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A large intracellular pool of inactive Na channel α subunits in developing rat brain

(ion transport/neurons/cell culture/posttranslational processing/anti-Na^+ channel antibodies)

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ABSTRACT **An intracellular pool of Na channel α subunits has been detected in developing brain cells *in vivo* and *in vitro* by phosphorylation with cAMP-dependent protein kinase, immunoprecipitation with specific antiserum, and $NaDodSO_4$ gel electrophoresis or by radioimmunoassay. These α subunits are membrane-bound, contain complex carbohydrate chains, and have an apparent molecular weight of 260,000 like mature α subunits. In contrast to mature α subunits, the intracellular subunits are not covalently attached to a β2 subunit, and they do not bind saxitoxin with high affinity. They comprise 67–77% of the total immunoreactive α subunit in developing rat brain cells but are not a prominent component in the adult brain. It is proposed that this intracellular pool of α subunits forms a ready reserve of preformed subunits for incorporation into the surface membrane during periods of active membrane biogenesis. The results suggest that disulfide linkage of the α and β2 subunits, insertion into the cell surface membrane, and attainment of a functional conformation are closely related late events in the biogenesis of the Na channel. These processes may regulate the number of functional Na channels in the developing brain.**

Voltage-sensitive Na channels mediate changes in Na conductance during action potentials in electrically excitable cells. Na channel-specific toxins such as saxitoxin (STX) and tetrodotoxin (TTX) have been used to identify and purify Na channels to near homogeneity from eel electroplax (1–4), rat brain (5–9) and skeletal muscle (10, 11), and chicken heart (12). Purified Na channels from each of these sources have a similar high molecular weight α subunit of 260 kDa. The purified Na channel from adult rat brain consists of three polypeptide subunits: α of 260 kDa, β1 of 36 kDa, and β2 of 33 kDa (7, 13). Each of these subunits is heavily glycosylated, and N-linked glycosylation is required for biosynthesis of functional neuronal Na channels (13, 14). In adult rat brain, the α and β2 subunits are covalently attached by disulfide bonds (7). These features of the structure of the rat brain Na channel indicate that it must undergo several steps of posttranslational processing and assembly before a mature cell-surface Na channel can be produced. In this report, we begin an analysis of the steps in this process by identifying an inactive intracellular form of the α subunit that is membrane-bound and glycosylated but is not covalently associated with β2 subunits. This form accounts for 67–77% of the α subunit in developing rat brain cells *in vivo* or *in vitro*.

EXPERIMENTAL PROCEDURES

Materials. Materials were obtained from the following sources: [γ-^{32}P]ATP from New England Nuclear; *Staphylococcus aureus* cells (SAC), TTX, and fluorodeoxyuridine from Calbiochem; purified catalytic subunit of cAMP-dependent protein kinase from Barbara Flug of the laboratory of E. G. Krebs of this department; Dulbecco's modified Eagle's medium (DME medium) and fetal calf serum from GIBCO; horse serum from KC Biological (Lenexa, TX); plastic culture plates from Falcon; poly(D-lysine) from Sigma; GF/C glass fiber filters from Whatman; and Sprague–Dawley rats from Tyler Laboratories (Bellevue, WA). Saxitoxin was tritiated and prepared as described (14).

Cell Culture. Brains dissected from 15-day rat embryos were triturated in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline with 5 mM glucose and were seeded at 100,000 cells per cm^2 on 35-mm or 60-mm plastic Petri dishes that had been precoated with poly(D-lysine). The initial growth medium consisted of DME medium with 5% fetal calf serum. All culture media contained 10 μg of penicillin and 20 μg of streptomycin per liter. Cultures were grown at 36°C in a 7.5% CO_2 atmosphere. When nonneuronal cells neared confluence, the cultures were treated with 15 μg of fluorodeoxyuridine and 45 μg of uridine per ml for 3 days. After that treatment, the growth medium was changed to DME medium with 10% heat-inactivated horse serum. Cells were used after 21–23 days *in vitro*.

