Palmitylation, Sulfation, and Glycosylation of the α Subunit of the Sodium Channel

ROLE OF POST-TRANSLATIONAL MODIFICATIONS IN CHANNEL ASSEMBLY*

(Received for publication, April 17, 1987)

John W. Schmidt and William A. Catterall
From the Department of Pharmacology, School of Medicine, University of Washington, Seattle, Washington 98195

Antibodies to the α and β2 subunits and site-directed antibodies that distinguish α subunits of the R1 and RII subtypes have been used to study the biosynthesis and assembly of sodium channels. The R1 sodium channel subtype is preferentially expressed in rat brain neurons in primary cell culture. Post-translational processing of α subunits includes incorporation of palmitoyl residues in thioester linkage and sulfate residues attached to oligosaccharides. The incorporation of [3H]palmitate into α subunits is inhibited by tunicamycin, indicating that it occurs in the early stages of biosynthesis but after co-translational glycosylation. Mature α subunits are attached to β2 subunits through disulfide bonds within 1 h after synthesis and up to 30% can be specifically immunoprecipitated from the cell surface with antibodies against the β2 subunits by 4 h after synthesis. The remaining α subunits remain in an intracellular pool. The α subunits synthesized in the presence of castanospermine and swainsonine have reduced apparent size. Castanospermine prevents incorporation of approximately 81% of the sialic acid of the α subunit and inhibits sulfation but not palmitoylation. Although inhibition of glycosylation with tunicamycin blocks assembly of functional sodium channels, castanospermine and swainsonine do not prevent the covalent assembly of α and β2 subunits or the transport of αβ2 complexes to the cell surface, and sodium channels synthesized under these conditions have normal affinity for saxitoxin. Thus, the extensive processing and terminal sialylation of oligosaccharide chains during maturation of the α subunit is not essential. A kinetic model for biosynthesis, processing, and assembly of sodium channel subunits is presented.

The voltage-sensitive sodium channel mediates sodium influx during the rising phase of sodium-dependent action potentials. It has been purified in functional form from eel electroplax, rat skeletal muscle, and rat brain (reviewed in Refs. 1-4). The sodium channels purified from adult rat brain are composed of three glycoprotein subunits (3): α (260 kDa), β1 (56 kDa), and β2 (33 kDa) (5, 6). The β1 subunits are associated with the α subunit noncovalently; the α and β2 subunits are disulfide-linked. The β2 subunit, but not the β1 subunit, can be removed from the purified sodium channel without loss of channel function (7, 8). High molecular weight mRNA from rat brain (9), α subunit mRNA purified by hybrid selection (10), and α subunit mRNA synthesized from cloned cDNA (11) can direct the synthesis of functional sodium channels in Xenopus oocytes suggesting that α subunits alone are able to form a functional sodium channel. Two different α subunit mRNAs have been cloned and fully sequenced from rat brain (12). They encode two distinct proteins, R1 and RII, with 87% primary structure identity.

In adult rat brain, most α subunits are present as the αβ2 disulfide-linked complex. In contrast, most α subunits are present in a form that is not disulfide-linked to β2 in neonatal rat brain (13). We refer to these as "free-α" subunits. We have used primary cultures of rat brain neurons as a model system to study the biosynthesis of the α subunit and the assembly of mature sodium channels (13, 14). The αβ2 complexes and functional sodium channels are preferentially localized at the cell surface while free-α subunits are present as an intracellular pool that does not bind the sodium channel ligand saxitoxin (15). Covalent assembly is a late event in α subunit processing, occurring after transport to the Golgi. Free-α subunits are not rapidly degraded and may be available as a precursor pool for assembly of functional sodium channels in growing neurons.

Pulse-chase experiments reveal a short-lived α subunit precursor (αp) of 224 kDa containing high mannose oligosaccharide chains (14). This initial precursor is converted first to an intermediate of 224 kDa containing complex oligosaccharide chains and then to an intermediate of 249 kDa which slowly approaches the mature size of 260 kDa (14). Inhibition of asparagine-linked glycosylation with the inhibitor tunicamycin prevents the formation of functional sodium channels in nerve and muscle cells (15-17), indicating that N-linked glycosylation is essential for sodium channel biosynthesis. In the presence of tunicamycin, rat brain neurons synthesize a core polypeptide (αo) of 203 kDa that is neither processed to forms with larger apparent size nor disulfide-linked to β2 subunits before being rapidly degraded (14).

Since inhibition of co-translational N-linked glycosylation has such profound effects, it is of interest to determine if other post-translational modifications might play a role in the biosynthesis of mature sodium channels, and if post-translational processing of the co-translationally attached oligosaccharide chains also is required for sodium channel biosynthesis. In this report, we show that the R1 sodium channel subtype is preferentially expressed in embryonic rat brain neurons in primary cell culture, describe palmitylation and sulfation of the α subunit of the sodium channel, present new results on the assembly of mature sodium channels, and investigate the requirement for post-translational processing.
of the oligosaccharide chains of the \( \alpha \) subunit by inhibition of specific reactions in the processing pathway via castanospermine or swainsonine.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tunicamycin, neuraminidase (Type X), protein A-Sepharose, wheat germ agglutinin, CNBr-activated Sepharose 4B, and fatty acid-free bovine serum albumin were from Sigma. Tetrodotoxin, fluorodeoxyuridine, castanospermine, and swainsonine were from Behring Diagnostics. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, WGA, wheat germ agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**Electrophoresis, Autoradiography, and Quantification**

STX Binding and Protein Assays—Specific binding of [\( ^{3}H \) ]saxitoxin was measured as described previously (15) in standard binding medium containing 150 mM NaCl, 5 mM KCl, 1.6 mM MgSO\(_4\), pH 7.4. Non-specific binding was determined in the presence of 1 \( \mu \)M tetrodotoxin and subtracted from the total binding to calculate specific binding. Total cell protein was measured by the method of Peterson (19).

