The Effects of Inhibiting Oligosaccharide Trimming by 1-Deoxynojirimycin on the Nicotinic Acetylcholine Receptor*

(Received for publication, May 13, 1986)

McHardy M. Smith‡, Sondra Schlesinger§¶, Jon Lindstrom***, and John Paul Merlie‡‡

From the Department of Pharmacology and §Department of Microbiology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110 and the ¶Department of Receptor Biology, The Salk Institute, San Diego, California 92138

The nicotinic acetylcholine receptor has a subunit stoichiometry of $\alpha_2\beta_2\gamma_2\delta_2$; all 5 subunits contain N-linked oligosaccharides. We investigated what role trimming of the oligosaccharides played in the post-translational processing of the subunits and assembly of the receptor by examining the receptor synthesized in the presence of an inhibitor of oligosaccharide trimming, 1-deoxynojirimycin. BC3H-1 cells express one-third fewer receptors when grown in the presence of 1-deoxynojirimycin. The receptor subunits that are expressed have decreased mobility by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating an inhibition of oligosaccharide trimming. In control cells, 40% of the translated $\alpha$ subunit acquires the capacity to bind $\alpha$-bungarotoxin with a half-time of 40 min before assembly with the other subunits; the rest is rapidly degraded. In 1-deoxynojirimycin-treated cells approximately the same amount of $\alpha$ subunit is translated as in control cells, but that $\alpha$ subunit is degraded more rapidly, and only 25% acquires the capacity to bind $\alpha$-bungarotoxin. From these results, we conclude that oligosaccharide processing either may aid in protecting the $\alpha$ subunit primary translation product from degradation or may be required for the conformational change or other post-translational modification(s) necessary for formation of the $\alpha$-bungarotoxin binding form of the $\alpha$ subunit, which is then protected from proteolytic degradation. The cell surface receptor that is expressed in the presence of 1-deoxynojirimycin, however, is not altered in its affinity for cholinergic ligands. Thus, we conclude that differential N-linked oligosaccharide trimming of the 2 $\alpha$ subunits does not appear to play a part in the differences in affinities of the 2 $\alpha$ subunits for cholinergic ligands.

The nicotinic acetylcholine receptor, a ligand-gated ion channel, is the best understood of the neurotransmitter receptors. Many of the biochemical properties of this integral membrane protein from both the vertebrate neuromuscular junction and from some fish electric organs have been extensively studied (for reviews see Popot and Changeux, 1984, and Karlin, 1980). The protein consists of four homologous polypeptide chains noncovalently associated in a stoichiometry of $\alpha_2\beta_2\gamma_2\delta_2$ (Raftery et al., 1980). The primary sequences of the subunits, predicted from sequencing cDNAs from several species, are known (see references in Popot and Changeux, 1984). The $\alpha$ subunits contain all or part of the binding sites for acetylcholine and the antagonist $\alpha$-bungarotoxin (BTX) (reviewed in Karlin, 1980), as well as a single consensus sequence for N-linked glycosylation (Noda et al., 1982; Boulter et al., 1985; Isenberg et al., 1986).

Several intermediates in receptor biosynthesis have been detected by immunoprecipitation (Fig. 1) (reviewed in Carlin and Merlie, 1986; Merlie and Smith, 1986). The $\alpha$ subunit acquires the capacity to bind BTX with high affinity significantly after translation of the polypeptide, but before subunit assembly. Because the $\alpha$ subunit synthesized in the presence of tunicamycin does not acquire the capacity to bind BTX with high affinity (Merlie et al., 1982) and the translation product of $\alpha$ subunit mRNA that has been mutated to remove the site of N-linked glycosylation does not bind BTX (Mishina et al., 1985), carbohydrate attachment is requisite for formation of the BTX binding site or requisite for proper folding to achieve the BTX-binding form of the $\alpha$ subunit. Furthermore, since the in vitro translated glycosylated $\alpha$ subunit does not bind BTX (Anderson andBlobel, 1981; Sebbane et al., 1983) and the in vivo translated glycosylated $\alpha$ subunit does not acquire the capacity to bind BTX for several minutes after translation (Merlie et al., 1981), we assume that the primary product of translation, $\alpha_0$, must be subjected to further post-translational modification(s) for in vivo to undergo the transition to the species that binds BTX, $\alpha$-BTX. Since oligosaccharide processing is one such modification which can influence the post-translational maturation of a protein (Schlesinger et al., 1984), we wished to determine if the initial steps of oligosaccharide trimming could affect the assembly of the subunits or formation of $\alpha$-BTX.