Cell and Membrane Preparation. A crude synaptosomal membrane fraction (P3) was prepared from rat brains as described (15). Cultured cells were gently washed off culture plates in buffer 1 and collected by gravity sedimentation or centrifugation at 1000 rpm in a clinical centrifuge. This preparation contained >95% intact cells as assessed by trypan blue exclusion. Cells were lysed by homogenization in 5 mM EDTA (pH 7.4) or by incubation with 0.5% saponin. All buffers used from the point of cell homogenization or solubilization through immunoprecipitation contained the following reagents as protease inhibitors: 2–5 mM EDTA, 1 μM pepstatin A, 100 μM phenylmethylsulfonyl fluoride, and 1 mM iodoacetamide.

STX Binding. Specific binding of STX to the Na channel was measured by the filter-binding assay previously described at 0°C in the presence of 10 nM STX (14, 15).

Immunoprecipitation. The rabbit antiserum used was raised against highly purified Na channel from adult rat brain (16). This antiserum reacts with the α subunit of the Na channel in electrophoretic immunoblots of $NaDodSO_4$ gels (unpublished data). Its interaction with membrane-bound and solubilized Na channel has been described (16). Antibody was bound to membranes in buffer 1 (130 mM choline chloride/50 mM Hepes/Tris/5.4 mM KCl/1.6 mM $MgSO_4$, pH 7.4) containing 5 mg of bovine serum albumin per ml at 4°C overnight. The membranes were then washed with buffer 1 to remove unbound antibody and solubilized by incubation with mixing for 30 min in 5% Triton X-100 in buffer 2 (50 mM Na_2HPO_4/20 mM KF/2.5 mM EDTA/25 mM Tris/75 mM NaCl, pH 7.4), a medium designed to inhibit phosphatase

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Abbreviations: STX, saxitoxin; TTX, tetrodotoxin; SAC, *Staphylococcus aureus* cells.

activity. To remove remaining membranes, samples were centrifuged for 10 min at 8000 × *g*. The resulting solubilized antibody–antigen complexes were immunoprecipitated by incubating with 10–20 μl of 10% (wt/vol) SAC for 30 min and were sedimented in a Beckman Microfuge. Solubilized Na channels were immunoprecipitated by incubation with antiserum for 4 hr followed by incubation with 20 μl of 10% SAC per μl of antiserum for 30 min and then were sedimented by centrifugation at 8000 × *g* for 1 min. SAC pellets were washed three times with 1% Triton X-100 in buffer 2. Immunoprecipitations were judged to be quantitative when additional antiserum failed to precipitate more α subunit from SAC supernatants.

Phosphorylation. Phosphorylation of immunoprecipitated Na channel was performed in essentially the same way as described for purified Na channel (16). SAC immunoprecipitates were suspended in 40 μl of phosphorylation buffer containing 5 mM $MgCl_2$, 20 mM Tris, 5 mM EGTA, 0.1% Triton X-100, and 500 ng of the catalytic subunit of cAMP-dependent protein kinase. The reaction was started by addition of 10 μl of phosphorylation buffer containing 5 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP and transfer to a 36°C water bath. After 2 min, 100 μl of ice-cold stop solution (100 mM NaH_2PO_4/20 mM EDTA, pH 7.4) was added. The SAC pellet was washed once in water and suspended at room temperature in $NaDodSO_4$ gel loading solution to release immunoprecipitated Na channel.

RIA. RIAs were performed as described in detail previously (17). Briefly, for the membrane RIA, membranes or intact cells were incubated with antiserum to allow adsorption of anti-Na channel antibodies. Membranes and bound antibody were removed by sedimentation in an Airfuge for 10 min at 180,000 × *g*. The supernatant was incubated with ^{32}P-labeled Na channel for 4 hr and then with SAC for 30 min. Anti-Na channel antibody remaining in the membrane-adsorbed supernatant was quantified by sedimentation of the SAC and determination of the amount of ^{32}P in the SAC immunoprecipitates.

