Biochimica et Biophysica Acta, 730 (1983) 161–172
Elsevier Biomedical Press

BBA 71609

SODIUM CHANNEL ACTIVITY IN BRAIN MEMBRANE FRACTIONS ISOLATED FROM RATS OF DIFFERENT AGES

ANDRÉS BUONANNO and RAIMUNDO VILLEGAS *

Centro de Biofísica y Bioquímica, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 1827, Caracas 1010A (Venezuela)

(Received August 10th, 1982)
(Revised manuscript received January 17th, 1983)

Key words: Na^+ channel; Synaptosome; Myelin; Aging; (Rat brain)

The Na^+ channel activity (tetrodotoxin sensitive $^{22}Na^+$ flux induced by veratridine and/or anemone toxin II) was studied in two fractions of brain cell plasma membranes, named A and B, isolated by the method of Gray and Whittaker ((1962) J. Anat. 96, 79–87) from rats 5, 10, 30 and 60 days old. The $^{22}Na^+$ flux was measured in membrane vesicles formed by the isolated membranes, in the absence of drugs (control), in the presence of veratridine, and in the presence of veratridine plus tetrodotoxin. Fraction A consists primarily of neuronal and glial membranes in rats of 5 and 10 days of age, while in the older rats this fraction becomes enriched in myelin. In Fraction A of 5-day-old and 10-day-old rats, veratridine (25 μM) increases the $^{22}Na^+$ flux 2.4- and 1.6-fold, respectively, and the increment continues to diminish with age, until it becomes negligible in the 60-day-old rats. Fraction B consists of synaptosomes and membrane vesicles, and at the four ages studied veratridine (25 μM) causes an increment of the $^{22}Na^+$ flux of about 2.5-fold. Fractions A and B from 10-day-old rats, and Fraction B from 60-day-old rats, which are sensitive to veratridine, also respond to anemone toxin II. When veratridine is used in presence of anemone toxin II (0.5 μM), the $K_{0.5}$ for veratridine is diminished and the maximum $^{22}Na^+$ flux is increased. The increments of $^{22}Na^+$ flux caused by veratridine and/or anemone toxin II in Fractions A and B are blocked by tetrodotoxin ($K_{0.5}$ approx. 5 nM). Fraction A from 60-day-old rats could be subfractionated by osmotic shock and sucrose gradient centrifugation to obtain three subfractions, two of which are enriched in axolemma and display Na^+ chennel activity. The other subfraction is enriched in myelin and shows no Na^+ channel actiivty. The plasma membrane preparations from young rats (up to 10 days) are devoid of myelin and are useful for studies of Na^+ channel activity.

Introduction

During the process of excitation and conduction of a nerve impulse, excitable membranes transiently change their permeability to Na^+ and K^+ [2]. The mechanism responsible for the changes in Na^+ conductance is called Na^+ channel. The channels are generally envisaged as proteins embedded in the membrane lipid bilayer. Three groups of neurotoxins have been found to interact specifically with the Na^+ channel. The first group is formed by the lipid-soluble polycyclic compounds batrachotoxin, grayanotoxin, veratridine, and aconitine which activate the sodium channel, thus increasing the transmembrane Na^+ current [3–5]. The second group is formed by polypeptide toxins obtained from scorpion and sea anemone venoms which have been shown to slow the process of inactivation [6–8]. Positive heterotropic

* Present address: Instituto Internacional de Estudios Avanzados, Apartado 17606, Caracas 1015A, Venezuela.

cooperativity has been observed for the binding of ^{125}I-labeled scorpion toxin in presence of the lipid-soluble polycyclic neurotoxins [9], as well as higher Na^+ fluxes in neuroblastoma when both types of toxins are utilized simultaneously [9–11]. The third group is formed by tetrodotoxin and saxitoxin which block the transmembrane current of Na^+ [12,13].

The use of radiolabelled neurotoxins has permitted the identification and isolation of peptides which bind neurotoxins with high affinity [14–17]. Although peptides which bind neurotoxins have been highly purified, this is not a sufficient criterion to conclude that these peptides represent the total complex which constitutes the Na^+ channel. Another approach has been to incorporate membrane fragments [18,19] and solubilized Na^+ channels [20–23] into lipid vesicles and to measure $^{22}Na^+$ fluxes stimulated by the lipid-soluble polycyclic neurotoxins which are abolished by tetrodotoxin or saxitoxin.

Many studies on Na^+ channels have been done using the synaptosomal fraction, Fraction B, obtained from adult rat brains by the method of Gray and Whittaker [1]. In this fraction, binding, stimulation of the $^{22}Na^+$ fluxes by neurotoxins, and depolarization of synaptosomes by the lipid-soluble polycyclic toxins and polypeptide toxins, as well as blockage of the $^{22}Na^+$ flux and repolarization by tetrodotoxin, have been observed [24,25]. However, little is known about the Na^+ channel activity of Fraction A, the fraction containing axolemma and also myelin in adult rats. The presence of myelin in the fractions obtained from adult rats has made it difficult to determine the Na^+ channel activity in this fraction.

In the present paper, the Na channel activity in membrane Fractions A and B obtained from the brain of rats 5 and 10 days old (where the presence of myelin is not yet detectable) was assayed and compared to the activity in the same fractions obtained from the brain of rats 30 and 60 days old (where myelin is present). Fraction A from 60-day-old rats was subfractionated into fractions enriched in myelin or in axolemma, and the Na^+ channel activity in these subfractions was assayed. Also, the sensitivity to the three types of neurotoxins, and the polypeptide composition of Fractions A and B at different ages were compared.

Materials and Methods

Materials. Veratridine was purified by the method of Kupchan et al. [26] from veratrine which was purchased from K and K (Plainview, NY); sea anemone toxin II (ATX II) was purchased from Ferring GMBH (F.R.G.), and (citrate-free) tetrodotoxin from Sankyo Co. Ltd. (Tokyo); cation exchange resin, Dowex 50W-X8, was purchased from Sigma (St. Louis, MO). $^{22}Na^+$ carrier-free was obtained from New England Nuclear at 1 mCi/ml (Boston, MA).