Cell Solubilization and Glycoprotein Isolation with Wheat Germ Agglutinin-Sepharose—Cells were rinsed with standard binding medium washed on the plates with 1 ml of standard binding medium and stored frozen until solubilization. Cells from individual cultures were solubilized in 350 \( \mu \)l of 5% Triton X-100 in Buffer N consisting of 50 mM NaHPO\(_4\), 20 mM KF, 2.5 mM EDTA, 25 mM Tris, 75 mM NaCl, pH 7.4, as described previously (13, 14). For isolation of wheat germ agglutinin-binding proteins, solubilized extracts were incubated in a rotating microfuge tube with 100 \( \mu \)l of wheat germ agglutinin-Sepharose for 90 min and washed to remove unbound proteins. Bound glycoproteins were eluted with 1 ml of 1% Triton X-100, 0.4 M NaCl, 50 mM HEPES-Tris, pH 7.4, containing 0.2 M N-acetylglucosamine and protease inhibitors as described previously (14). This eluted fraction is referred to as the wheat germ agglutinin-binding protein pool. For isolation of a wheat germ agglutinin-non-binding protein pool supernatant of the wheat germ agglutinin adsorption and a 0.5-ml wash of the wheat germ agglutinin pellet were pooled as described (14).

**Antibodies and Immunoprecipitation**—Four rabbit anti-sodium channel antibodies were used for experiments described here. The 4675 antisem and anti-sodium channel antibodies are directed against the \( \alpha \) subunit and have previously been characterized (13, 14, 20, 21). Anti-\( \alpha \) subunit antibodies were affinity purified from the 4675 and 7035 antisem as described previously (22). Similar results are obtained with these two anti-\( \alpha \) subunit antisera. Anti-SP114 antisem was prepared against a synthetic peptide of a sodium channel acidic sequence (residues 467-486) that is unique to the \( R_{0} \) sodium channel subtype (23). Anti-SP11 antisem was prepared against a synthetic peptide with the amino acid sequence (residues 465-481) for the corresponding segment of the \( R_{0} \) sodium channel subtype (25). These antisera specifically precipitate the homologous channel subtype (25).

Monoclonal antibody 1G11 was generated following immunization of a mouse with purified sodium channels and is directed against the \( \alpha \) subunit. For the experiments described here, 1G11 antibodies were from an ascites fluid.

For preparation of blocked 7035 anti-\( \alpha \) subunit antibodies, approximately 5 pmol of sodium channels which had been purified through the final sucrose gradient centrifugation step (24) were incubated with 10 pmol of affinity-purified 7035 antibodies equivalent by radioimmunoassay to 1 \( \mu \)l of whole antisem. For preparation of blocked 2843 anti-\( \beta \) subunit antibodies, 2 pmol of sodium channel was used to block 1 \( \mu \)l eq of antisem. The blocked antibodies were >95% blocked as determined by radioimmunoassay.

For immunoprecipitation of sodium channels from solubilized cells and from wheat germ agglutinin-binding and -nonbinding protein pools, the following amounts of antibody reagents were used per rat brain neuron culture: 5 \( \mu \)l of 4675 anti-\( \beta \) antibodies or 4675 anti-\( \alpha \) subunit antibodies, 1 \( \mu \)l of 7035 antisem or 1 \( \mu \)l eq of 7035 anti-\( \alpha \) antibodies, or 2 \( \mu \)l of monoclonal 1G11 ascites fluid. One mg of protein A-Sepharose was added to each mixture for each mg of ascites fluid; 0.5 mg was used per \( \mu \)l equivalent of affinity-purified antibodies. Sodium channel extracts were incubated with antibodies on ice for 16-24 h, followed by incubation with protein A-Sepharose for 1 h.

Cell-surface immunoprecipitates were obtained by incubation of intact cells in culture dishes with 25 \( \mu \)l of 7035 or 50 \( \mu \)l of 2843 antisem in standard binding medium as previously described (14).

**Phosphorylation of Immunoprecipitates—Sodium channel \( \alpha \) subunits in immunoprecipitates were labeled with \( ^{32}P \) by phosphorylation with the catalytic subunit of CAMP-dependent protein kinase as described previously (13).

Electrophoresis, Autoradiography, and Quantification of Radioactivity in Gel Slices—Samples were prepared for SDS-PAGE in sample buffer containing 30 mM Tris, 10 mM EDTA, 3% SDS, 5% sucrose, pH 7.4. For reduction, a 1:1 mixture of sample and buffer, samples were adjusted to 5 \( \mu \)M \( \beta \)-mercaptoethanol in SDS sample buffer. For reduction and alkylation, samples were adjusted to 5 \( \mu \)M \( \beta \)-mercaptoethanol in sample buffer at pH 9.0 and boiled for 5 min. The hot samples were adjusted to 15 mM iodoacetamide, cooled at room temperature for 30 min, and adjusted to pH 7.4 in a solution of HCl before loading on SDS-PAGE as described previously (13) using 3-10% linear polyacrylamide gradients.

In previous experiments, we have established the apparent molecular masses of the mature \( a_{2} \) complex and the mature \( \alpha \) subunit as 300 and 260 kDa, respectively (5, 14). These two values, plus the

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Footnotes:

1. The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WGA, wheat germ agglutinin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

migration position of myosin (205 kDa), were used as markers for interpolation of apparent molecular mass values for the other α subunit forms described in these experiments. The 3–10% gradient gels used here allow for separation of α, α2 (203 kDa) and the αβ2 complex (300 kDa) over about 10% of the length of the gel and result in comparatively sharp bands, even though the α subunit appears to have considerable microheterogeneity. α Subunit forms differing in size by 15 kDa can be reproducibly resolved in the same sample under these conditions. Differences in average size of 4 kDa can be resolved by comparison of separate samples.