For RIA of solubilized samples, antiserum was incubated with solubilized Na channel for 16 hr, incubated with ^{32}P-labeled channel for 4 hr, and immunoprecipitated with SAC as for the membrane RIA.

$NaDodSO_4$ Gel Electrophoresis. Electrophoresis was based on the method of Maizel (18) using a stacking gel of 3% acrylamide and a running gel with a linear 3–8% acrylamide gradient. Samples in gel loading buffer (3% $NaDodSO_4$/30 mM Tris/2 mM EDTA/5% sucrose/0.01% bromophenol blue, pH 9.0) were adjusted to the indicated concentration of 2-mercaptoethanol and boiled for 5 min to reduce disulfide bonds. Iodoacetamide was then added to a final concentration of 15 mM and incubated for 20 min at room temperature. pH was adjusted to 7.4 (by addition of HCl) just before samples were loaded on the gels. Gels were fixed and stained with Coomassie blue, dried, and placed on Kodak X-OMAT film for 1–24 hr. Gel slices of interest were dissolved in hydrogen peroxide, and ^{32}P was determined by liquid scintillation spectrometry.

Protein Determination. Protein was determined by Peterson's (19) modification of the Lowry assay with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Neonatal Rat Brain Contains Free α Subunit. Previously, the polypeptide components of the Na channel have been detected by their specific binding of neurotoxins that recognize only functional forms of the channel. The rapid and selective phosphorylation of the α subunit by cAMP-dependent protein kinase and the availability of specific antisera against the Na channel (16) now provide means for detection and characterization of inactive forms of the channel. Na channels were solubilized from rat brain membranes, partially purified by immunoprecipitation, and phosphorylated with [γ-^{32}P]ATP and cAMP-dependent protein kinase. Analysis of the labeled α subunit by $NaDodSO_4$ gel electrophoresis and autoradiography shows a single labeled band of 300 kDa in the absence of reducing agent, a clearly resolved doublet of bands of 300 kDa and 260 kDa after partial reduction with 1 mM 2-mercaptoethanol, and a single band of 260 kDa after complete reduction with 5 mM 2-mercaptoethanol (Fig. 1*A*). Immunoprecipitation of these proteins was completely blocked by pure Na channels, confirming their identity (ref. 16; unpublished results). These results show that essentially all of the α subunit in a crude synaptosomal fraction of adult rat brain is present as a disulfide-linked complex with the β2 subunit as previously concluded from analysis of purified Na channels and photoaffinity-labeled Na channels in brain membranes (7). Free α subunits—that is, α subunits not disulfide-linked to a β2 subunit—are not detected.

In contrast, a similar analysis of the α subunit in 16-day postnatal rat brain reveals the presence of a substantial concentration of free α subunit in the absence of reducing agent (Fig. 1*B*). Reduction with 2-mercaptoethanol converts all of the α-β2 complex to free α subunit as in adult brain (Fig. 1*B*). Approximately 50% of the α subunit is free in 16-day rat brain. The number of functional Na channels per mg of rat or mouse brain protein as measured by STX or TTX binding is <10% of the adult level at birth and increases to the adult level by postnatal day 21 (20–22). We find that the ratio of α-β2 complex to free α subunit increases progressively during this time (data not shown). The disulfide-linked α-β2 complex may represent the functionally active form of the Na channel.