Isolation of brain membranes. Female Sprague-Dawley rats of 5, 10, 30 and 60 days of age were decapitated, and their brains immediately used to obtain plasma membrane Fractions A and B following the method of Gray and Whittaker [1]. All procedures were carried out at 0–4°C as follows: 10 g of the brain tissue were homogenized in 100 ml of solution A (0.32 M sucrose, 5 mM Tris-HCl, pH 7.0) with 10 strokes of a Potter-Elvehjem homogenizer at 800 rpm. The homogenate was centrifuged at $1000 \times g$ for 10 min, and the resulting supernatant centrifuged at $17\,000 \times g$ for 1 h. The membrane pellet (P_2) was resuspended in 15 ml of solution A, and 5 ml placed in each tube of a Beckman rotor SW 25.2 which contained 27 ml of 0.8 M and 1.2 M sucrose solution buffered with 5 mM Tris-HCl, pH 7.0. The gradient was centrifuged at $65\,000 \times g$ for 2 h to obtain three fractions; Fraction A, which lies on the 0.32–0.8 M sucrose interphase and has been described to contain myelin and axon fragments; Fraction B which lies on the 0.8–1.2 M sucrose and is enriched in synaptosomes, and Fraction C, the pellet, which contains mostly free mitochondria. Fractions A and B were carefully collected with a Pasteur pipette, diluted to approx. 0.3 M sucrose with 5 mM Tris-HCl, pH 7.0, and pelleted at $100\,000 \times g$ for 45 min. The pellets were resuspended with solution A and stored at −70°C.

Subfraction of fraction A. Fraction A from 60-day-old rats, which is composed of myelin and axolemma, was subfractionated into its two components using a modification of the method of De Vries et al. [27]. One volume of Fraction A suspension from 60-day-old rats containing 30 mg of protein in 0.32 M sucrose, 5 mM Tris-HCl, pH 7.0, was rapidly diluted with 25 volumes of 10 mM

EDTA, 5 mM Tris-HCl, pH 7.0, homogenized with 10 strokes in a Potter-Elvehjem homogenizer (type C), and stirred for 45 min at 4°C. The membranes were pelleted at 50 000 × *g* for 30 min and resuspended in 20 ml of 1.0 M sucrose, 5 mM Tris-HCl, pH 7.0. These 20 ml of membrane suspension were layered on top of 20 ml of a 1.2 M sucrose, 5 mM Tris-HCl, pH 7.0 and over them 20 ml of 0.8 M sucrose, 5 mM Tris-HCl, pH 7.0, were carefully pipetted. The gradient was centrifuged at 65 000 × *g* for 75 min (4°C) in a Beckman rotor SW 25.2. The three phases obtained were collected carefully using Pasteur pipettes, diluted with 5 mM Tirs-HCl, pH 7.0 to obtain 0.32 M sucrose solutions, and then centrifuged at 100 000 × *g* for 20 min. The pellets were finally resuspended in 100 mM KH_2PO_4, 1 mM $MgSO_4$, pH 6.8, and used immediately for $^{22}Na^+$ transport assays.

Assay for sodium channel activity. Membrane vesicles were prepared as described previously by Kanner [28], by rapidly diluting 0.5 ml of the plasma membranes (10–15 mg protein/ml) suspended in solution A with 6.5 ml of solution B (100 mM KH_2PO_4, 1 mM $MgSO_4$, pH 6.8), and incubating for 5 min at 37°C. The membranes were centrifuged at 50 000 × *g* for 10 min, and the pellets resuspended in solution B at 4°C to obtain 10 to 15 mg of protein/ml.

To measure $^{22}Na^+$ influx, 10 μl of the membrane suspension were rapidly mixed in tubes containing 190 μl of 100 mM choline chloride (20–30°C), 1 mM NaCl labelled with 0.2 μCi of $^{22}Na^+$, and the different neurotoxins or their solvents. Three types of samples were assayed: one had the solvents of the neurotoxins (control), the second had veratridine and/or sea anemone toxin, and the third had veratridine and/or sea anemone toxin plus tetrodotoxin. At different times after the addition of membrane vesicles to the radioactive solutions, 175 μl of the mixtures were passed through small Dowex 50W-X8 cation-exchange columns [29], and eluted with 2 ml of 0.5 M sucrose in 10 mM Tris-HCl, pH 7.0 into scintillation vials. The radioactivity trapped inside the vesicles present in the eluent collected from the columns was determined in a liquid scintillation counter (Packard Tri-Carb 3255).

The Dowex columns were prepared as follows. The ion exchange resin (Dowex W50-X8) which was purchased in the hydrogen form, was washed with distilled water and Trizma base to obtain the resin in Tris form (pH 7.5). The columns were made in Pasteur pipetts which contained approx. 2 ml of resin. In order to minimize the nonspecific adsorption of proteins to the resin, the columns were pretreated with 100 μl of bovine serum albumin 0.1 g of protein/ml and washed with 3 ml of 0.5 M sucrose, 10 mM Tris-HCl, pH 7.0.

Electron microscopy. Brain slices or pellets of brain plasma membranes from 10-day-old or 60-day-old rats were fixed in a 3% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4 and osmolarity of 270 mosmol/l compensated with sucrose. The samples were postfixed in 1% OsO_4 in the same phosphate buffer, then dehydrated in an ethanol series, and embedded in Epon 812. Fine sections double stained with uranyl acetate and lead citrate were observed in a Siemens Elmiskop 101 electron microscope.