RESULTS

Expression of Sodium Channel Subtypes in Rat Brain Neurons in Primary Cell Culture—Two different mRNAs which encode sodium channel α subunits with 87% primary structure identity have been cloned and sequenced from rat brain (23). We have developed sequence-directed antibodies corresponding to predicted amino acid residues 465–481 of the R1 subtype and residues 467–486 of the R11 subtype (23). This segment is missing in the electrophores sodium channel and contains only 5 widely spaced residues which are homologous between R1 and R11. Our anti-R1 and anti-R11 antibodies immunoprecipitate these sodium channel subtypes specifically (23). To examine which of the two sodium channel subtypes is expressed in embryonic rat brain neurons in cell culture, sodium channels were solubilized, isolated by immunoprecipitation with either anti-SP111 or anti-SP11l1, radiolabeled by phosphorylation with cAMP-dependent protein kinase, and analyzed by SDS-PAGE and autoradiography. As illustrated in Fig. 1, anti-SP111 antibodies are effective in immunoprecipitating α subunits (lane 1) while anti-SP11l1 antibodies are not (lane 2). Both free-α and αβ2 complexes are immunoprecipitated (Fig. 1), indicating that a single α subunit subtype is responsible for these two pools of α subunits.

Incorporation of [3H]Palmitate into Newly Synthesized α Subunits—Newly synthesized sodium channel α subunits undergo several stages of post-translational processing which cause changes in the apparent size (14). Many plasma membrane glycoproteins have covalently attached palmitic acid in amide or thioester linkages (25–27) and a preliminary report indicates that the purified sodium channel from eel electroplax contains substantial covalently attached fatty acid that is predominantly palmitate (28). Rat brain neurons were grown in the presence of [3H]palmitate for 24 h as described under "Experimental Procedures" and sodium channel α subunits were isolated by immunoprecipitation and analyzed by SDS-PAGE and autoradiography. Polypeptides migrating at the positions of both free-α and αβ2 complexes incorporated [3H]palmitate (Fig. 2A, lane 1). These polypeptides were not immunoprecipitated with antibodies that had been blocked by prior incubation with purified sodium channels confirming the identification of these polypeptides as α and αβ2 (Fig. 2A, lane 2). The [3H]palmitate is removed from α by incubation of the SDS gel with 1 M hydroxylamine, pH 7.4, prior to autoradiography (Fig. 2B, compare lanes 1 and 2). Removal of palmitate under these conditions indicates attachment by thioester linkage to cysteine residues (25).

We have previously observed that block of co-translational N-glycosylation by growth of cells in the presence of tunicamycin prevents synthesis of lectin-binding forms of the α subunit (14). In order to measure the extent of incorporation of [3H]palmitate into pre-existing sodium channels, rat brain neurons were grown overnight in tunicamycin and then palmitate incorporation into WGA binding forms of α was measured during a subsequent 24-h pulse. Incorporation of palmitate into pre-existing α subunits was low (Fig. 2A, lane 3), indicating that most palmitylation of sodium channels occurs during initial biosynthesis and processing. Weak labeling of mature α subunits is detected in the presence of tunicamycin. This low level of incorporation must be due to palmitylation of sodium channels that were synthesized before addition of tunicamycin, since tunicamycin completely blocks synthesis of glycosylated channels (14).

In order to examine the effect of tunicamycin on palmitoylation of newly synthesized sodium channels, the incorporation of [35S]methionine and [3H]palmitate into α subunit precursors was measured in a 4-h pulse in the presence and absence of tunicamycin (Fig. 2C). Under control conditions, α subunits labeled with [35S]methionine were observed (Fig. 2C, lane 1) and the incorporation of [3H]palmitate into the newly synthesized subunits was easily detected (Fig. 2C, lane 3). We have previously reported that the core α subunit polypeptides made in the presence of tunicamycin (α, 203 kDa) are not processed to larger forms and are degraded within several hours after synthesis (14). Incorporation of [35S]methionine into α is observed in the presence of tunicamycin (Fig. 2C, lane 2), but no incorporation of [3H]palmitate into this polypeptide is detected (Fig. 2C, lane 4). These results indicate that block of N-glycosylation completely prevents palmitoylation of newly synthesized α subunits. Since palmitoylation of several other proteins takes place in the Golgi (26, 27), palmitoylation may be inhibited because α is not transported from the endoplasmic reticulum to the Golgi.

It might be expected that palmityl residues would alter the apparent molecular weight of sodium channel α subunits in SDS-PAGE by 'binding excess SDS (28). In order to assess the contribution that palmitoylation makes to the apparent molecular weight of the α subunits under our gel conditions, palmitoyl residues were removed by treatment for 2 h with 1 M hydroxylamine, pH 7.4, and the decylated α subunits were analyzed by SDS-PAGE. In several experiments, deacylation
either had no effect on apparent molecular weight or reduced it by less than 4 kDa, the limit of reliable estimate of size change in our experiments. Thus, palmitylation does not make a major contribution to the apparent size of α subunits when analyzed under our conditions of gel electrophoresis.