Free α Subunits Are in an Intracellular Pool in Cultured Rat Brain Cells. Embryonic rat brain cells in primary cell culture develop Na channels over a similar time course and to a similar extent as *in vivo* (23). After 2–3 weeks *in vitro*, our cultures consist of small aggregates of neuronal somata with extensively developed networks of neurites growing over a monolayer of nonneuronal cells and contain an average of 850 fmol of STX binding sites per mg of protein. The form of the α subunit in these cells was examined by solubilizing the cells

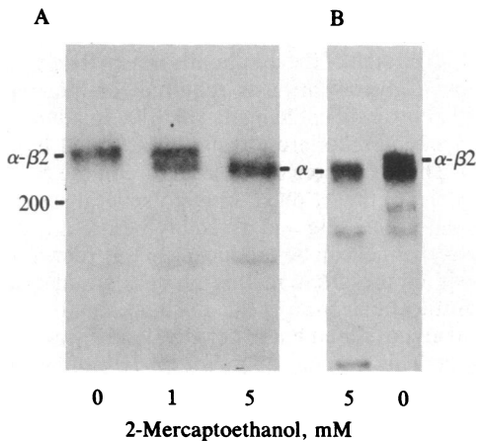

FIG. 1. Disulfide-linked and free α subunits in neonatal and adult rat brain. Rat brain p3 membranes from day 16 (*B*) and adult (*A*) rat brains were prepared and solubilized as described. Immunoprecipitates were prepared and phosphorylated, and the α subunit was resolved by $NaDodSO_4$ gel electrophoresis. The autoradiograms of the gels are shown. Samples were reduced with the indicated concentration of 2-mercaptoethanol (mM). The position of Coomassie blue-stained myosin heavy chain is shown (M_r 200,000, shown ×10^{-3}).

in Triton X-100, immunoprecipitating Na channels, phosphorylating the α subunit with cAMP-dependent protein kinase, and analyzing the labeled subunits by $NaDodSO_4$ gel electrophoresis with and without reduction by 2-mercaptoethanol. Like neonatal rat brain *in vivo*, the embryonic rat brain cells developing *in vitro* have a large pool of free α subunit (Fig. 2, lanes 5 and 6). Approximately 79% of the α subunit is in the free pool.

The binding of antibodies in our antiserum to the α subunit is only very slowly reversible. The complex of antibody and solubilized Na channel does not dissociate appreciably in 24 hr. Antibody bound to Na channels in intact cells is quantitatively retained through washing for 1 hr. In order to determine the subcellular location of the α-β2 complex and free α subunit, intact rat brain cells were incubated with antiserum to allow interaction with α subunits available at the cell surface but not those located inside the cell. The cells were then solubilized, and the form of the cell-surface Na channel was examined by immunoprecipitation, phosphorylation, and $NaDodSO_4$ gel electrophoresis with or without reduction. The results show that most of the cell-surface α subunit is present as an α-β2 complex (Fig. 2, lanes 1 and 2).

After immunoprecipitation of cell-surface α subunit, the soluble supernatant was incubated with antiserum, and the form of any remaining, intracellular α subunits was examined by the same procedure. An average of 77% of the total ^{32}P-labeled α subunit is recovered in the intracellular pool and nearly all of that is free α subunit, not disulfide-linked to β2 (Fig. 2, lanes 3 and 4). We conclude that free α subunits are primarily, if not entirely, intracellular, while the α-β2 complex is primarily located at the cell surface. Formation of the disulfide bond between the α and β2 subunit may be a late event in the posttranslational processing of the Na channel, which occurs concomitant with insertion into the plasma membrane.

Intracellular α Subunits Are Inactive. STX is membrane-impermeant and inhibits mature Na channels only from the extracellular surface (24). In previous studies of Na channel turnover in cultured neuroblastoma cells, we developed conditions for hypotonic lysis of cells that were effective in measuring intracellular STX binding sites (14). No intracellular STX binding sites were observed under normal growth conditions, but intracellular STX binding sites accumulated if lysosomal degradation was blocked (14). We have applied similar lysis conditions to determine whether the intracellular pool of α subunits is competent to bind STX. For intact cells,