1-Deoxynojirimycin (DNJ) is an inhibitor of the enzymes glucosidase I and II (Saunier et al., 1982). These enzymes trim the 3 glucose residues from the core oligosaccharide after it has been transferred to the nascent polypeptide (reviewed in Kornfeld and Kornfeld, 1985). The effects of DNJ-dependent inhibition of trimming on the subsequent post-translational processing of different proteins are varied. DNJ blocked secretion of IgD but not IgM (Peyrieras et al., 1983). In the presence of DNJ, the rate of secretion of $\alpha_0$ proteinase inhibitor was unchanged, but the amount secreted was halved.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by United States Public Health Service National Institute of Neurological and Communicative Disorders and Stroke Grant 5 T32 NS07057 and by a postdoctoral fellowship from the Muscular Dystrophy Associations of America.

§ Supported by grants from the National Institutes of Health.

¶ Supported by grants from the National Institutes of Health, the Muscular Dystrophy Associations of America, the Alexander S. Onassis Public Benefit Foundation, and the Los Angeles and California Chapters of the Myasthenia Gravis Foundation.

†† Supported by grants from the National Institutes of Health and the Muscular Dystrophy Associations of America. To whom correspondence may be addressed.
Effects of Deoxynojirimycin on Nicotinic Ach Receptor

Gross et al., 1983). In contrast, DNJ slowed the rate of secretion of α-antitrypsin and α-antichymotrypsin but apparently did not change the amount secreted (Lindstrom and Kong, 1984). Our results show that DNJ did not inhibit the formation of α2γ; however, in the presence of DNJ, the precursor form of the α subunit was degraded more rapidly than in its absence. In addition, the receptor expressed in the presence of DNJ, although altered in its oligosaccharide side chain, shows the same affinity for carbamylcholine and d-tubocurarine as receptor synthesized in control cells.

EXPERIMENTAL PROCEDURES

Materials—[35S]Metionine (1000-1500 Ci/mmol) was from American Corp. Formalin-fixed Staphylococcus aureus cells (Immunoprecipitin) were from Bethesda Research Laboratories. DNJ was obtained from Drs. H. D. Schlumberger and D. Schmidt (Bayer Corporation, West Germany). Synthesis of [3H]toboga toxin (3H-BTX) and sources of other materials have been described previously (Merlie and Lindstrom, 1983; Merlie and Sebbane, 1981).

Labeling of Cells—Receptor pretranslational processing was studied in cells labeled sequentially with [3H]BTX and [35S]methionine. Seven-day-old 10-cm cultures of BC3H-1 cells, grown as described by Merlie and Lindstrom (1983), were incubated in complete growth medium with 1-5 nM [3H]BTX for 2 h at 36°C to label surface-exposed receptor. Cells labeled with [3H]methionine and [35S]methionine protocols were used. For long labeling experiments, 0.1-0.2 μM of [35S]methionine was added directly to the medium and the plate incubated at 36°C for 2 h. For pulse-chase labeling, the medium was removed and the cell layer rinsed twice with warmed Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium (methionine-free Dulbecco's modified Eagle's medium without methionine. Two ml of labeling medium methionine) were added, and the cells were incubated at 36°C for 5 min. At the end of this pulse period (time 0) the radioactive medium was removed. The number of conditions for immunoprecipitation for assembled BTX is determined by the presence of DNJ. However, as the number of washings of the Immunoprecipitin pellet increased, recovery of prelabeled surface receptor [3H]BTX complexes increased, recovery of prelabeled surface receptor [3H]BTX complexes decreased. For this reason recovery was monitored by comparing the amount of recovered prelabeled [3H]BTX receptor complexes with the amount of [3H]BTX receptor complexes initially added.