Incorporation of $^{35}$SO$_4$ into Newly Synthesized α Subunits—Many glycoproteins contain sulfate groups on their carbohydrate moieties (29, 30). To examine sulfation of the α subunit of the sodium channel, rat brain neurons in primary cell culture were grown for 24 h in growth medium containing $^{35}$SO$_4$, the α subunits were isolated by immunoprecipitation with affinity-purified antibodies, and incorporation of $^{35}$SO$_4$ was measured by SDS-PAGE and autoradiography as described under “Experimental Procedures.” $^{35}$SO$_4$ was detected in the α subunit band (Fig. 3, lane 2). If the antibodies used for immunoprecipitation were first blocked by incubation with purified sodium channels, no labeled protein is detected with the size of the α subunit (Fig. 3, lane 1), confirming identification of the $^{35}$SO$_4$-labeled protein as the α subunit of the sodium channel. Thus, the α subunit is modified by palmitylation, sulfation, and glycosylation during early post-translational processing during assembly into mature sodium channels.

The Time Course of Appearance of αβ2 Complexes at the Cell Surface—αβ2 Complexes are preferentially localized at the cell surface while free-α subunits are preferentially localized in the intracellular compartment (13, 14). However, the anti-α subunit antibodies used in these previous studies co-immunoprecipitate the larger pool of free-α subunits along with αβ2 so that careful analysis of SDS gels before and after reduction of disulfide bonds is required to determine the amount of αβ2 complex present. We have therefore re-examined the preferential localization and the time course of appearance of αβ2 complexes at the cell surface by direct immunoprecipitation with antibodies against the β2 subunit. Incubation of intact cells with anti-β2 antiserum, followed by washing to remove unbound antibody, solubilization of membranes, isolation of antigen-antibody complexes, phosphorylation, and SDS-PAGE analysis shows that these antibodies bind only αβ2 complexes at the cell surface (Fig. 4A, lane 3). Addition of anti-α antiserum to the detergent-solubilized supernatant from the cell-surface immunoprecipitation allows immunoprecipitation of the remaining intracellular α subunits, which are mostly free-α subunits (Fig. 4A, lane 4). For comparison, control experiments were performed with the anti-α subunit antiserum 7035. This antiserum also immunoprecipitates only αβ2 complexes from the cell surface (Fig. 4A, lane 2), while free-α subunits are accessible to antibodies for immunoprecipitation only after detergent lysis of the cells (Fig. 4A, lane 1). Thus, immunospecific isolation of sodium channels from rat brain neurons in cell culture with anti-α subunit antisera 4675 (13) or 7035 (Fig. 4A) or with anti-β2 subunit antiserum 8249 (Fig. 4A) shows that αβ2 complexes are preferentially localized at the cell surface while free-α subunits are intracellular.

Anti-β2 antibodies selectively isolate newly synthesized αβ2 complexes from cells that have been pulse-labeled with $[^{35}$S]methionine and incubated in chase medium for an additional 20 h to allow subunit assembly (Fig. 4B). Under nonreducing conditions, a single labeled polypeptide that migrates at the
complexes at the cell surface was determined by immunoprecipitation with anti-n subunit antibodies for isolation of the intracellular position of the αβ2 complex (300 kDa) is observed (Fig. 4B, lane 1). The amount of [35S]methionine in β2 subunits is too small to be detected by this technique (14). Antibodies against β2 subunits that have been blocked by pre-incubation with purified sodium channel [lane 3] or without (lane 2) reduction of disulfide bonds. One-third of the sample was reacted with 8243 antisera that had been blocked by preincubation with purified sodium channel (lane 1). C, rat brain neurons were pulse-labeled with 200 μCi/ml [35S]methionine and incubated in chase medium for the indicated times. The cells were then incubated with 8243 antisera for isolation of cell surface αβ2 complexes and further processed with 1G11 monoclonal antibodies to immunoprecipitate the remaining α subunits. The results were quantified by determination of [35S] in gel slices and the percent of the total [35S]-α recovered in the surface immunoprecipitates was plotted.

The time course of appearance of newly synthesized αβ2 complexes at the cell surface was determined by immunoprecipitation and quantified by determination of [35S] methionine in gel slices. Increasing amounts of αβ2 complex are detected at the cell surface from 1 to 4 h after synthesis. The fraction of newly synthesized α subunits that is covalently assembled with β2 subunits and inserted into the cell surface approaches 30%. The time course and extent of appearance of newly synthesized αβ2 complexes on the cell surface is similar to the time course for overall assembly of αβ2 complexes analyzed previously (14). Therefore, these results with anti-β2 subunit antibodies confirm our previous analysis of the delayed covalent assembly of αβ2 complexes and show that αβ2 is detected at the cell surface concomitant with assembly.

Effects of Castanospermine and Swainsonine on Biosynthesis and Assembly of the α Subunit—Inhibition of N-linked glycosylation with tunicamycin prevents all of the changes in apparent size that occur during processing of the α subunit precursors and inhibits the synthesis of metabolically stable α subunits and their incorporation in mature sodium channels (14, 15). We have used inhibitors of two specific oligosaccharyl-processing enzymes which act at early and late steps in the processing pathway to investigate the requirement for processing reactions in biosynthesis and assembly of mature sodium channels in the plasma membrane. Castanospermine inhibits glucosidases I and II of the endoplasmic reticulum (31). These enzymes remove capping glucose residues from newly attached asparagine-linked oligosaccharide chains as the first step in processing of N-linked oligosaccharide chains. Swainsonine inhibits mannosidase II of the Golgi (32, 33). This enzyme removes terminal mannose residues from one of the two branches of biantennary oligosaccharide chains, which is required for further processing and sialylation of that branch.

Rat brain neurons were pulse-labeled for 1 h in medium containing [35S]methionine and incubated for an additional hour in chase medium. α Subunits were isolated by immunoprecipitation and analyzed by SDS-PAGE and autoradiography. Under control conditions, most labeled α subunits are processed to an apparent size of 249–260 kDa during the chase period while a smaller population of α subunit precursors (ααp, 224 kDa) is also detected (Fig. 5A, lane 1). In contrast, newly synthesized sodium channels have an apparent size of 236 kDa in the presence of castanospermine or swainsonine (Fig. 5A, lanes 2 and 3). The sizes of these glycoprotein precursors are intermediate between αp (224 kDa) which is expected to contain trimmed high mannose chains and mature α (260 kDa) which contains fully processed complex oligosaccharide chains. These results show that both castanospermine and swainsonine effectively inhibit processing of sodium channel precursors.