Gel electrophoresis. Electrophoresis of membrane fractions was carried out in 9% polyacrylamide gels in the presence of 0.1% sodium dodecyl sulfate with the discontinuous buffer system and gel formulation of Laemmli [30]. The proteins were dissociated by heating at about 100°C for 2 min, in presence of 1% sodium dodecyl sulfate and 2-mercaptoethanol. Protein determinations were done by the procedure of Lowry et al. [31].

Results and Discussion

Electron microscopy of brain slices and of fractions A and B.

Previous studies showed that myelin formation in the brain of rats is not detectable until these are older than 10 days, and that after this age, the accumulation of brain myelin plotted as a function of the postnatal age of the rat increases linearly [32]. To confirm that the brain of rats of 10 and 60 days of age differed morphologically with respect to myelin formation, slices of the basal region and cortex of the brain were observed by electron microscopy. When the basal region of 10-day-old and 60-day-old rats were compared, myelin was observed only in the 60-day-old rats (Figs. 1a and 1b). In addition to the numerous neuronal bodies, axons, and synaptic connections observed in the brain cortexes at both ages, a few myelinated

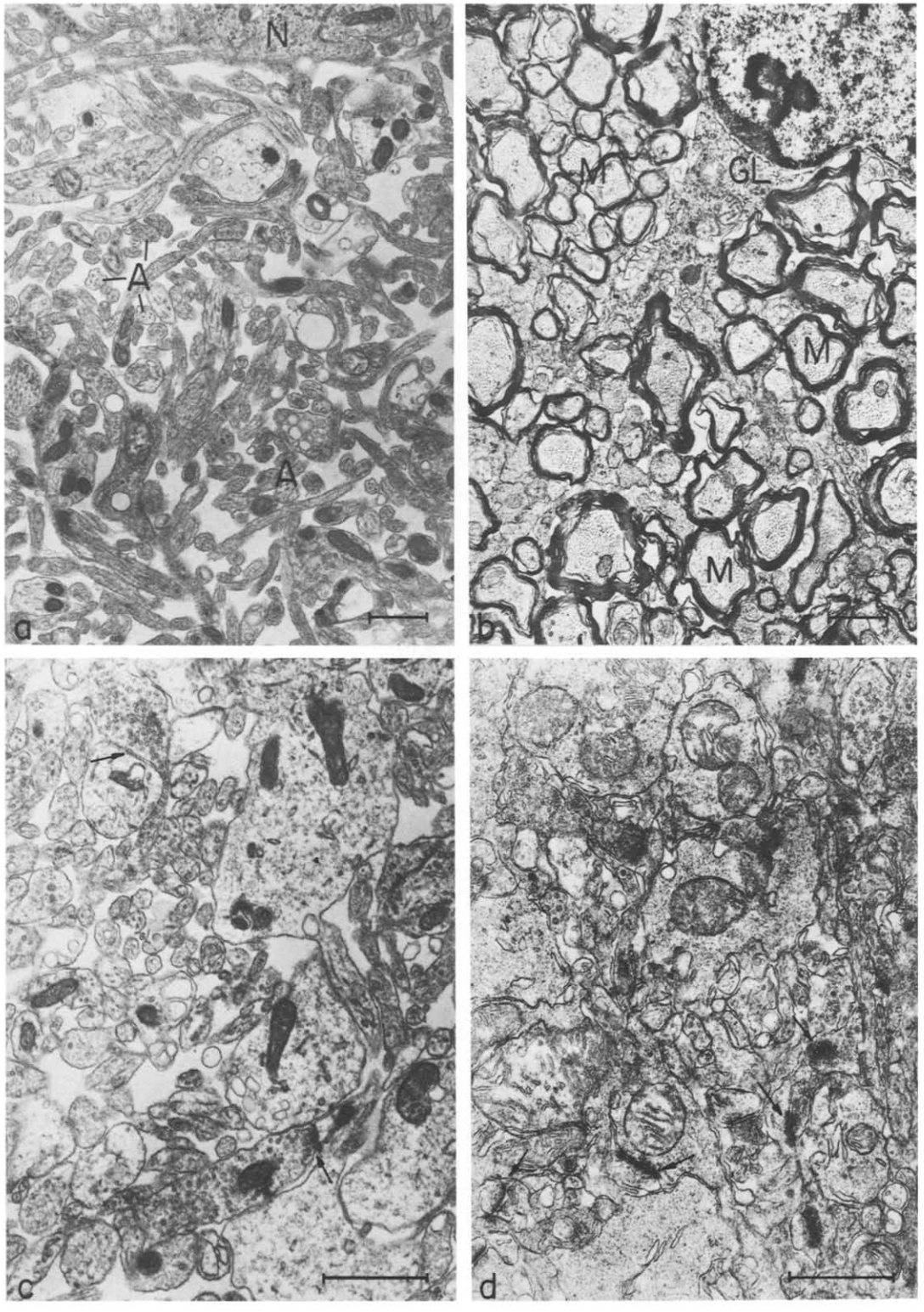

Fig. 1. Electron micrographs of the basal region and brain cortex of rats 10 and 60 days old. (a) In the basal region of the 10-day-old rats, neurons (N), axons which have not been myelinated (A), and glial processes which mingle loosely with the neuronal processes are observed. (b) The basal region of 60-day-old rats shows numerous closely packed, myelinated axons (M) and interfascicular glial cells (GL). In the neuropile of the brain cortexes of (c) 10-day-old and (d) 60-day-old rats, several synapses (arrows) are observed. Note that in the 60-day-old rats the processes are closely packed. The bars represent 1 μm.

