Differential Binding Activity of Erythrocyte Ankyrin to the Alpha-Subunits of Na⁺, K⁺-ATPases from Rat Cerebral and Axonal Membrane

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ABSTRACT. Ankyrin is an important key protein transferring the signal between the inside and outside of eukaryotic cells, because of its ability to bind both to ionic channels of the plasma membranes and to cytoskeletal proteins. In this study, we investigated new ankyrin binding proteins in rat cerebral membrane. Five main proteins in the extract of the demyelinated membranes were bound to erythrocyte ankyrin as examined by affinity chromatography. One of the proteins had a molecular weight of 97 kD that was almost identical with that of the alpha-subunit of Na⁺, K⁺-ATPase. ¹²⁵I-labeled erythrocyte ankyrin was bound to the alpha-subunit of cerebral Na⁺, K⁺-ATPase, which contains both alpha and alpha(+) subunits. The binding experiment also showed that 70% of the total erythrocyte ankyrin bound to cerebral Na⁺, K⁺-ATPase. On the other hand, erythrocyte ankyrin binds less to Na⁺, K⁺-ATPase prepared from rat brain stem axolemma which contained only alpha(+) subunit. These results suggest that erythrocyte ankyrin may bind with high affinity to a cerebral Na⁺, K⁺-ATPase isoform with alpha subunit, but with much lower affinity to axonal Na⁺, K⁺-ATPase containing alpha(+) subunit.

The cytoskeletal proteins of plasma membranes function to stabilize the membranes and to control the distribution of the channels and receptors specific for each tissue membrane. Among cytoskeletal proteins, erythrocyte ankyrin is one of the most extensively studied and has been found to bind to anionic channels and spectrin (2, 11, 32). On the other hand, bovine brain ankyrin was found to bind to tubulin (4), fodrin (4), the cytoplasmic domain of erythrocyte anion channel (4), and voltage dependent Na⁺ channel (28). The association of ankyrin with these cytoskeletal proteins suggests that ankyrin plays a role in transmitting signals between the inside and outside of the cells through its intramembranous structure.

In rat brain, there are two isoforms of ankyrin. Ankyrin_B is a broadly distributed isoform, and ankyrin_R is a restrictedly distributed isoform which shares epitopes with erythrocyte ankyrin (15, 21). Kordeli *et al.* (15) reported that ankyrin_B is distributed in glial and neuronal cells of the gray matter of the central nervous system, while ankyrin_R is located at the perikarya of neuron cells, the specific initial segments of axon, the nodes of Ranvier of myelinated nerves and the axolemma of unmyelinated axons. These specific locations of two isoforms of ankyrin suggest its role as linking membrane

components at the localized places.

Na⁺, K⁺-ATPase is identified in many tissues. In the nervous system, three types of alpha-subunit of Na⁺, K⁺-ATPase are reported (10, 12, 26). Na⁺, K⁺-ATPase in the kidney epithelial cells binds to erythrocyte ankyrin (14, 23). However, it is unknown whether erythrocyte type ankyrin can bind to Na⁺, K⁺-ATPase in the nervous system. In this report we isolated Na⁺, K⁺-ATPase from two different parts of rat brain, namely the cerebral membrane and brain stem axonal membrane (axolemma), and investigated their binding ability toward erythrocyte ankyrin.

MATERIALS AND METHODS

Purification of ankyrin from erythrocytes. Ankyrin was prepared from erythrocyte membrane of human blood cells by the method of Tyler *et al.* (32). Briefly, ankyrin was extracted from spectrin-depleted vesicles with 1 M KCl, followed by ion exchange chromatography on DE-52 cellulose and by sucrose gradient centrifugation. The purified protein was dialyzed against 5 mM sodium phosphate buffer (pH 7.6) containing 1 mM sodium EDTA, 20 mM KCl and 0.5 mM DTT and stored at –80°C. The ankyrin preparation in each step was analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Fairbanks *et al.* (7).