In all cases, at the end of the pulse and at least 1 ml of extraction buffer supplemented to 0.5 M NaCl and 0.5 M sucrose was added to each immunoprecipitation followed by centrifugation in the Eppendorf model 5413 for 3 min. The pellet was resuspended and pelleted in extraction buffer supplemented to 0.5 M sucrose and 0.5 M NaCl but without bovine serum albumin and resuspended and pelleted in phosphate-buffered saline with 0.1% Triton X-100. Finally the pellet was resuspended in SDS sample buffer (Laemmli, 1970) and placed in a boiling water bath for 2 min. Samples were subjected to SDS-polyacrylamide gel electrophoresis on a 10% acrylamide, 0.2% N,N'-biacrylamide acrylamide gel and buffer system described by Laemmli (1970). The gels were processed for fluorography (Laisney and Mills, 1975) and exposed to Kodak XAR-5 film under conditions in which band intensity was proportional to radioactivity and exposure time.

Immunoprecipitation—Whole cell pellets were solubilized in extraction buffer (0.1 M NaCl, 0.02 M sodium borate, 0.015 M EDTA, 0.015 M EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 10 μM N-ethylmaleimide, 0.5% bovine serum albumin, 0.1% sodium dodecyl sulfate (SDS), and 0.5% Triton X-100, pH 9) for 5 min on ice.

Insoluble material was removed by centrifugation for 3 min in an Eppendorf centrifuge, model 5413, at 4°C. To detect the various forms of the α subunit, identical aliquots of the soluble extracts were incubated on ice for 1 h with either antibody or BTX (see Fig. 1) and then for an additional hour with Immunoprecipitin. To detect the γ subunit, the antibody mAb 61 (Taarts et al., 1986; Merlie and Lindstrom, 1983) was used in combination with Immunoprecipitin preabsorbed with rabbit-anti-rat IgG. To detect assembled receptor (experimentally defined as α subunit immunoprecipitated with an anti-β antibody) anti-β mAb 148 (Guilick and Lindstrom, 1983) was used in combination with Immunoprecipitin preabsorbed with rabbit-anti-rat IgG. Total α subunit that could bind BTX was detected by adding 50 nm BTX to the solubilized extract for 1 h followed by the addition of anti-BTX antibodies and Immunoprecipitin (this protocol is abbreviated Tx-anti-Tx). Immunoprecipitation of surface-reconstituted receptor was also accomplished using BTX followed by anti-BTX antibodies. However, in this case the BTX had been bound by adding it to intact cells prior to harvest, so that only [3H]-labeled receptors that had been exposed to the cell surface during the chase were detected. Thus, surface receptors were immunoprecipitated by incubating the extract, from cells previously incubated with BTX, with anti-BTX antibodies for the first h, and adding Immunoprecipitin for the second h (abbreviated surface Tx-anti-Tx). In all cases, nonspecific immunoprecipitation was determined by either inclusion of 100-fold excess BTX (for Tx-anti-Tx) or by use of a monoclonal antibody which is not known to be present in the receptor. In all cases, [35S]labeled receptor that had been exposed to the cell surface during the chase were detected. Thus, surface receptors were immunoprecipitated by incubating the extract, from cells previously incubated with BTX, with anti-BTX antibodies for the first h, and adding Immunoprecipitin for the second h (abbreviated surface Tx-anti-Tx). In all cases, nonspecific immunoprecipitation was determined by either inclusion of 100-fold excess BTX (for Tx-anti-Tx) or by use of a monoclonal antibody which is not known to be present in the receptor.
common amount of trichloroacetic acid-precipitable $^{35}$S added to the immunoprecipitation. These corrections assume that the recovery by immunoprecipitation of the intracellular forms of the $\alpha$ subunit is identical to the recovery of the internal standard, $^{125}$I-BTX-labeled surface receptor. We have previously shown that this assumption is reasonable since in sequential immunoprecipitations, less than 10% of the $\alpha$ subunit immunoprecipitated in the first cycle can be recovered by a second cycle of immunoprecipitation (Merlie et al., 1982). With these corrections, the units on panels in Fig. 3 are directly comparable. In determining the concentration dependence of the effects of DNJ on the formation of the various forms of the $\alpha$ subunit (Fig. 4), a similar procedure for averaging between experiments, described above, was used. In each experiment, the normalized peak areas of $\alpha$ subunit immunoprecipitated from the 2 control plates were averaged, and this average was set at 100%. The peak areas of $\alpha$ subunit immunoprecipitated at each concentration of DNJ were expressed as a percentage of that mean of the two control plates from that experiment. Data from the five experiments were then averaged as the percent of control.