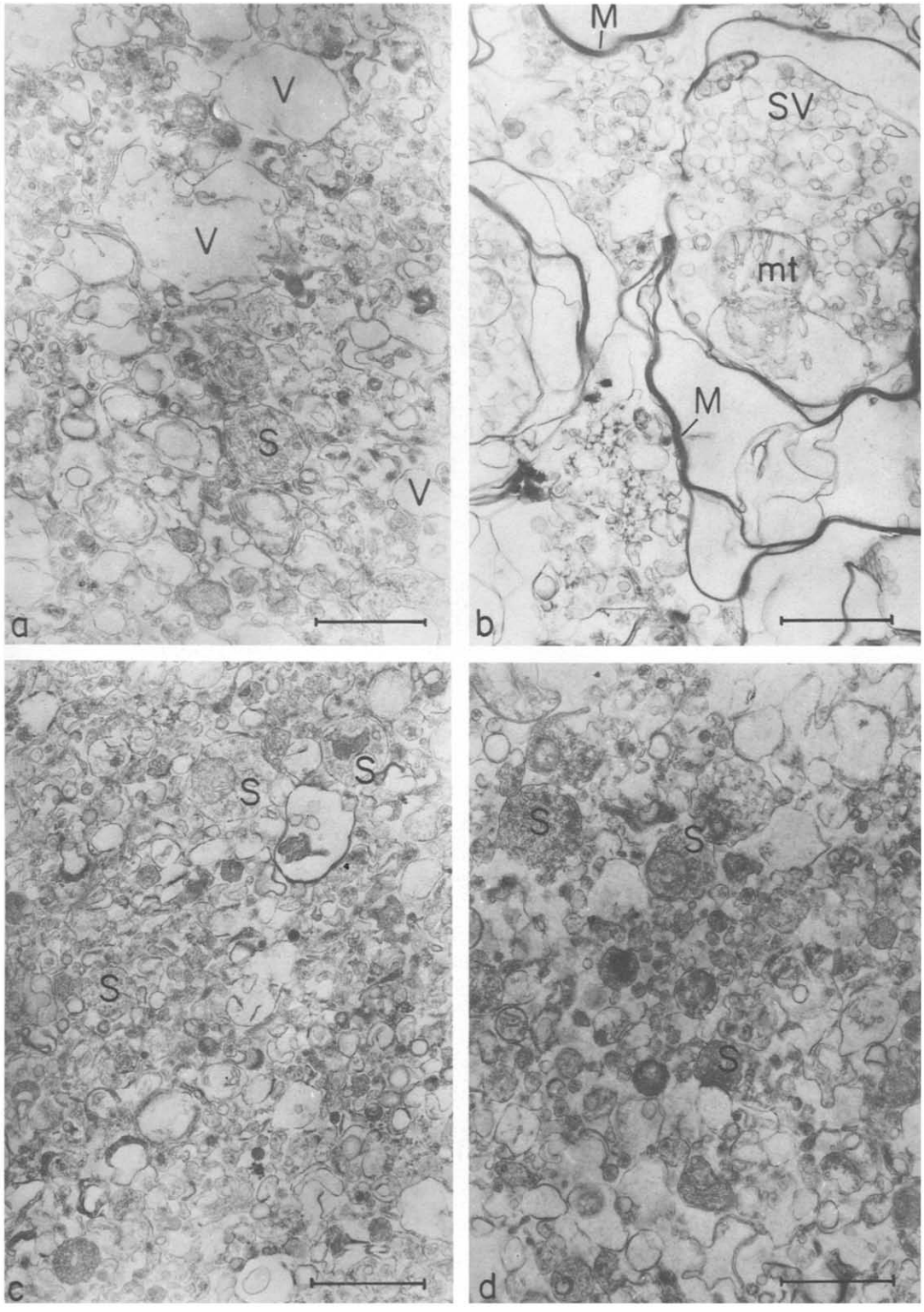

Fig. 2. Electron micrographs of fractions A and B of 10 and 60 days old rats. (a) Fraction A from 10-day-old rats shows empty membrane vesicles of different sizes (V) and ocassionally synaptosomes (S). (b) in contrast, fraction A from 60-day-old rats is enriched in myelin fragments (M) which entrap small vesicles of approx. 0.1 μm (SV) and a few mitochondrial remnants (mt). Fraction B of (c) 10-day-old and (d) 60-day-old rats are similar and show synaptosomes (S) and numerous vesicles of different sizes. The bars represent 1 μ.

axons were seen only in the samples from 60-day-old rats (Figs. 1c and 1d).

In 1962 Gray and Whittaker [1] developed a method to isolate brain plasma membranes which was based on the homogenization of mammalian brains and the fractionation of the plasma membranes by a combination of differential and density gradient centrifugations. By using this method, two membrane fractions were obtained from adult rats, one enriched in myelin and axons fragments (fraction A), and another enriched in synaptosomes (fraction B).

In order to compare fractions A and B obtained from the brains of rats 5, 10, 30 and 60 days old, these were examined by electron microscopy. Fraction A from 5-day-old and 10-day-old rats contained vesicles of different sizes, and in this fraction myelin was absent (Fig. 2a). In contrast, fraction A from 30-day-old to 60-day-old rats was enriched in myelin, which trapped in its interior vesicles with diameters of approx. 0.1 μm (Fig. 2b). The composition of fraction B was similar at the four ages studied; it contained synaptosomes and vesicles with diameters of approx. 0.1 μm (Figs. 2c and 2d).

$^{22}Na^+$ flux in membrane vesicles

In order to determine the Na^+ channel activity in fractions A and B, $^{22}Na^+$ flux was measured as indicated above, in absence of drugs (control), in presence of veratridine, and in presence of veratridine plus tetrodotoxin. The Na^+ channel activity refers to the increase of $^{22}Na^+$ flux caused by veratridine which can be abolished by tetrodotoxin. The identification of Na^+ channels relies on the specificity of tetrodotoxin to block the veratridine-induced $^{22}Na^+$ flux through the Na^+ channel.

The Na^+ channel activity was measured at room temperature (20–23°C) because the veratridine-stimulated $^{22}Na^+$ flux was not observed at 0°C. The effect of veratridine has been reported to be temperature sensitive in crab axons, where the effect of this alkaloid on the membrane potential was hardly observed below 8°C [33].