Ankyrin-affinity chromatography. Ankyrin binding pro-

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teins in brain were examined by affinity chromatography using erythrocyte ankyrin coupled to Affi-Gel 10 (Bio-Rad Laboratories Co., Richmond, CA). This ankyrin agarose column has the ability to bind erythrocyte spectrin. Rat brain was homogenized in 10 mM sodium phosphate buffer (pH 7.5) containing 0.32 M sucrose, 1 mM sodium EGTA, 0.2 mM PMSF, 0.2 mM DTT and aprotinin (20 TIU/l). The homogenate was centrifuged at $28,000 \times g$ for 10 min, then at $30,000 \times g$ for 30 min. The pellet was demyelinated by centrifugation at $30,000 \times g$ for 20 min after being suspended in the same volume of 10 mM sodium phosphate buffer (pH 7.5) containing 1 M sucrose, 1 mM sodium EGTA as described by Davis *et al.* (4). The demyelinated membranes were washed twice in 10 mM sodium phosphate buffer (pH 7.6) containing 1 mM sodium EGTA and then solubilized in 1% (v/v) Triton X-100 solution containing 5 mM $MgCl_2$, 1 mM sodium EGTA, 75 mM KCl, 10 mM imidazole-HCl (pH 7.5) and 0.5 M NaCl. The suspension was stirred for 10 min on ice and then centrifuged successively at $30,000 \times g$ for 25 min and $100,000 \times g$ for 60 min. The supernatant was dialyzed against the loading buffer containing 0.05% Tween 20, 10 mM sodium phosphate (pH 7.5), 1 mM sodium EGTA, 0.1 M NaCl and 1 mM NaN_3 . The dialysate was applied to the ankyrin-agarose column and washed with the loading buffer until the absorbance at 280 nm became zero and the bound proteins were eluted with 0.8 M KI dissolved in the same loading buffer. The eluted proteins were dialyzed against the loading buffer and solubilized in Laemmli's sample buffer (17). SDS-PAGE was carried out in a 5–16% linear gradient acrylamide gel (20) containing 0.1% SDS and then silver-stained (34).

Preparation of Na^+ , K^+ -ATPase from rat cerebra and brain stem. Wistar rats (150 g) were purchased from Nippon Bio-Sup center (Tokyo, Japan) and killed by anaphylaxis with CO_2 . The cerebra and brain stem were dissected out anatomically. The partial purification of Na^+ , K^+ -ATPase from cerebra was conducted by the gentle extraction with SDS according to the procedure of Siegel *et al.* (27). In this paper, this Na^+ , K^+ -ATPase preparation was called cerebral Na^+ , K^+ -ATPase. Another type of Na^+ , K^+ -ATPase was isolated from rat brain stem axolemma according to the method of Sweadner (30), which is designated as axonal Na^+ , K^+ -ATPase in this paper. Freshly prepared enzyme was stored at $-80^\circ C$ after suspending in 10 mM sodium phosphate buffer (pH 7.5) containing 0.32 M sucrose, 1 mM sodium EDTA and 0.2 mM PMSF, and used for binding assays. Na^+ , K^+ -ATPase activity was measured according to the method of Esmann (6). The activity of axonal Na^+ , K^+ -ATPase was 1.0–1.4 $\mu moles/min/mg$, which was twice to five times higher than that of cerebral Na^+ , K^+ -ATPase.

Preparation and affinity purification of antibodies against erythrocyte ankyrin. Erythrocyte ankyrin eluted from DE-52 cellulose column was electrophoresed on 5.6% polyacrylamide gel by the method of Fairbanks (7) and electroeluted. New Zealand white rabbit was immunized by multisited intradermal boosting injections of the purified ankyrin emulsified

with complete Freund's adjuvant, followed by one intradermal boosting injection of the protein in incomplete Freund's adjuvant 4 weeks after the first injection. The antiserum was affinity-purified using an ankyrin-sepharose column. The bound antibodies were eluted with 0.1 M glycine-HCl (pH 2.8) and its specificity evaluated by immunoblot as described below.

Immunoblot analysis. After the electrophoresis, the proteins were transferred to nitrocellulose membrane (0.22 μm , Schleicher & Schuell, Dassel, Germany) according to the method of Towbin (32). The method of blocking and incubation was conducted as described by Shibayama *et al.* (25). The nitrocellulose membrane was blocked by incubation in 5% non-fat dry milk-TBS (20 mM Tris-HCl, pH 8.0, 0.85% NaCl) for 30 min at $37^\circ C$, and then incubated with the affinity-purified antibody against human erythrocyte ankyrin (0.03–3.00 $\mu g/ml$) for 18 h at $4^\circ C$. The membrane was washed three times with 0.05% Tween 20-TBS and incubated with alkaline phosphatase-conjugated anti rabbit IgG (Fc fragment, 1:7500, Promega Co., Madison, WI). After 2 h incubation, the membrane was washed as described above and the color was developed by using 5'-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium chloride chromogen system (3). The reaction was stopped by addition of 3% trichloroacetic acid.

