ion influx upon the rise in action potential in neurons, skeletal muscles, cardiac and neuroendocrine cells (1, 3, 5, 9, 11), and are composed of a large α -subunit of 260 kDa as the principal component, which consists of four domains of internal homology. The major actions of the channels reside in the primary structure of this subunit. The purified α -subunit protein has been found to be associated with one or two additional proteins such as $\beta 1$ and $\beta 2$ subunits, although the functional significance of these subunits is unclear.

cDNA clones isolated from rat brain encoded

three different α -subunit mRNAs and those have been sequenced as rat sodium channel subtype I-III (R_I - R_{III}) (2, 16, 18, 23). The multiple sodium channel subtypes have been found to be expressed not only in organ-specific way (30) but also in tissue-specific (11, 13). Specific subcellular localization has also been shown in rats (29).

Anti-peptide antibodies against the fragments of sodium channels were used to identify the localization of sodium channels or of each sodium channel subtype in rat brain and spinal cord (12, 13, 29). Further, the binding sites for sodium channel-directed neurotoxins (25, 26) and functions of specific sites (4, 8, 21) were investigated. In rat brain, subtype II (R_{II}) was shown to be approximately 80% of total sodium channels, and was preferentially expressed in the rostral areas of central nervous system (13, 29).

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Expression of R_{III} seems to be restricted to embryonic and neonatal period (10). Other subtypes of sodium channel have been reported in other organs and tissues (6).

Voltage-dependent sodium channels have also been identified in several neuroendocrine cells which are derived from the neural crest (9). These cells include C-cells, chromaffin cells and pheochromocytoma cells.

We have prepared two different antibodies, one of which, RY 137, was raised in rabbits against synthetic peptide corresponding to a conserved sequence within R_I and R_{II} [Na channel- α 1686–1705 according to sequence shown by Noda *et al.* (23): KREVGIDDMFNFETFGNSMI], and the other, RY 143, against peptide corresponding to subtype-specific region for R_{II} (Na channel- α 467–485: ASAESRDFSGAGGIGVFSE). The characteristics of those antibodies in the immunohistochemical staining of rat adrenal medulla and in biological activities on neuroblastoma cells were described in this report.

MATERIALS AND METHODS

Synthesis of Peptide Immunogen

Peptides related to α -subunit of rat sodium channels [rat Na channel- α (1686–1705): KREVGIDDMFNFETFGNSMI; rat Na channel- α (467–485): ASAESRDFSGAGGIGVFSE] were synthesized by solid phase methodology with the Fmoc-strategy using an automated peptide synthesizer (PerSeptive model 9050 plus, USA) in the manner described previously (22, 32). Briefly, the peptide chain was elongated on polyethylene glycol-polystyrene resin (0.80 g). Fmoc-amino acid derivatives (4 equimoles) were activated in the presence of *o*-(7-azabenzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate (HATU) (4 equimoles) and diisopropylethanolamine (DIEA) (6 equimoles). The coupling was performed at room temperature for 60 min. After construction of the desired protected peptide resin, the protected peptide resin (1.21 g) was treated with trifluoroacetic acid (24 mL) in the presence of *m*-cresol (0.6 mL), 1, 2-ethanedithiol (1.8 mL) and thioanisole (3.6 mL) at 25°C for 120 min, followed by treatment of 20% piperidine/DMF. The resin was removed by filtration, anhydrous ethylether was added to the filtrate to give the crude product. The crude peptide was purified by reverse phase HPLC on a column of YMC-Pack D-ODS-5 (2.0 $\times$ 25.0 cm)

using 0.01 N HCl/CH₃CN (75/25–60/40, v/v, 30 min) as eluent at a flow rate of 7 mL/min to yield the desired peptides. Purity of the peptides was assessed by analytical HPLC on a column of YMC-Pack D-ODS-5 (0.46 $\times$ 25.0 cm) using 0.01 N HCl/CH₃CN (95/5–40/60, v/v, 30 min) as eluent at a flow rate of 1 mL/min, and after hydrolysis of synthetic peptides in 6 N HCl/1% phenol at 110°C for 24 h, the amino acid analysis of the ensuing hydrolysates were performed using a Beckman System 7300 (Beckman, USA).