The curves representing the uptake of $^{22}Na^+$ as a function of time in membrane vesicles are presented in Fig. 3 for fraction A and in Fig. 4 for fraction B. Although the uptake of $^{22}Na^+$ in presence of veratridine was approximately the same in fraction A at the four ages studied, the basal uptake was higher in the 30-day-old and 60-day-old rats. The increment in the basal uptake of $^{22}Na^+$ coincides with the enrichment of myelin in fraction A of the older rats.

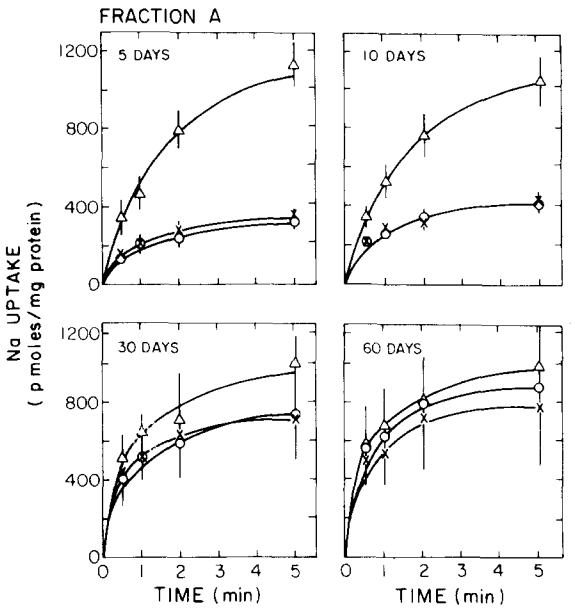

Fig. 3. Influx of $^{22}Na^+$ in brain membrane vesicles from fraction A isolated from rats of 5, 10, 30 and 60 days of age. Influx of $^{22}Na^+$ was measured at 23°C in the absence of toxins (○), in presence of 25 μM veratridine (△), and in the presence of 25 μM veratridine plus 1 μM tetrodotoxin (×). The data points represent the means of five experiments, and the bars show the standard deviation when larger than the dimensions of the symbol. The curves were drawn by eye.

In order to compare the Na^+ channel activity in fractions A and B from rats 5, 10, 30 and 60 days old, the measurements of $^{22}Na^+$ influx were taken at 30 s where uptake was approximately linear with respect to time. These results are summarized in Table I. In fraction A from 5-day-old to 10-day-old rats, 25 μM veratridine increased the $^{22}Na^+$ flux 2.4- and 1.6-fold, respectively; this increment diminished with age to 1.2- and 1.1-fold in the 30-day-old and 60-day-old rats, respectively. In fraction B at the four ages studied, 25 μM veratridine increased the $^{22}Na^+$ flux about 2.5-fold. The increment in $^{22}Na^+$ flux by veratridine in both fractions was abolished by tetrodotoxin.

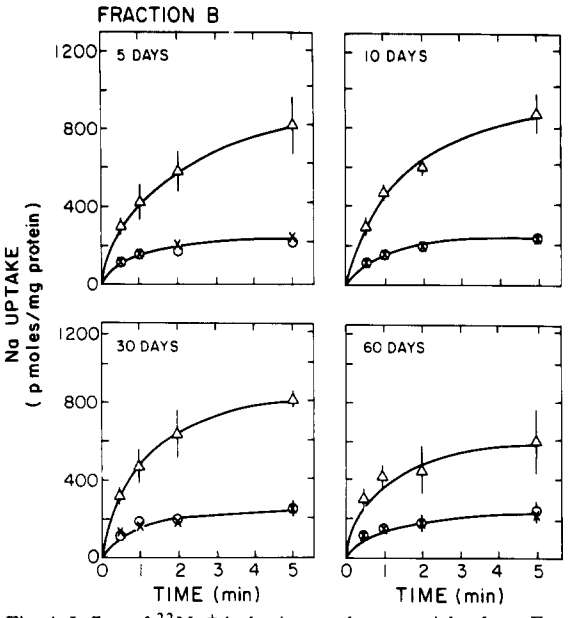

Fig. 4. Influx of $^{22}Na^+$ in brain membrane vesicles from Fraction B isolated from rats of 5, 10, 30 and 60 days of age. Influx of $^{22}Na^+$ was measured at 23°C in the absence of toxins (○), in the presence of 25 μM veratridine (△), and in the presence of 25 μM veratridine plus 1 μM tetrodotoxin (×). The data points represent the means of five experiments, and the bars show the standard deviation when larger than the dimensions of the symbol. The curves were drawn by eye.

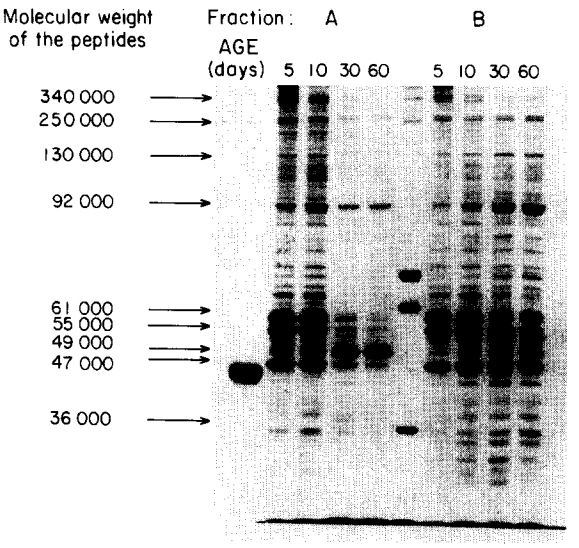

Fig. 5. Electrophoretic patterns of brain membrane proteins of fractions A and B obtained from rats of 5, 10, 30 and 60 days of age. The proteins (40 μg) were separated on a 9% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue G-250. Molecular weights were determined by using the protein standards displayed in the unidentified columns in the left side (ovalbumin) and in the middle of the figure (bovine serum albumin, thyroglobulin, ferritin, catalase and lactate dehydrogenase).