Binding assay. The purified ankyrin (0.2–0.6 μg) and partially purified Na^+ , K^+ -ATPase (10–50 μg) were incubated in 100 μl of 10 mM sodium phosphate buffer (pH 7.5) containing 0.15 M KCl for 90 min at $4^\circ C$ and then centrifuged for 30 min at $180,000 \times g$ as described in Koob *et al.* (14). After the centrifugation, the supernatant and the pellet were carefully separated. After the pellet was suspended in the same volume of supernatant with 10 mM sodium phosphate buffer (pH 7.5), one tenth volume of $10 \times$ Laemmli's sample buffer was added, and heated for 15 min at $85^\circ C$. SDS-PAGE was conducted on a 5–16% linear gradient acrylamide gel. Proteins were detected either by Coomassie blue staining or by immunoblot with anti ankyrin. Quantitative densitometry of the developed sheet and stained gel was performed using Densito Pattern Analyzer EPA-3000 (Maruzen Petrochemical Co., Tokyo, Japan).

Overlay of ^{125}I -labeled ankyrin. Isolated ankyrin was labeled with 500 μCi $Na^{125}I$ using iodogen according to the instruction manual of Pierce Co. (Rockford, IL., USA). Unbound ^{125}I was removed by gel filtration using Sephadex G-25. Na^+ , K^+ -ATPase fraction was electrophoresed as described above, then transferred onto nitrocellulose. After the sheet was blocked for 15 min in immunoblot buffer containing bovine serum albumin (40 mg/ml), 150 mM NaCl, 10 mM sodium phosphate (pH 7.5), 1 mM sodium EDTA, 1 mM NaN_3 and 0.2% (v/v) Triton X-100, pH 7.5, it was incubated with ^{125}I labeled ankyrin in the above immunoblot buffer for 14 h at $4^\circ C$. The sheet was washed 5 times with the immunoblot buffer without BSA, dried and autoradiographed on X-ray film (Kodak XAR-5, Eastman Kodak Co., Rochester, NY) at

–80°C. As a control experiment, binding of ¹²⁵I labelled ankyrin to spectrin was ascertained.

Other method. Protein concentrations were determined by the method of Lowry (18) using bovine albumin as a standard. Affi-gel 10 was purchased from Bio-Rad Laboratories (Richmond, CA., USA). BrCN-activated sepharose was obtained from Pharmacia-LKB Uppsala, Sweden). All reagents were of analytical grade.

RESULTS

Purification of ankyrin. Ankyrin was extracted from spectrin-depleted erythrocyte membranes with 1 M KCl, followed by chromatography on DE-52 cellulose and by sucrose gradient centrifugation as described in Materials and Methods. Samples of each purification step were analyzed by SDS-PAGE and shown in Fig. 1. The ankyrin thus purified showed one major band with a minor band of its proteolytic fragment (lane E in Fig.

Fig. 1. Purification of ankyrin from erythrocyte membranes: samples were subjected to 5.6% polyacrylamide gel electrophoresis by the method of Fairbanks (6) and stained with Coomassie blue.

Lane A; human erythrocyte membranes (ghost). Lane B; spectrin extracted from the erythrocyte membranes with 0.2 M EDTA. Lane C; spectrin-depleted membranes. Lane D; ankyrin eluted from DEAE cellulose with 0.1–0.2 M KCl. Lane E; ankyrin isolated by sucrose gradient centrifugation. 1.1 and 2.1 indicated alpha- and beta- spectrin, respectively. 2.1 indicates ankyrin.

1), although 0.4 mM diisofluorophosphate, aprotinin (20 TIU/l) and 0.2 mM PMSF were added to all solutions used for preparation. This ankyrin was coupled to Affi-Gel 10 or BrCN-activated sepharose as described in Materials and methods.