To estimate the mature sizes and examine the assembly of sodium channel α subunits of rat brain neurons grown in the presence of swainsonine, cultures were pulse-labeled with [35S]methionine for 1 h and incubated in chase medium for 20 h. Sodium channel α subunits made in the presence of swainsonine are processed to a mature size of approximately 248 kDa, slightly smaller than sodium channels of control cells (260 kDa) (Fig. 5B, lanes 3 and 4). αβ2 complexes from control and swainsonine-treated cultures were immunoprecipitated by anti-β2 subunit antibodies (Fig. 5B, lanes 1 and 2). The apparent size of the αβ2 complexes from swainsonine-treated cells was approximately 283 kDa compared to 300 kDa for control cells, but a similar fraction of α subunits was assembled with β2 subunits in the presence of swainsonine. Similar experiments were carried out with castanospermine. After pulse-labeling and incubation for 20 h in
To examine the amounts of αβ2 complexes in inhibitor-treated cultures directly, αβ2 complexes were isolated by immunoprecipitation with anti-α subunit antibodies and analyzed by SDS-PAGE (Fig. 6B). Comparable amounts of 32P-labeled α subunits were immunoprecipitated from control and inhibitor-treated cells by anti-α subunit antibodies. These results support the conclusion from the [35S]methionine-labeling experiments that the two inhibitors of oligosaccharide processing do not alter the assembly of αβ2 complexes.

To determine if the αβ2 complexes of castanospermine- and swainsonine-treated cells are preferentially localized at the cell surface, intact cells were incubated with anti-α antisem and processed to isolate cell-surface αβ2 complexes (Fig. 6C). Immunoprecipitated α subunits were then labeled with 32P by phosphorylation with CAM-dependent protein kinase. Normal amounts of 32P-labeled cell-surface αβ2 complexes are present in castanospermine- and swainsonine-treated cells. Thus, both assembly of the disulfide-linked αβ2 complex and insertion into the cell surface membrane proceed normally despite the inhibition of oligosaccharide processing by castanospermine and swainsonine. These results show that castanospermine and swainsonine alter the normal oligosaccharide processing of the sodium channel without altering the steady state level of α subunits, the formation of αβ2 complexes, or their transport to the cell surface.
Post-translational Modification of Sodium Channels

Effect of Castanospermine on Palmitylation and Sulfation of the α Subunit—As described above, sodium channel α subunits synthesized in the presence of tunicamycin are not palmitylated. In contrast, castanospermine treatment does not reduce incorporation of $^{35}$Smethionine into α subunits (Fig. 7A, lanes 1 and 2) and does not reduce palmitylation of α subunits (Fig. 7A, lanes 3 and 4). Evidently, palmitylation of the α subunits requires addition of N-linked oligosaccharide chains, but does not require processing to their mature form.

In contrast to palmitylation, castanospermine treatment blocks sulfation of the α subunit essentially completely (Fig. 7B). These results support our conclusion that the incorporation of $^{35}$SO$_2$ into the α subunit reflects sulfation of carbohydrate residues (29).

Effects of Castanospermine and Swainsonine on Saxitoxin Binding—We have previously shown that growth of rat brain neurons in the presence of tunicamycin results in a loss of saxitoxin-binding sites with approximately the same first order kinetics as the degradation of $^{35}$Smethionine-labeled αβ2 complexes (14). Pulse-chase experiments showed that α3 is rapidly degraded after synthesis and is not assembled with β2 subunits or inserted into the cell surface. Since the incompletely glycosylated sodium channels from castanospermine- and swainsonine-treated cells accumulate at normal levels and are incorporated into the cell surface (Figs. 5 and 6), it was of interest to determine if they achieve a functional conformation as measured by saxitoxin binding. Cells grown in the presence of castanospermine or swainsonine for 3 days had an average of 95 (±3) and 91% (±6) of the control level of saxitoxin-binding sites, in contrast to the exponential loss of saxitoxin-binding sites in the presence of tunicamycin (Fig. 8A). Specific binding of saxitoxin to sodium channels from control and castanospermine-treated cells was measured over a range of concentrations to determine whether inhibition of oligosaccharide processing had any effect on the affinity for saxitoxin. The data indicate that sodium channels of control and castanospermine-treated cells have the same affinity for saxitoxin (Fig. 8B). Evidently, the normal processing of sodium channel oligosaccharide side chains is not required for formation and metabolic stability of sodium channels with the normal affinity for saxitoxin.

Glycosidase Digestion of α Subunits—The results with glycosylation inhibitors suggested that most of the 57 kDa increase in apparent size of α subunits from α1 of 203 kDa to mature α of 260 kDa might be accounted for by oligosaccharide addition and processing. The difference in size between α1 and α2 (21 kDa) could be due to co-translational addition of core asparagine-linked chains and the difference in size between α subunits made in the absence and presence of castanospermine (26 kDa) could be due to post-translational processing of N-linked oligosaccharides. To examine this question more directly, we have used specific glycosidases to remove oligosaccharide components and examine the corresponding changes in apparent size by SDS-PAGE.