Anti-synthetic peptide antibody

Production of antibodies was carried out in the following manner. Synthetic peptide (1.5 mg) without conjugation with macromolecular protein was stirred with a 50% (w/v) polyvinylpyrrolidone (MW 25,000–30,000, Merck, Darmstadt, Germany) in 1.5 mL of saline for 2.5 h at 25°C. The mixture was emulsified with 1.5 mL of Freund's complete adjuvant (Calbiochem Behring, La Jolla, CA, USA) for 10 min in an ice bath. The emulsion was injected intradermally into two Japanese white female rabbits (about 2.5 kg). For primary immunization, each rabbit received a portion of the emulsion containing approximately 1 mg of peptide. Booster injections were given at two-week intervals using half the dose of the immunogen used for the primary immunization. After the fourth injection, the rabbits were bled from the marginal ear vein at ten days after each booster injection. Serum was then subjected to a titer check. Thus, RY 137 which used rat Na channel- α (1686–1705) KREVGIDDMFNFETFGNSMI as immunogen, and RY 143 which used rat Na channel- α (467–485) ASAESRDFSGAGGIGVFSE as immunogen were obtained.

Immunohistochemistry

Male Wistar rats weighing about 200 g were used for the immunohistochemical examination. The animals were deeply anesthetized by an intraperitoneal injection of pentobarbital, and perfused with a physiological saline followed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The adrenal gland was removed and immersed in the same fixative for additional 6 h. The fixed tissues were dipped in 30% sucrose solution for overnight at 4°C and quickly frozen in liquid nitrogen. Frozen sections about 20 μ m

Fig. 1 Immunohistochemistry for Na channel II in the adrenal gland of rats. The immunoreactivity with RY 143 is found selectively in medullary endocrine cells, while the cortex (asterisk) is free from any positive reaction (a). In some chromaffin cells, the intense reactivity with RY 143 appears to be localized at the cell membrane (arrows in b). Antiserum RY 137 also stains cell bodies of chromaffin cells, but more intense reactivity is found in nuclei of the cells (c) a $\times 200$; b and c $\times 400$

thick were prepared in a cryostat (CM 3050; Leica, Nussloch, Germany) and mounted on poly-L-lysine-coated glass slides.

Immunostaining of sodium channel was carried out according to the avidin-biotin complex (ABC) method. The sections were treated with 0.3% Triton X-100 in 0.1 M phosphate buffered saline (pH 7.3) for 1 h and then with 0.3% H_2O_2 in methanol for inhibition of endogenous peroxidase activity. After treatment with a blocking serum for 30 min, the sections were incubated with rabbit serum against rat Na channel II (RY 137, RY 143) diluted in 1: 3,000 overnight at room temperature. The sections were then incubated with biotinylated goat anti-rabbit immunoglobulins and ABC (Histofine kit; Nichirei, Tokyo, Japan). The antigen-antibody reaction was visualized by incubation in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% 3,3'-diaminobenzidine and 0.002% H_2O_2 .

Cell Assay

Mouse neuroblastoma cell line (Neuro-2a, ATCC CCL-131) was maintained as previously described (20). Cells were plated into 96-well microtiter plates at the concentration of 1×10^5 cells per well in 200 μ L RPMI 1640 (Sigma, USA) with 5% FCS. After incubation for 24 hours at 37°C, the cells received 10 μ L each of 10 mM ouabain (Sigma, USA), 1 mM veratridine (Sigma, USA), 600 pg/mL of brevetoxin PbTx-1 (Calbiochem, USA), and then antisera and control serum which had been serially diluted by 4-fold were added. The cells were further incubated for 5 hours and the cells were assayed with MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium) (Sigma, USA) as described by Manger *et al* (20).

RESULTS AND DISCUSSION.

In order to obtain antibodies against α -subunit of rat sodium channels using synthetic peptides corresponding to partial sequences of the protein, the (1686–1705) and (467–487) sequences of rat sodium channel were selected as immunogens. These sequences are supposed to contain most hydrophilic regions in the α -subunit of rat sodium channels. These synthetic peptides were purposely used as immunogens without conjugation to a macro-molecule, since the macro-molecule conjugation may result in some chemical and conformational modifications of the immunogen peptide (32).