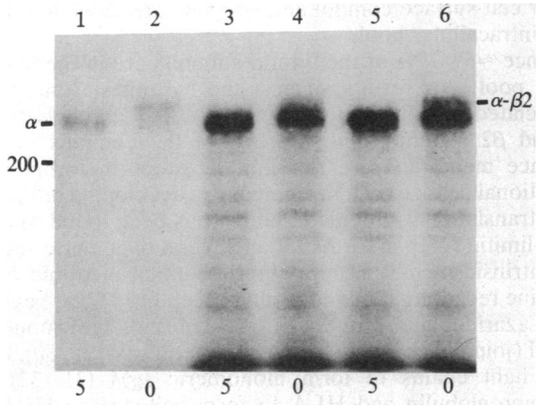

FIG. 2. Disulfide-linked and free α subunits in cell-surface and intracellular membranes. Day 23 cultures of rat brain cells were incubated with antiserum prior to solubilization to allow immunoprecipitation of cell-surface α subunits (lanes 1 and 2). The remaining α subunit was immunoprecipitated with additional antiserum (lanes 3 and 4). Total cellular α subunit is shown in lanes 5 and 6.

74% of the α-β2 complex is accessible to antibody before solubilization, but only 21% of the free α subunit is accessible (Table 1). After hypotonic lysis, the percentage of free α subunit accessible to antibody before detergent solubilization increases 2.9 ± 0.6-fold to 61%. In contrast, the percentage of α-β2 complex accessible to antibody is not increased significantly by hypotonic lysis, consistent with its proposed location at the cell surface. Since most of the antibodies in our antiserum react with the extracellular surface of the α subunit (see below), the 2.9-fold increase in α subunit accessible to antibody should be accompanied by a similar increase in STX binding, if intracellular α subunits are competent to bind STX with high affinity. However, no increase in STX binding was observed upon hypotonic lysis (Table 1). Similar results to those in Table 1 were also obtained when cells were lysed with 0.5% saponin as described by Fambrough and Devreotes (25). On the basis of these results, we conclude that the intracellular pool of free α subunits is not competent to bind STX.

The Intracellular Pool Accounts for Most of the Immunoreactive α Subunits in Rat Brain Cells. The experiments described so far rely on phosphorylation to quantitate the cell surface and intracellular pools of α subunits. If these two pools are phosphorylated to different extents by unlabeled phosphate *in vivo*, this technique will give a misleading estimate of the fraction of α subunits in each. To determine the fraction of α subunits in the intracellular pool more rigorously, we designed two different RIA protocols. In the first experiment, we compared the ability of intact cells and hypotonically lysed cells to bind anti-α subunit antibody and remove it from solution. Increasing numbers of intact brain cells were incubated with 0.5 μl of antiserum, and the antibody–cell complex was removed by sedimentation. The anti-α subunit antibody remaining in solution was then quantitated by immunoprecipitation of ^{32}P-labeled α subunit. Fig. 3*A* shows that the immunoprecipitation of ^{32}P-labeled α subunit can be reduced >85% by adsorption to intact cells with 50% reduction by 3 nM STX binding sites. These results show that >85% of the antibody reactivity in our antiserum

Table 1. Accessibility of STX binding sites and immunoreactive α subunits in intact and lysed cells

Assay	Intact cells	Lysed cells	Ratio
[^{3}H]STX binding, %	100	98	0.98 ± 0.2
^{32}P-labeled α subunits accessible to Ab			
α-β2 complex, %	74	83	1.1 ± 0.1
Free α subunit, %	21	61	2.9 ± 0.6