Peptide composition of fractions A and B

The peptide composition of both fractions, as determined by SDS-polyacrylamide gel electrophoresis, are shown in Fig. 5. When equal quantities of protein were analyzed, the polypeptide pattern of fraction A was observed to vary with age. Samples from 5-day-old and 10-day-old rats were relatively enriched in high molecular weight peptides (over 47 000), while samples from 30-day-old and 60-day-old rats were relatively enriched in low molecular weight peptides (under 60 000). Pre-

TABLE I

INITIAL ^{22}Na INFLUX IN BRAIN MEMBRANE VESICLES FROM FRACTIONS A AND B OF RATS 5, 10, 30 AND 60 DAYS OLD

The $^{22}Na^+$ influx was measured at 23°C, 30 s after the addition of the membrane vesicles to the media containing $^{22}Na^+$. Relative activity refers to the influx of $^{22}Na^+$ in presence of veratridine/the influx of Na^+ in presence of veratridine plus tetrodotoxin.

	^{22}Na influx (pmol/mg protein per min)							
	Fraction A				Fraction B			
Age (days)	5	10	30	60	5	10	30	60
Control	285	440	825	1122	234	246	249	246
Veratridine (25 μM)	713	699	1031	1150	605	614	644	629
Veratridine (25 μM) + tetrodotoxin (1 μM)	302	431	889	1016	237	239	257	247
Relative activity	2.4	1.6	1.2	1.1	2.6	2.6	2.5	2.6

vious work by De Vries et al. [27] showed that axolemma-enriched fractions from rat central nervous system contained mostly high molecular weight peptides, while the myelin-enriched fractions mainly had peptides of low molecular weight. These results suggests that fraction A from 5-day-old and 10-day-old rats is enriched in axolemma, and with age, myelin begins to predominate in this fraction. However, further work is necessary to determine which changes in the peptide patterns are due to variations in the proportions of plasma membranes composing fraction A and which are due to variations in the peptides found in membranes at different ages. The polypeptide patterns obtained for fraction B at the four ages studied were similar; the samples showed a relative enrichment in high molecular weight peptides (over 47 000).

Subfractionation of a myelin-enriched fraction

Fraction A obtained from 30-day-old and 60-day-old rats consists mainly of myelin fragments, and some axon fragments. In order to remove myelin from the axolemma in this fraction, and to show that the Na^+ channel activity resides in the axolemma enriched fraction, fraction A from 60-day-old rats was subfractionated by a modification of the method described by De Vries et al. [27]. The method is based on separating the axolemma from myelin by submitting these membranes to an osmotic shock in presence of chelating agents, and separating the two membranes on sucrose density gradients.

The three subfractions obtained by this method were designated, F1 (which floats on 0.8 M sucrose), F2 (which lies on the 0.8–1.0 M sucrose interphase), and F3 (which lies on the 1.0–1.2 M sucrose interphase). The Na^+ channel activity in these subfractions were assayed. There was no increment in $^{22}Na^+$ flux when F1 was exposed to 100 μM veratridine, while a 1.6- and 1.5-fold veratridine-induced increment in $^{22}Na^+$ flux was observed in subfractions F2 and F3, respectively (Fig. 6). When subfractions F1 and F2 were mixed in similar proportions to those obtained in the sucrose gradients, the veratridine induced increment of $^{22}Na^+$ flux was lost. The veratridine stimulated $^{22}Na^+$ flux was abolished by tetrodotoxin.

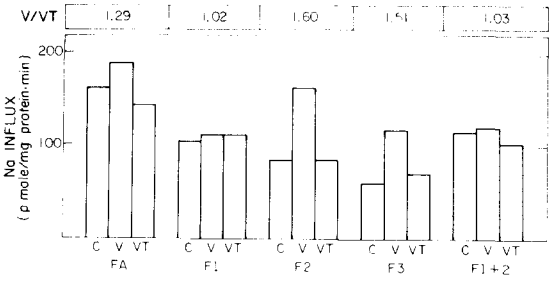

Fig. 6. Influx of $^{22}Na^+$ in membrane vesicle subfractions obtained from fraction A of 60-day-old rats. Fraction A (FA) was exposed to an osmotic shock and subfractionated by centrifugation in a sucrose gradient into three subfractions: F1, F2, and F3. F 1 + 2 is the mixture of subfractions 1 and 2. Influx of $^{22}Na^+$ was measured for 1 min at 23°C in absence of toxins (C), in presence of 100 μM veratridine (V), and in the presence of 100 μM veratridine plus 1 μM tetrodotoxin (VT). V/VT refers to the $^{22}Na^+$ influx measured in presence of 100 μM veratridine, devided by the $^{22}Na^+$ influx measured in presence of 100 μM veratridine plus 1 μM tetrodotoxin.

The peptide patterns observed by SDS-polyacrylamide gel electrophoresis of fraction A and the three subfractions (Fig. 7) were in agreement with those obtained by De Vries et al. [27], which show that F1 is enriched in low molecular weight peptides (myelin), while F2 and F3 are enriched in high molecular weight (axolemma). The peptide composition of F2 and F3 are similar to those obtained in fraction A of 5-day-old and 10-day-old rats which are devoid of myelin.

Response of fractions A and B to neurotoxins

The comparative sensitivity of Na^+ channels to veratridine, sea anemone toxin II and tetrodotoxin was studied in membrane fractions A and B obtained from the brain of 10-day-old and 60-day-old rats. Fractions A and B of 10-day-old rats, and fraction B of 60-day-old rats showed similar responses to the neurotoxins. The curves obtained from fraction B of 10-day-old rats are shown to illustrate these effects. The negligible response of fraction A from 60-day-old rats to the neurotoxins may be due to its enrichment in myelin. The $K_{0.5}$ values, which refer to the concentration of toxin causing 50% of its maximal effect on $^{22}Na^+$ influx, are given in Table II.