Ankyrin binding proteins in the extract from brain membrane. The rat demyelinated membrane fraction was extracted with 1% Triton X-100 and loaded on the column of ankyrin-agarose gel. After extensive washing of the column with the loading buffer, the binding proteins were eluted with 0.8 M KI and electrophoresed on a SDS polyacrylamide gel (Fig. 2). As shown in lane 3 in Fig. 2, 5 major proteins were bound to the ankyrin-agarose column and eluted with 0.8 M KI. The protein bands with molecular weights of 235 kD and 240 kD were detected and assumed to be alpha and beta fodrin, respectively, as reported by Bennett *et al.* (4). One of the bound proteins had a molecular weight of 97 kD that was almost similar to that of the alpha-subunit of rat brain Na⁺, K⁺-ATPase, a major component of the

Fig. 2. Binding of brain membrane proteins to erythrocyte ankyrin: The solubilized proteins from demyelinated membranes were loaded on an ankyrin-agarose column and eluted with 0.8 M KI as described in Materials and Methods. Proteins were electrophoresed on a 5–16% linear gradient polyacrylamide gel by the method of Matudaira (20). Lane 1; Demyelinated membrane from rat brain. Lane 2; the proteins extracted with 1% Triton X-100 containing 5 mM MgCl₂, 1 mM sodium EGTA, 75 mM KCl, 10 mM imidazole-HCl and 0.5 M NaCl (pH 7.5). The gels of lanes 1 and 2 were stained with Coomassie blue. Lane 3; Proteins eluted with 0.8 M KI by the above buffer. The gel was stained with silver nitrate.

brain membranes. In order to examine whether this 97 kD protein might correspond to the α -subunit of Na^+ , K^+ -ATPase, we isolated Na^+ , K^+ -ATPase from rat brain, and then examined whether it binds to the erythrocyte ankyrin or not.

Specificity of affinity-purified ankyrin antibody. The antiserum against human erythrocyte ankyrin was affinity purified as described in Materials and Methods. The antibody eluted by 0.1 M glycine-HCl, pH 2.8, from ankyrin-sepharose column reacted only with ankyrin in ghosts (lane 3 in Fig. 3).

Characterization of cerebral Na^+ , K^+ -ATPase. Na^+ , K^+ -ATPase was partially purified from rat cerebra by the method of Siegel (27). The average activity of this cerebral Na^+ , K^+ -ATPase from six experiments was 0.36 $\mu\text{moles/min/mg}$ of protein. In the presence of 1 mM ouabain, a specific inhibitor of Na^+ , K^+ -

ATPase, the Na^+ , K^+ -ATPase activity was decreased to 20% as compared to that without ouabain, indicating it was Na^+ , K^+ -ATPase.

The partially purified cerebral Na^+ , K^+ -ATPase showed several bands on the gel as assayed by SDS-PAGE. Three main bands were detected at the molecular weights of 97 kD, 50 kD and 32 kD. The duplicate bands detected around the molecular weight of 97 kD, was identical to that of the α -subunit of Na^+ , K^+ -ATPase (lane 2P, 3P in Fig. 4). Since the α -subunit of Na^+ , K^+ -ATPase is known to be phosphorylated in the presence of Na^+ and K^+ in a few seconds (29), phosphorylation of the Na^+ , K^+ -ATPase was examined by incubation for 10 seconds in 100 μl of reaction mixture containing 140 mM NaCl, 60 μM [γ - ^{32}P]-ATP, 30 mM Tris-HCl (pH 7.1), 20 mM KCl, 3 mM MgCl_2 . As a result, only the 97 kD band was specifically phosphorylated under these conditions (data not shown). When the isolated Na^+ , K^+ -ATPase was applied to SDS-PAGE and immunoblotted with antibodies against rat Na^+ , K^+ -ATPase α -1 fusion protein purchased from UBI (New York, USA), the band at the molecular weight at 97 kD was detected (data not shown). From these re-

Fig. 3. The specificity of affinity-purified anti-ankyrin: The human erythrocyte ghost was electrophoresed as in Fig. 1 and transferred to a nitrocellulose membrane, followed by incubation with affinity purified antibody and development as described in Materials and Methods.

Lane 1; Coomassie blue stained pattern of erythrocyte ghost. Lane 2; Immunoblot with rabbit IgG (0.3 $\mu\text{g/ml}$). Lane 3; Immunoblot with affinity purified anti-ankyrin (0.3 $\mu\text{g/ml}$).

Fig. 4. Co-sedimentation of erythrocyte ankyrin with cerebral Na^+ , K^+ -ATPase: Erythrocyte ankyrin (7 μg) was incubated with Na^+ , K^+ -ATPase from rat cerebra (42 μg) in 10 mM phosphate buffer (pH 7.5) containing 0.15 M KCl at 4°C for 18 hr. and centrifuged. The supernatant and pellet were solubilized in Laemmli's sample buffer and analyzed by a 5–16% linear gradient polyacrylamide gel electrophoresis and stained with Coomassie blue. Unstained duplicate gel was assayed by immunoblotting with affinity-purified anti-ankyrin.