Two peptides prepared in the present study gave yields of 15.5% and 16.0%, respectively. The amino acid ratios in acid hydrolysates of these rat sodium channel-related peptides agree well with the expected values, respectively. On analytical reverse phase HPLC using CH₃CN (5–60%), (30 min, 1.0 mL/min) in 0.01 N HCl, both synthetic peptides, such as rat Na channel (1686–1705) and (467–485) showed sharp single peaks at 21.0 min and 16.8 min, respectively. Using these two synthetic peptide immunogens, anti-rat Na channel- α (1686–1705) serum RY 137 and anti-rat Na channel- α (467–485) serum RY 143 were raised in rabbits after 5th immunization and used for immunostaining at a dilution of 1 : 3,000.

Adrenal medulla demonstrated immunoreactivity against both antisera. Figure 1c shows that the nuclei of chromaffin cells were strongly stained by RY 137, which recognizes conserved region of R_I and R_{II} between domain 4 segment 5 (D4S5) and D4S6, while the cytoplasm of

chromaffin cells was immunoreactive, more intensely in cell membrane, for RY 143, which was raised against peptide corresponding to R_{II}-specific sequence between D1S6 and D2S1, as shown in Figure 1a, b.

The cell burst of mouse neuroblastoma cells, Neuro-2a, which was induced by co-treatment of ouabain, veratridine, and brevetoxin PbTx-1, was inhibited by both antisera. Inhibition was observed with upto 1 : 384 dilution of RY 137 and 1 : 98, 304 dilution of RY 143 (Table 1).

R_I and R_{II} have been shown to be major subtypes of α -subunit of sodium channel in adult rat brain (11, 13, 29). Expression of R_I and R_{II} exhibited distinct spatial and temporal patterning, indicating their separated role in the brain (12). Subtype-specific localization of sodium channel was also demonstrated by *in situ* hybridization of mRNAs (10, 24). Region-specific anti-peptide antibodies have previously been used to distinguish the distribution of each channel subtype. R_{II}, which occupies approximately 80% of total sodium channels, was preferentially expressed in the rostral areas, while R_I was expressed homogeneously in the central nervous system (13, 29).

In adrenal medulla, whose chromaffin cells are known to be the same embryonic origin with sympathetic ganglionic neurons (19), existence of multiple and species-specific types of sodium channel has been suggested (7, 14, 31). One of them, human neuroendocrine sodium channel (hNE-Na), has been cloned and its amino acid sequence has been demonstrated (17). This new member of sodium channel family was shown to be expressed in bovine adrenal medulla (17, 31).

Table 1 Survival of neuroblastoma cells after the treatment of ouabain, veratridine and brevetoxin PbTx-1 by the addition of anti-rat sodium channel antisera

	ΔOD_{570} following MTT assay* ¹						
	dilution factor for antisera* ²						
	96	384	1536	6144	24576	98304	393216
NRS* ³	0.1148	0.1498	-0.0922	-0.0922	-0.4272	-0.1382	-0.4482
RY 137	0.3028 * ⁴	0.2578	0.1518	-0.0552	0.0868	0.0178	-0.4112
RY 143	0.0708	0.4228	0.4038	-0.0592	0.5068	0.3118	-0.1592

Mouse neuroblastoma cell line, Neuro-2a, was maintained and subjected to the cell assay as described in Materials and Methods. *¹; The data shown is the difference from the OD value obtained in the cell group which received only ouabain, veratridine and brevetoxin PbTx-1. *²; Dilution factors indicate the final dilution of serum in the wells. *³; Normal rabbit serum. *⁴; The values above 0.2000 were judged as positive and were shown in bold letters.

Outer cellular part of hNE-Na, which in turn corresponds to peptide antigen for antibody RY 137 in R_{II}, has well-conserved sequence of amino acids, whereas the area corresponding to antigen peptide for antibody RY 143 is heterogenous between R_{II} and hNE-Na (17, 23).

The neuroblastoma cell burst is known to be caused by the enhanced Na⁺ influx and then water inflow with the stimulation of veratridine and brevetoxin in the combination of sodium pump blockade with ouabain (20). Inhibition of the burst suggests that the antisera blocked excess influx of sodium ion into the cells in a pharmacological or a physical manner. Since the antibody RY 143 recognizes the inner cellular part of α -subunit of R_{II}, the cell burst inhibition may be caused first by the opening of sodium channel with the toxins to allow transfer of the antibodies into the cells with water, followed by the antibody binding to the channel to restrain further influx of sodium ion and water and then swelling of the cells.

The present study demonstrated that the region-specific antibodies described above were useful for detection and characterization of sodium channels in rat neuroendocrine cells.

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