The hyperbolic curve obtained for $^{22}Na^+$ flux as a function of veratridine concentration reached

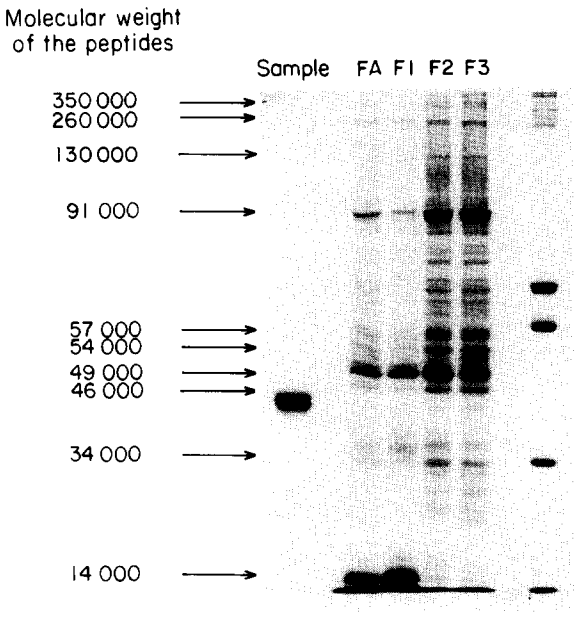

Fig. 7. Electrophoretic patterns of brain membrane proteins of Fraction A (FA) and its subfractions: F1, F2, F3, obtained from rats of 60 days of age. The proteins (40 μg) were separated on a 9% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue F-250. Molecular weights were determined by using the protein standards displayed in the unidentified columns in the left side (ovalbumin) and in the right side of the figure (bovine serum albumin, thyroglobulin, ferritin, catalase and lactate dehydrogenase).

a plateau at about 50 μM veratridine. However, at 200 μM veratridine a 20 to 40% decrease in $^{22}Na^+$ flux was observed as compared to the flux obtained with 100 μM veratridine (Fig. 8).

An increase in $^{22}Na^+$ flux was also produced by sea anemone toxin II in the fractions studied (Fig. 8), which was smaller than that of veratridine. The $K_{0.5}$ values obtained for anemone toxin II in the three fractions studied were between 1 and 3 μM. These results are in agreement with previous studies done with neuroblastoma cells which demonstrated that anemone toxin [34] and scropion toxin [35] used alone are poor activators of Na^+ channels. When neuroblastoma cells or synaptosomes are incubated with polypeptide toxins in presence of the polycyclic lipid-soluble toxins, the effect of the lipid-soluble toxins on $^{22}Na^+$ flux are synergistically increased [34]. The synergistic effect between veratridine and anemone toxin II was observed in the fractions studied and is illustrated in Fig. 9. A decrease in the $K_{0.5}$ value for veratridine and an increase in $^{22}Na^+$ flux was observed when concentrations of anemone toxin II were used, which by itself, only produced a small effect on $^{22}Na^+$ flux (Table II). The increment of $^{22}Na^+$ flux by veratridine, and veratridine plus sea anemone toxin II was abolished by tetrodotoxin. These results demonstrate the presence of the three

TABLE II

APPARENT DISSOCIATION CONSTANTS ($K_{0.5}$) FOR VERATRIDINE, SEA ANEMONE TOXIN II, VERATRIDINE IN PRESENCE OF 0.5 μM ANEMONE TOXIN II, AND TETRODOTOXIN IN FRACTIONS A AND B OF 10-DAY-OLD RATS AND FRACTION B OF 60-DAY-OLD RATS

The $K_{0.5}$ value refers to the concentration of toxin causing 50% of the maximal effect on the $^{22}Na^+$ flux.

	$K_{0.5}$							
	Veratridine (μM)		Anemone toxin II (μM)		Veratridine (μM) in presence of 0.5 μM anemone toxin II		Tetrodotoxin (nM) [a]	
Age (days)	10	60	10	60	10	60	10	60
Fraction A	20	–	1	–	4	–	5	–
Fraction B	17	9	2.5	1	2	2	6	6

[a] The $K_{0.5}$ value for tetrodotoxin was determined by measuring its effect on the $^{22}Na^+$ influx increment caused by 100 μM veratridine.

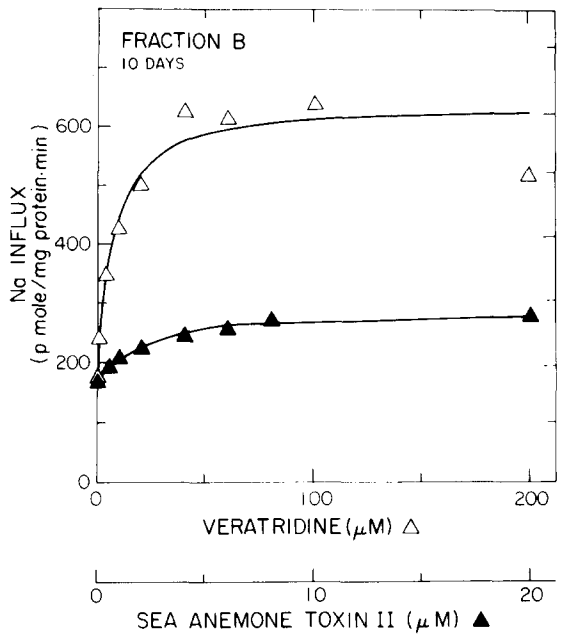

Fig. 8. The effect of different concentrations of veratridine (△) or sea anemone toxin II (▲) on the Na^+ influx in brain membrane vesicles from fraction B of 10-day-old rats. Influx of $^{22}Na^+$ was measured at 23°C, 30 s after the addition of the vesicles to the solution containing $^{22}Na^+$. The continuous lines are least-squares fits to the data.