Sodium channels were isolated from rat brain neurons by immunoprecipitation, radioactively labeled by phosphorylation, and incubated with different glycosidases. Analysis of the resulting samples by SDS-PAGE resolves decreases in the apparent size of the α subunit after treatment. Removal of sialic acid residues by treatment with neuraminidase reduces the size of α to 239 ± 3 kDa (S.D., n = 12) (Fig. 9A, lane 3). Thus, terminal sialic acid residues appear to make a major contribution to the size of the α subunit. Digestion of desialylated α with endoglycosidase-F results in a further decrease in apparent size (Fig. 9A, lane 2). The apparent size of the α subunits recovered after endoglycosidase digestion is 220 kDa, as observed previously for purified adult brain α (6). The recovery of α subunits after digestion with endoglycosidase-F is not quantitative, so it is not certain that completely deglycosylated α is recovered. In contrast to the major change in apparent size of the α subunit due to removal of the desialy-
With 1G11 monoclonal antibodies. The immunoprecipitated protein kinase, incubated with enzymes as described below, and phosphate, 50 mM NaCl, 25 mM sodium citrate, pH 5.0, for 4 h at 37 °C. The samples were then incubated for 24 h at 37 °C with no enzyme treatment. Lane 2, immunoprecipitated α subunits were incubated with 0.1 unit of neuraminidase in 1% Nonidet P-40, 100 mM sodium chloride, 50 mM NaCl, 25 mM sodium citrate, pH 5.0, for 4 h at 37 °C. The samples from the neuraminidase digestion was adjusted to 0.5% SDS and 1% β-mercaptoethanol and boiled for 5 min. Five percent Nonidet P-40 was added and the pH was adjusted to 6.0 by addition of 0.1 M EDTA, pH 7.4. Endoglycosidase F (0.3 munits) was added and the samples were incubated for 24 h at 37 °C. Lane 3, immunoprecipitated α subunits were incubated with neuraminidase as described for lane 2. Lane 4, immunoprecipitated α subunits were incubated with 0.1 unit of neuraminidase in 1% Nonidet P-40, 100 mM Tris maleate, pH 5.0, for 4 h at 37 °C. The samples were denatured in SDS and diluted into Nonidet P-40 solution as described for lane 2. Glyceranase (0.3 units) was added and the samples were incubated for 24 h at 37 °C. The O-glycanase preparation used was shown to be active in control experiments with fetuin as substrate. Rat brain neurons were grown overnight in normal growth medium and incubated with enzymes as described for lane 2. The cells were pulse-labeled with [35S]methionine with no additions (lanes 3 and 4) or with 50 µg/ml castanospermine (lanes 1 and 2) as described under “Experimental Procedures,” and incubated overnight in chase medium. α Subunits were isolated by immunoprecipitation with 1G11 monoclonal antibodies. The immunoprecipitates were split and the immunoprecipitated α subunits were incubated with neuraminidase as described for lane 2. α Subunits were incubated with neuraminidase as described for lane 2. α Subunits were incubated with neuraminidase as described for lane 2.

**Fig. 9.** Glycosidase digestion of sodium channel α subunits from rat brain neurons. A, Rat brain neurons were collected and solubilized, and α subunits were isolated by immunoprecipitation with 1G11 monoclonal antibodies. The immunoprecipitated α subunits were labeled with 32P by phosphorylation with CAMP-dependent protein kinase, incubated with enzymes as described below, and analyzed by SDS-PAGE and autoradiography. Lane 1, immunoprecipitated α subunits were denatured in 0.5% SDS, 1% β-mercaptoethanol and boiled for 5 min. Five percent Nonidet P-40 was added and the pH was adjusted to 6.0 by addition of 0.1 M EDTA, pH 7.4. Endoglycosidase-F (0.3 munits) was added and the samples were incubated for 24 h at 37 °C. Lane 3, immunoprecipitated α subunits were incubated with neuraminidase as described for lane 2. Lane 4, immunoprecipitated α subunits were incubated with 0.1 unit of neuraminidase in 1% Nonidet P-40, 100 mM Tris maleate, pH 5.0, for 4 h at 37 °C. The samples were denatured in SDS and diluted into Nonidet P-40 solution as described for lane 2. Glyceranase (0.3 units) was added and the samples were incubated for 24 h at 37 °C. The O-glycanase preparation used was shown to be active in control experiments with fetuin as substrate. B, Rat brain neurons were grown overnight in normal growth medium and incubated with enzymes as described for lane 2. The cells were pulse-labeled with [35S]methionine with no additions (lanes 3 and 4) or with 50 µg/ml castanospermine (lanes 1 and 2) as described under “Experimental Procedures,” and incubated overnight in chase medium. α Subunits were isolated by immunoprecipitation with 1G11 monoclonal antibodies. The immunoprecipitates were split and the immunoprecipitated α subunits were incubated with neuraminidase as described for lane 2. α Subunits were incubated with neuraminidase as described for lane 2.

**Figure 10.** Kinetic model of sodium channel α subunit processing. A kinetic model for processing and assembly of mature sodium channel complexes is presented. This model is fit to experimental data in the Miniprint Section and the indicated values for rate constants in hour−1 are derived. Deg., degradation.

**Discussion**

*Post-translational Processing of the α Subunit of the Sodium Channel*—In previous studies we have shown that the sodium channel α subunit undergoes extensive post-translational processing of its asparagine-linked oligosaccharide chains and described the time course of processing of sodium channel α subunits from WGA nonbinding to WGA-binding form, processing to the mature size, and formation of disulfide-linked α92 complexes (14). Experiments with anti-92 antibodies presented in this report have confirmed and extended our previous studies by measuring the assembly and insertion into the cell surface of α92 complexes directly. In addition, we have collected further data (34) defining the kinetics of α subunit processing and assembly more precisely using the methods described previously in Ref. 14. Fig. 10 summarizes a minimal model that describes all of these results. Quantitative evaluation of this kinetic scheme using mathematical models for the kinetics of α subunit processing results in good fits of the pulse-chase kinetic data (see Miniprint Section). The values for the rate constants in Fig. 10 are derived from these fits to the Equations presented in the Miniprint Section. α Subunits are initially synthesized as a 224-kDa glycoprotein precursor which does not bind to wheat germ agglutinin. This form is processed with a half-time of 0.5 h (Fig. 10, k1) to a wheat germ agglutinin-binding form of the same apparent size. This processing step is expected to coincide with addition of N-acetylglucosamine residues and transport from the endoplasmic reticulum to the mid-Golgi. After attainment of WGA binding capacity, α subunits are processed in a single step with a half-time of 0.4 h (Fig. 10, k2) to a form with an apparent size of 249 kDa. This form increases in apparent size with a half-time of 0.6 h (Fig. 10, k3) to finally reach the apparent size of 249 kDa.