**Competition Binding Assays**—Because of the slow dissociation rate of $^{125}$I-BTX from the receptor, it is not practical to assess competitor binding affinity by equilibrium binding assays; instead we used initial rate binding assays which were carried out essentially as described by Sine and Taylor (1979). Cells in 24-well plates were grown in the absence or presence of DNJ for 3 days. At the end of this time, the growth medium was removed from the cells and replaced with 0.45 ml of room temperature DMEM buffered with 20 mM Hepes (pH 7.5) containing 1 $\mu$M BTX to define nonspecific binding, various concentrations of the competitors d-tubocurarine or carbamylcholine, or nothing to define total binding. After a 10 min, 0.15 ml of $^{125}$I-BTX in DMEM was added to a final concentration of 10 nM, and the cells were incubated for a further 10 min. The cells were washed with 4 changes of phosphate-buffered saline, the $^{125}$I solubilized with 0.2 N NaOH, and counted in a $\gamma$ counter. Total and nonspecific binding were determined in triplicate, while individual wells were used to determine the binding of $^{125}$I-BTX in the presence of each concentration of competitor.

**RESULTS**

**DNJ Decreased Cell Surface Expression of Receptor**—The first step in analyzing the effects of the glucosidase inhibitor, DNJ, on receptor biosynthesis and on the binding of cholinergic ligands to the receptor produced in the presence of DNJ was to determine the effects of varying concentrations of DNJ on receptor number. Cells were treated with DNJ for 2 days and then assessed for the quantity of surface receptor, by $^{125}$I-BTX binding, and for incorporation of $^{35}$S)methionine into protein as described under "Experimental Procedures." At concentrations of DNJ higher than 0.2 mM, BC3H-1 cells expressed a third fewer receptors than control cells (Fig. 2A). The concentration of DNJ necessary for a half-maximal effect on receptor expression was approximately 0.1 mM. This concentration for a half-maximal effect is somewhat lower than that seen by Lemansky et al. (1984) and may be due to differences in the experimental protocols or to the cell lines studied. Neither the half-life of pre-existing receptor nor receptor synthesized in the presence of DNJ were altered from control values by DNJ treatment (Fig. 2B). The incorporation of $^{35}$S)methionine into trichloroacetic acid-insoluble material was not inhibited by more than 7%, averaged over 5 experiments, at any of the concentrations of DNJ tested. Thus, at these concentrations, DNJ caused a decrease in receptor expression without causing a decrease in total protein synthesis or a decrease in the half-life of the receptor, suggesting a specific effect on the post-translational synthesis of the receptor complex.

**DNJ Decreased Oligosaccharide Trimming of the $\alpha$ Subunit**—The effect of DNJ on $\alpha$ subunit oligosaccharide trimming was monitored by examining the alteration in $M$, as

![Fig. 2. BC3H-1 cells express a third fewer acetylcholine receptors after 2 days growth in DNJ but receptor half-life is not altered in the presence of DNJ. A, cultures of BC3H-1 cells were grown in the presence of the indicated concentrations of DNJ for 2 days. At the end of this time cell surface acetylcholine receptors/dish were measured by incubating cells at 36 °C for 2 h with $^{125}$I-BTX. The free $^{125}$I-BTX was washed away, the cells harvested, and the cell-associated $^{125}$I-BTX counted. In each of 5 experiments, total binding in the absence of DNJ was measured with 2 dishes of BC3H-1 cells, and binding in the presence of the indicated concentration of DNJ was measured in a single dish. The amounts of $^{125}$I-BTX bound to control cells were averaged within each experiment, and that value was set at 100%. The percent of $^{125}$I-BTX binding in the presence of the stated concentrations of DNJ was calculated, and those values were averaged across the 5 experiments. Thus, the points represent the mean ± S.D. of 10 observations in the absence of DNJ and 5 observations at each concentration of DNJ except at 2.5 mM which was used in only 3 of the 5 experiments and thus n ~ 3. B, the half-life of preformed receptor and newly synthesized receptor in the presence of DNJ was examined. DNJ was (filled symbols) or was not added (open symbols) at 0 h to a final concentration of 1 mM to 35-mm dishes of BC3H-1 cells 5 days after plating. One set of the plates was labeled in 8 mM $^{125}$I-BTX for 1 h before the addition of DNJ (circles); the other set was labeled at 24 h (squares) after DNJ addition. At the end of the $^{125}$I-BTX labeling period, the medium was removed and replaced with conditioned growth medium containing 0.25 $\mu$M BTX. Triplicate plates were harvested at various times after labeling, and the cell-associated $^{125}$I-BTX was determined, as described under "Experimental Procedures." The data are plotted as the mean of the replicates; the standard deviation of each group was seldom more than 6% of the mean. Nonspecific binding, assessed by addition of 0.8 $\mu$M BTX during the labeling period, was approximately 2 fmol of $^{125}$I.
Effects of Deoxynojirimycin on Nicotinic ACh Receptor