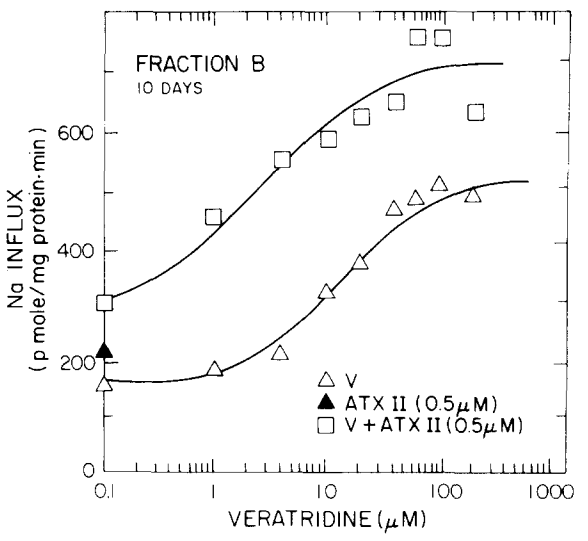

Fig. 9. The effect of different concentrations of veratridine, in the absence or presence of 0.5 μM sea anemone toxin II (ATX II), on the Na^+ influx in brain membrane vesicles from fraction B of 10-day-old rats. The influx was measured at 23°C, 30 s after the addition of the vesicles to the solution containing $^{22}Na^+$. The curves represent the influx induced by veratridine in the absence (△) and in the presence (□) of 0.5 μM anemone toxin II. The effect of 0.5 μM anemone toxin II (▲) on $^{22}Na^+$ influx is also shown.

specific receptor sites associated with Na^+ channels in electrically excitable cells.

The $K_{0.5}$ values for tetrodotoxin in the fractions studied were obtained by measuring the $^{22}Na^+$ flux in membrane vesicles which were exposed to 100 μM veratridine plus varying concentrations of tetrodotoxin. The results for fraction B of 10-day-old rats are illustrated in Fig. 10. The $K_{0.5}$ value for tetrodotoxin in the fractions studied was approx. 5 nM (Table II). These values coincide with those calculated for the effect of tetrodotoxin in axons [12], axolemma preparations [36], and neuroblastoma cells [9].

The absence of Na^+ channels in myelin

It should be pointed out that the decrease in the neurotoxin-sensitive $^{22}Na^+$ influx in fraction A coincided with the progressive enrichment of myelin in this fraction, and that preparations of myelin (F1) did not show any response to the neurotoxins. Although neuronal cells have been characterized by having voltage-dependent Na^+

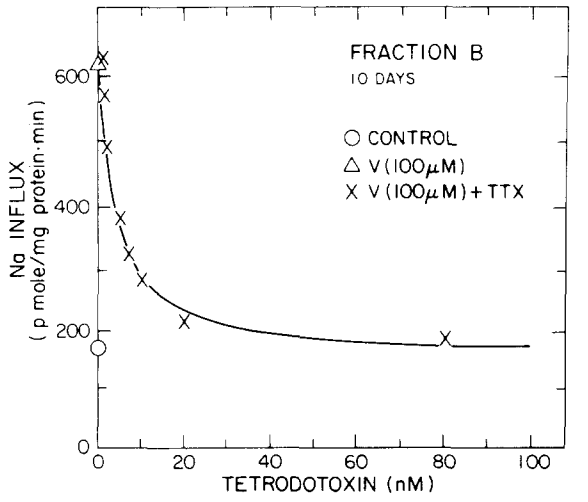

Fig. 10. The effect of different tetrodotoxin concentrations of the veratridine-induced $^{22}Na^+$ influx in brain membrane vesicles from fraction B of 10-day-old rats. The influx of $^{22}Na^+$ was measured at 23°C, 30 s after the addition of the vesicles to the solution containing $^{22}Na^+$, in the absence of drugs (○), in the presence of 100 μM veratridine (△), and in the presence of 100 μM veratridine plus different concentrations of tetrodotoxin (×). The continuous line is a least-squares fit to the data.

channels which interact specifically with three groups of neurotoxins, Na^+ pathways sensitive to neurotoxins have also been detected in squid Schwann cells [37], as well as in human 'glial-like' cells in culture [38]. Although myelin originates from glial cells [39,40], the lack of response of myelin-enriched fractions and myelin preparations (F1) to the neurotoxins, suggests that myelin is a specialized membrane which does not have Na^+ channels.

The presence of Na^+ channels in axolemma-enriched preparations

In the present work, we have demonstrated that the axolemma present in axolemma-enriched fractions isolated from rat brain maintained Na^+ channel activity. This activity is a characteristics of this specialized membrane. Similarity in veratridine and tetrodotoxin sensitivity, as well as the synergistic activation of the Na^+ channel by veratridine and anemone toxin, indicate that fraction A from 5-day-old and 10-day-old rats have Na^+ channel activity with pharmacological properties, similar to other axolemmal preparations. These membranes are devoid of myelin and useful for studies of Na channel activity. Furthermore, removal of myelin from fraction A of 60-day-old rats allowed us to demonstrate Na^+ channel activity.

Fraction B (synaptosomes and membrane vesicles) from all four age groups studied showed Na^+ channel activity.

Acknowledgement

We would like to express our gratitude to Dr. Gloria M. Villegas and Mr. Freddy Sánchez for the electron microscopy work; to Dr. Flor V. Barnola for fruitful discussions; to Mrs. Teresa Proverbio and Mr. Marin for their help in the preparation of the rat brain membranes; to Mr. H. Parada for the purification of the veratridine; and to Miss Isabel Otaegui and to Mrs. Sol van Praag for their secretarial assistance.

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