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3 Portions of this paper (including part of “Discussion” and Figs. 11-14) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 87M-1251, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
mature value of 260 kDa. The experiments described here provide new information on the processing reactions which are responsible for these changes in the size of the α subunit. Removal of sialic acid with neuraminidase reduces the size of α by 21 kDa. Block of oligosaccharide processing with castanospermine plus removal of sialic acid with neuraminidase reduces the size of α by 28 kDa. It seems likely, therefore, that processing of core oligosaccharide chains in the Golgi that is blocked by castanospermine accounts for at least 7 kDa of apparent size and addition of sialic acid accounts for approximately 21 kDa of the apparent size of mature α. Thus, modifications of the co-translationally added oligosaccharide chains account for at least 28 kDa of the 36-kDa difference in apparent size between α, and mature α.

Measurements of the carbohydrate content of the α subunits purified from adult rat brain also support this conclusion (6, 35, 36). Fifteen to 31% (6, 35, 36) of the mass of the purified protein is carbohydrate, and 9.4% (35) or 16.5% (36) is sialic acid. This content of carbohydrate (40–78 kDa) is sufficient to account for the difference in electrophoretic mobility between α, (203 kDa) and mature α (260 kDa).

Membrane glycoproteins may also be modified by O-linked glycosylation, sulfation, and fatty acylation. O-Glycanase, which cleaves the core oligosaccharide of desialylated O-linked oligosaccharide chains, had no detectable effect on the size of the α subunit suggesting a low level of O-linked glycosylation of α and little or no effect of O-linked glycosylation on its size. Our results provide biosynthetic evidence for sulfation of the newly synthesized α subunit. Inhibition of this reaction by castanospermine indicates that it represents attachment of sulfate to oligosaccharide residues in the Golgi. The contribution of sulfation to the apparent size of the α subunit has not been independently measured, but the presence of charged sulfated moieties may contribute to the 7-kDa increase in apparent size during processing of the core oligosaccharides.

Newly synthesized sodium channel α subunits also incorporate palmitoyl residues. Release of covalently attached palmitate by mild treatment with hydroxylamine indicates that it is attached to thioester linkages (37). The sites of attachment of palmitate to membrane proteins via thioester linkages are usually cysteine residues in or near transmembrane segments (38). α Subunits have 24 proposed transmembrane segments containing 12 cysteine residues (12), and chemical analysis of the sodium channel from eel electroplax indicates a high content of covalently attached fatty acid (28). Palmitoylation of newly synthesized sodium channels is inhibited by tunicamycin but not by castanospermine. These results are consistent with attachment of palmitate in the Golgi as has been proposed for other membrane proteins (27). Block of co-translational glycosylation by tunicamycin is likely to prevent attainment of an appropriate conformation of the newly synthesized α subunit for transport to the Golgi and thereby inhibit palmitoylation.

Addition of hydrophobic domains like covalently attached palmitoyl residues might cause abnormal binding of SDS and alter the mobility of the α subunit in SDS-PAGE. It has been proposed (28) that this may be the basis for the unusually high electrophoretic free mobility observed for sodium channel α subunits from all sources studied (24, 39, 40). Under the conditions of our experiments, we do not observe a significant effect of removal of covalently attached palmitate on the apparent molecular weight of the α subunits. Thus, palmitoylation does not make a major contribution to the changes in apparent molecular size we have observed during α subunit processing. However, these results do not exclude a more prominent effect of palmitylation on the electrophoretic behavior of the α subunit in Ferguson plot experiments (24, 39, 40), or on the electrophoretic mobility of the α electroplax sodium channel whose electrophoretic free mobility is much greater than that of the α subunit of the rat brain sodium channel (24, 39).

Assembly of Sodium Channels in Rat Brain Neurons—Previous studies with antibodies against the α subunits have shown that biogenesis of sodium channels in rat brain neurons involves synthesis of the individual subunits, post-translational processing of their polypeptide and oligosaccharide components, and assembly of a heterotrimeric complex which is inserted into the cell surface. Development of antibodies against the β2 subunits has provided an independent test of these conclusions. The results described in Fig. 4 show that antibodies against the β2 subunit immunoprecipitate αβ2 complexes from the cell surface, but not free α subunits in the intracellular pool. During pulse-chase experiments, these αβ2 complexes are observed within the first hour after synthesis of the polypeptides and their concentration approaches a maximum of approximately 70% of the newly synthesized α subunits with a half-time of 1.2 h (Fig. 10, k,s) by 4 h after synthesis. The remaining 30% of these α subunits do not form a disulfide-linked complex with β2 subunits and do not appear on the cell surface within several hours after synthesis. Assembly of newly synthesized α subunits into a mature sodium channel complex containing disulfide-linked β2 subunits therefore is a rate-limiting step in the biogenesis of cell-surface sodium channels.