Lane 1; Erythrocyte ankyrin alone. Lane 2; Brain Na^+ , K^+ -ATPase alone. Lane 3; Erythrocyte ankyrin plus cerebral Na^+ , K^+ -ATPase. S = supernatant, P = pellet.

sults, the 97 kD band was determined to be the alpha-subunit of Na⁺, K⁺-ATPase. Before the binding experiment, whether the partially purified cerebral Na⁺, K⁺-ATPase might contain brain or erythrocyte ankyrin was examined by using immunoblot method with affinity-purified anti-ankyrin. The result demonstrated that this Na⁺, K⁺-ATPase (40 µg) did not contain any protein reactive with the antibody against erythrocyte ankyrin (data not shown). Therefore, we used this cerebral Na⁺, K⁺-ATPase for the binding assay to erythrocyte ankyrin.

Cosedimentation of cerebral Na⁺, K⁺-ATPase with erythrocyte ankyrin. As described in Materials and Methods, erythrocyte ankyrin (7 µg) and cerebral Na⁺, K⁺-ATPase (33 µg) were incubated in 10 mM sodium phosphate buffer (pH 7.5) containing 0.15 M KCl for 90 min on ice. After the incubation, the suspension was centrifuged at 100,000 × g for 30 min and the resulting

supernatant and pellet were electrophoresed on a 5–16% acrylamide gradient gel. As is evident from lane 1-P in Fig. 4 (Coomassie blue staining and immunoblot), erythrocyte ankyrin was not sedimented by this centrifugation, while cerebral Na⁺, K⁺-ATPase was pelleted completely (Coomassie blue staining, lane 2-P in Fig. 4). When erythrocyte ankyrin was incubated with cerebral Na⁺, K⁺-ATPase and centrifuged under the same conditions as above, appreciable amounts of ankyrin were sedimented (immunoblot, lane 3-S and 3-P in Fig. 4). The percentage of sedimented erythrocyte ankyrin as estimated by densitometer was approximately 70% of total ankyrin. These results indicated that erythrocyte ankyrin binds to cerebral Na⁺, K⁺-ATPase. However, cerebral Na⁺, K⁺-ATPase contained alpha and beta subunits with other minor contaminated proteins, and the question arises as to which subunits or proteins erythrocyte ankyrin binds.

¹²⁵I labeled ankyrin overlay. In order to answer the above question, cerebral Na⁺, K⁺-ATPase was electrophoresed on SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, and incubated with ¹²⁵I labeled erythrocyte ankyrin. The autoradiogram in Fig. 5 showed that the strongest radioactive band had a molecular weight of 97 kD, indicating that erythrocyte ankyrin binds to the alpha-subunit of cerebral Na⁺, K⁺-ATPase.

Cosedimentation of axonal Na⁺, K⁺-ATPase with erythrocyte ankyrin. Two isoforms of Na⁺, K⁺-ATPase could not be separately isolated. However, according to Sweadner (30), Na⁺, K⁺-ATPase containing only the alpha(+) subunit can be prepared from axolemma from brain stem. Therefore, Na⁺, K⁺-ATPase from axolemma was prepared and incubated with erythrocyte ankyrin for 90 min in 10 mM sodium phosphate buffer (pH 7.5) containing 0.15 M KCl at 4°C, and then the solution was centrifuged as described above. Both supernatant and pellet were electrophoresed on a 5–16% acrylamide gradient gel, transferred to a nitrocellulose membrane and immunoblotted with affinity purified antibody against erythrocyte ankyrin. For comparison, the cerebral Na⁺, K⁺-ATPase was incubated with erythrocyte ankyrin at the same time, and analyzed by the same procedure. As is evident from Fig. 6, only a small amount (18%) of erythrocyte ankyrin sedimented with axonal Na⁺, K⁺-ATPase (lane 2P), while most (82%) of erythrocyte ankyrin sedimented with cerebral Na⁺, K⁺-ATPase (lane 4P). This suggests that two isoforms of Na⁺, K⁺-ATPase localizing in different domains of neuronal membranes binds to its specific ankyrin isoform.