For STX binding, rat brain cells were gently removed from the plate, and a portion was hypotonically lysed as described. STX binding was then measured, and binding to intact cells is designated as 100%. For $NaDodSO_4$ gel analysis, intact and lysed cells were prepared similarly. Each preparation was incubated with antiserum, washed by centrifugation, solubilized, immunoprecipitated with SAC, phosphorylated, and subjected to $NaDodSO_4$ gel electrophoresis with or without reduction of disulfide bonds by 2-mercaptoethanol as described in the legend to Fig. 2 and *Experimental Procedures*. This procedure detects α subunit accessible to antibody (Ab) before solubilization. To detect the α subunit that is inaccessible to antibody before solubilization, the solubilized supernatant from the SAC immunoprecipitation was incubated with antiserum again and processed as above. ^{32}P in gel slices corresponding to the α-β2 complex and the free α subunit was determined. For each sample, ^{32}P in the free α subunit was estimated from the α-subunit band in the unreduced gel lane. The ^{32}P in the α-β2 complex was estimated as the difference between cpm in the α-β2 band in an unreduced lane and that in a neighboring reduced lane. Data entries in the table are the percentage of each species that is accessible to antibody before solubilization.

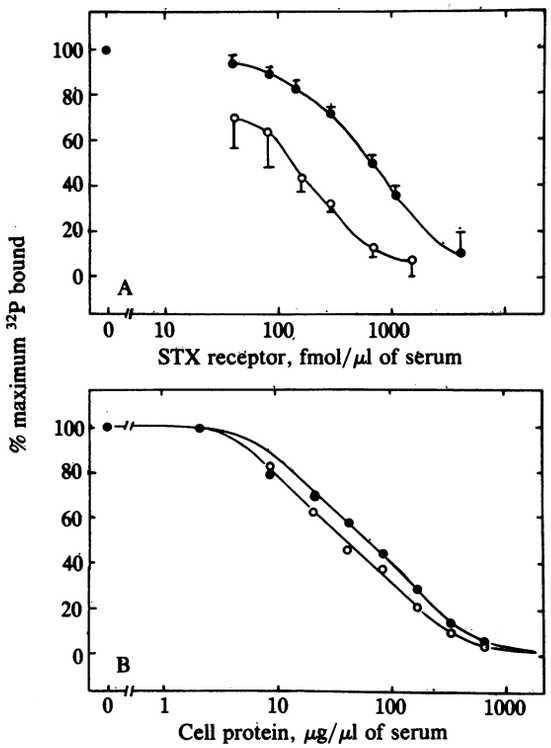

FIG. 3. Estimation of cell surface and intracellular α subunits by RIA. (*A*) Membrane RIAs of intact (●) and lysed (○) cells were performed as described. Amounts of membranes assayed are expressed as STX binding sites. Results are averages of three separate experiments, and SEMs are shown. (*B*) Cell cultures were incubated with immune serum (●) and processed to remove surface α subunits as described. Control cells were incubated with preimmune serum (○). Solubilized cells were analyzed by RIA as described. Amounts of solubilized cells are expressed as μg of protein. For all RIAs, the ^{32}P precipitated nonspecifically by preimmune serum was subtracted from each point to give the data shown.

is directed against antigens on the extracellular surface of the Na channel that are available in intact cells. The corresponding experiment carried out with hypotonically lysed cells (Fig. 3*A*) gives 50% reduction in immunoprecipitation by 0.65 nM STX binding sites. In three experiments, hypotonic lysis shifted the antibody adsorption curve 4.4 ± 1.0-fold. Since almost all of the immunoreactivity of the serum is directed against cell-surface antigens, the increased adsorption of antibody by lysed cells cannot be due to exposure of the intracellular face of the Na channel. Therefore, these results indicate that hypotonic lysis exposes new α subunits in an intracellular pool that does not bind STX. An intracellular pool accounting for 77% of the immunoreactive α subunit is consistent with these data.