Degradation of Sodium Channel α Subunits—In previous studies, we found that the apparent half-lives of the cell-surface αβ2 complexes and the intracellular pool of free α subunits were 30 and 30 h, respectively. In fitting the model of Fig. 10 to experimental data (see Miniprint Section and Ref. 41), a degradation rate constant of 0.022 h⁻¹ (31-h half-life) was used for all α subunit forms. In this kinetic model, the slower apparent turnover of the αβ2 complexes results from their slow replenishment from the larger free-α pool with a half-time of 231 h (Fig. 10, k,s). The apparent turnover of the free-α pool is slightly accelerated by this pathway as well. A model which incorporates these rate constants fits the experimental data closely (see Miniprint Section).

Role of Post-translational Modifications in Sodium Channel Assembly—Previous results showed that core glycosylation is required for normal subunit stability (14, 15). In contrast, castanospermine, an inhibitor of the first N-linked oligosaccharide processing step, does not have detectable effects on sodium channel biosynthesis. The difference in the effects of tunicamycin and castanospermine on sodium channel biosynthesis is striking. We assume that core glycosylation provides a required structural feature for normal folding of the α subunit and possibly a signal for transport to the Golgi. Once this critical step is achieved, post-translational processing reactions, including sulfation, that are inhibited by castanospermine apparently do not play an essential role in subunit assembly and transport to the cell surface.

Growth in the presence of castanospermine also does not alter the binding of saxitoxin to its receptor site on the extracellular surface of the channel. It remains to be determined if sodium channels made in the presence of castanospermine have normal ion transport and voltage-dependent gating. Since castanospermine prevents the incorporation of most of the sialic acid, it must have a major effect on the surface charge of the extracellular surface of the sodium channel. Negative surface charges on the sodium channel are expected to increase the local concentration of sodium near
the extracellular opening of its transmembrane pore and to increase the local concentration of a cationic ligand like saxitoxin near its receptor site. The lack of effect of inhibition of sialylation with castanospermine on saxitoxin binding indicates that the saxitoxin receptor site is located at a distance from the negative surface charges contributed by sialic acid residues or is insulated from their effects by the protein structure.

Acknowledgments—We thank Laurie Maechler for excellent technical assistance in preparation of cell cultures and the IGII monoclonal antibody, Dawn Merrick and Dr. Dalia Gordon for providing the anti-SP11 and anti-SP11 polyclonal antibodies, William Downey for providing polyclonal antisera, and Laurie Hoff for help in manuscript preparation.

REFERENCES


SUPPLEMENTARY MATERIAL TO
(Title of Article), Subtitle, and Corresponding Author's Name of the Sodium Channel)
Role of Posttranslational Modifications in Channel Assembly
K. W. Schmidt and W. A. Catterall

Materials and Methods of the Role of Posttranslational Modifications in Channel Assembly

Results

Fig. 11. Observation of WGA α5→ α2 WGA α3. a, b, The thief remains were pooled (Fig. 11). The data points for WGA α3 were for the indicated time. a, b, A wide range of data were produced by kα = kα + kα (kα = 0.023 [Fig. 11a]). The curves were made for the following: kα = kα + kα (kα = 0.023 [Fig. 11a]). The curves were made for the following: kα = kα + kα (kα = 0.023 [Fig. 11a]).

Fig. 12. Conversion of α2 to α3. a, b, Cells were pooled with 0.2 g of packed material for 0.5 h and harvested for the indicated times. The data points for WGA α3 were for the indicated time. a, b, A wide range of data were produced by kα = kα + kα (kα = 0.023 [Fig. 11a]). The curves were made for the following: kα = kα + kα (kα = 0.023 [Fig. 11a]).
Time-course and Events of Disassembly of α- and δ2 Subunits. Models in which free α and α in complexes in δ2 complexes do not exchange for the failure of initial or subunits to stay in complexes for the short time shown in Fig. 20. In solutions of 250 mM salt to be in complexes of δ2 complexes, free α δ2 complexes are also disassembled to δ2 complexes, but at a slower rate. This model includes reactions 1, 5, and 6:

\[
\frac{d[\text{Free/α}]}{dt} = (k_\text{δ2} - k_\text{4δ2}) [\text{δ2}] \cdot [\text{Free/α}]
\]

(10)

\[
\frac{d[\text{δ2}]}{dt} = -k_\text{δ2} [\text{δ2}] + k_\text{4δ2} [\text{δ2}] + k_\text{Free/α} [\text{Free/α}]
\]

(11)

\[
\frac{d[\text{4δ2}]}{dt} = k_\text{δ2} [\text{δ2}] + k_\text{4δ2} [\text{δ2}] - k_\text{Free/α} [\text{Free/α}]
\]

(12)

For the initial conditions: [Free/α] = 100, [δ2] = 100, [Free/α] = 10, [δ2] = 10, [3δ2] = 10

We found that αδ2 complexes do not last longer than 5h. The model was used to generate the curves shown in Fig. 13. These curves were fitted by a numerical approximation using the Runge-Kutta method (implemented in Fortran, for the Apple Macintosh). For the results shown, a step size of 0.01 hour was used.

Fig. 13. The disassembly of some free α to αδ2 complexes. Cells were pulse labeled for 5 h and chased for the indicated times. Results from the model are normalized to 100% - the maximum amount of free α (left-hand side) of 250 mM δ2 complexes. The data points for the free α (circles) and αδ2 complexes (squares) are reproduced from Fig. 13. Values were normalized to 100% maximum for each form.

Values for the rate constants in Fig. 13 were derived from Figs. 11, 12. Degradation rate constants kδ2 and k4δ2 were set at 0.002 h⁻¹ based on Fig. 14. The value of k4δ2 is taken as the average value of k4δ2 in Fig. 11, 12. The value of kδ2 is the average from Figs. 11, 12, and 13. Values for k4δ2 and k4δ2 come from Fig. 13. The value of k4δ2 is from Fig. 14.