Fig. 5. Binding of ¹²⁵I-ankyrin to the alpha-subunit of cerebral Na⁺, K⁺-ATPase: Partially purified cerebral Na⁺, K⁺-ATPase was electrophoresed on SDS-PAGE and transferred to a nitrocellulose membrane, followed by incubation with ¹²⁵I-ankyrin for 90 min at 4°C.

Lane 1; Molecular weight marker proteins. The number represents molecular weights in thousands. Lane 2; Coomassie blue stained pattern of the partially purified cerebral Na⁺, K⁺-ATPase. Lane 3; Autoradiogram.

DISCUSSION

The present study clearly demonstrated that erythro-

Fig. 6. Co-sedimentation of either cerebral or axonal Na^+ , K^+ -ATPase with erythrocyte ankyrin: Either cerebral or axonal Na^+ , K^+ -ATPase was incubated with erythrocyte ankyrin as described in Fig. 4. The suspension was centrifuged, and supernatant and pellet were analyzed as in Fig. 4.

Line 1; axonal Na^+ , K^+ -ATPase alone. Lane 2; axonal Na^+ , K^+ -ATPase plus erythrocyte ankyrin. Lane 3; cerebral Na^+ , K^+ -ATPase alone. Lane 4; cerebral Na^+ , K^+ -ATPase plus erythrocyte ankyrin. S=supernatant, P=pellet.

cyte ankyrin can bind to the alpha-subunit of rat cerebral Na^+ , K^+ -ATPase. This result corroborates that of Gundersen *et al.* (9) who reported that a macromolecule complex consisting of kidney type Na^+ , K^+ -ATPase, erythrocyte type ankyrin and fodrin exists in the retinal pigment epithelium cells, a kind of neuronal cell. The alpha subunits of rat brain Na^+ , K^+ -ATPase have molecular weights of approximately 95 kD and 92 kD (29), and the amino acid sequences of the alpha-subunits of the isoforms of Na^+ , K^+ -ATPase have 75–80% homology between them (12). Since large segments of the alpha subunit of Na^+ , K^+ -ATPase are exposed to the cytoplasm (12), it is possible that the alpha subunit of Na^+ , K^+ -ATPase binds to ankyrin present in the cytoskeletal network.

The Kd of the binding reaction for erythrocyte ankyrin and cerebral Na^+ , K^+ -ATPase was approximately 100–600 nM. On the other hand, the Kd of the binding for brain ankyrin and brain membrane was reported to be 20–60 nM (13). Judging from these results, the specificity of this binding of erythrocyte ankyrin and cerebral Na^+ , K^+ -ATPase was lower than that of brain ankyrin.

We also demonstrated in this paper that axonal Na^+ , K^+ -ATPase had a much lower affinity to erythrocyte ankyrin as compared to cerebral Na^+ , K^+ -ATPase. Two isoforms of ankyrin are present at different locations in axon and neuronal cells (5, 22). This result suggested that the alpha subunit of Na^+ , K^+ -ATPase may bind more to ankyrin_R than ankyrin_B. The distribution of ankyrin_R was partly studied. Ankyrin_R is reported to be present at the nodes of Ranvier in the axolemma (5, 21)

but not in neuronal or glial cells (15). However, kidney type Na^+ , K^+ -ATPase containing the alpha-subunit is also present at the nodes of Ranvier (1). These results suggest the possibility that the alpha-subunit of Na^+ , K^+ -ATPase and ankyrin_R are present at the nodes of Ranvier binding each other, and that the alpha(+) subunit of Na^+ , K^+ -ATPase exists at the paranodal and internodal regions of axons or dendrites and nonmyelinated axon, binding to another isoform of ankyrin, ankyrin_B. In fact, Kunimoto *et al.* (16) demonstrated that ankyrin_B was present primarily in dendrites and unmyelinated axons in adult cerebellum. Next, we have to investigate the type of Na^+ , K^+ -ATPase and ankyrin existing at the paranodal and internodal regions of dendrites and nonmyelinated axons. Also it remains to be determined whether the alpha(+) subunit of Na^+ , K^+ -ATPase can bind to ankyrin_B or not. If specific antibodies to the alpha and alpha(+) subunits of Na^+ , K^+ -ATPase and ankyrin_B were available, the study of the localization of these proteins will be achieved. This will help our understanding of the locomotion and location of the proteins playing important roles in the neuronal function.

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