In the second RIA protocol, we compared the block of immunoprecipitation of ^{32}P-labeled α subunit by unlabeled α subunits solubilized from whole cells (Fig. 2*B*) or from the intracellular pool. Solubilized α subunits from the intracellular pool were prepared by exposure of intact cells to antiserum, solubilization in Triton X-100, and removal of cell surface α subunits by adsorption to SAC. Analysis of the resulting supernatant by immunoprecipitation, phosphorylation, and NaDodSO$_4$ gel electrophoresis verified that the α-β2 complex present on the cell surface had been effectively removed, while the free α subunit of the intracellular pool remained. Inhibition of immunoprecipitation by this supernatant fraction (Fig. 3*B*) was then compared to inhibition by solubilized whole cells. Removal of the cell surface α subunits by prior immunoprecipitation caused only a 1.5-fold shift in the RIA curve. This result indicates that 67% of the α subunits are in the intracellular pool and only 33% are removed with the cell-surface pool. Thus, estimates made by radioactive labeling and by two different RIA procedures agree that 67–77% of the α subunits in cultured embryonic brain cells are present in an inactive intracellular pool consisting primarily of free α subunits.

Nature and Functional Role of the Intracellular Pool of Free α Subunits. Our results define several molecular characteristics of the free α subunits. They comigrate with the α subunit of the native Na channel, indicating that glycosylation of the intracellular protein is substantially complete. Consistent with this conclusion, the free α subunits in the intracellular pool bind quantitatively to wheat germ agglutinin-Sepharose (unpublished results), indicating that they contain complex carbohydrate chains with *N*-acetylglucosamine residues. The subunits are sedimented at 80,000 × *g*, indicating that they are membrane-bound as expected of a newly synthesized intracellular form of a cell-surface glycoprotein. The free α subunits are not disulfide-linked to β2 subunits, a characteristic we have used to estimate their relative amount. However, since all of our measurements were made under denaturing conditions by specific phosphorylation of the α subunit, we do not know whether the free α subunits are noncovalently associated with β1 and/or β2 subunits *in vivo*. Methods must be developed to label and specifically immunoprecipitate intracellular β subunits in order to examine this point.

Our experiments do not define the role of the intracellular α subunits, but many of their characteristics suggest that they may represent a long-lived pool of precursors to functional cell-surface Na channels. They are present in large amounts in embryonic and neonatal rat brain when the synthesis of new neuronal membrane is very active. They are not a prominent component of adult brain (Fig. 1*A*), which is much less active in the synthesis of new neuronal membrane. In pulse–chase experiments, they are heavily labeled by [^{35}S]methionine before labeled α subunits can be detected on the cell surface, consistent with a role as a precursor to the mature α subunit (unpublished data). The turnover time of the intracellular pool of α subunits is relatively long, comparable to that of mature Na channels that have reached the cell surface. The intracellular pool may act as a ready reserve for rapid incorporation of channels into the cell surface during active membrane biogenesis. Pulse–chase experiments that follow the complete life cycle of the Na channel subunits and the other components of the vesicular pool will be required to resolve this issue and to examine whether other cell-surface components are similarly concentrated in this intracellular pool.

Since ≈67–77% of the total α subunits are in the intracellular pool, our results suggest the hypothesis that events associated with formation of the disulfide bond between the α and β2 subunits and insertion of the complex into the surface membrane are rate-limiting steps in formation of functional cell-surface Na channels in developing brain cells. Posttranslational subunit assembly has been found to be the rate-limiting step in formation of several oligomeric secreted or intrinsic membrane proteins, including nicotinic acetylcholine receptors (26) and insulin receptors (27) as reviewed by Lazarides and Moon (28). Assembly of IgM monomers and J (joining) chains to form pentameric IgM (29, 30), heavy and light chains to form monomeric IgM (31, 32), and β_2-microglobulin and HLA to form cell-surface HLA (33) involves formation of a complex of dissimilar subunits as an essential, rate-limiting step. In each of these examples, the larger of the two components is produced in excess and remains in the intracellular compartment until associated with the smaller subunit. Considered in the light of these observations, the present results raise the interesting possibility that posttranslational assembly and/or disulfide link-

age of preformed α subunits with one or both of the β subunits may be an important point of regulation in biogenesis of functional Na channels in the brain.

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