determined by SDS-polyacrylamide gel electrophoresis. Cells were treated for 2 days with the same range of concentrations of DNJ used above, they were labeled for 2 h with [35S]methionine, and total α subunit was immunoprecipitated using mAb 61 followed by SDS-polyacrylamide gel electrophoresis. The α subunit synthesized in the presence of DNJ had decreased mobility relative to α synthesized in the absence of DNJ (Fig. 3, also shown in Fig. 6).

As can be seen in Fig. 3, there was less α subunit immunoprecipitated by mAb 61 from cells treated with DNJ. In several experiments, we examined whether DNJ treatment decreased the amount of all of the forms of the α subunit. These various forms of the α subunit are defined by their susceptibility to immunoprecipitation by the protocols described under “Experimental Procedures”; to recapitulate, total α subunit was immunoprecipitated with mAb 61, α subunit which had acquired the capacity to bind BTX was immunoprecipitated by the Tx-anti-Tx immunoprecipitation protocol, and assembled receptor was detected by determining how much α subunit was co-immunoprecipitated with β subunit by an anti-β subunit mAb. The fluorogram in Fig. 3 and fluorograms from similar experiments were quantitated to determine the effects of these concentrations of DNJ on the amount of each of the forms of the α subunit (Fig. 4). DNJ decreased the amount of [35S]-labeled α subunit immunoprecipitated by these various protocols from cells pulsed for 2 h (Fig. 4) with a similar concentration dependence to the effects on 125I-BTX binding (Fig. 2A).

**Binding of Cholinergic Ligands to Receptor Synthesized in the Presence of DNJ Was Not Altered**—It was apparent from the fluorograms in Fig. 3 that the α subunit synthesized in the presence of DNJ migrated more slowly by SDS-polyacrylamide gel electrophoresis than control (also shown in Fig. 6). Since after 2 days in DNJ greater than 95% of the receptor would have been composed of these altered subunits (Fig. 2B), we examined the affinity of the receptor synthesized in the presence of DNJ for cholinergic ligands. Agonists bind to the two α subunits in a cooperative manner, with Hill coefficients greater than 1 while some cholinergic antagonists bind to the 2 α subunits with Hill coefficients of less than 1 (Sine and Taylor, 1979). Cells were grown in the absence or presence of DNJ and than assessed for the capacity of a cholinergic agonist or antagonist to inhibit the binding of 125I-BTX. Under these conditions, DNJ treatment did not alter the capacity of the agonist, carbamylcholine, or the antagonist, d-tubocurarine, to inhibit the binding of 125I-BTX (Fig. 5).

Carbamylcholine inhibited the binding of 125I-BTX with a Hill coefficient of greater than 1, and d-tubocurarine inhibited the binding with a Hill coefficient of less than 1, independent of DNJ treatment. Thus, while DNJ treatment did increase the Ms of the subunits, it did not alter the affinity of the receptor for these 2 ligands.

**Fig. 4. The amounts of the forms of the α subunit immunoprecipitated after labeling for 2 h are reduced by DNJ.** Fluorograms of the experiment shown in Fig. 3 and similar experiments were scanned under conditions in which the peak area was proportional to the amount of 35S applied to the gel and the exposure time. Immunoprecipitations were carried out using the anti-total antibody, mAb 61, the Tx-anti-Tx immunoprecipitation protocol, or an anti-β subunit antibody, mAb 148, and quantitating the amount of α subunit co-immunoprecipitated with β subunit. The points represent the amount of α subunit recovered by each immunoprecipitation protocol, averaged as a percentage of the control amount of α subunit versus the DNJ concentration. The error bars represent the standard deviation of at least 5 observations at each concentration of DNJ, except at 2.16 mM, where there were as few as 1.

**Fig. 5. DNJ does not alter the affinity of receptors for cholinergic ligands.** Cell cultures were grown in the absence (open symbols) or presence (closed symbols) of 1 mM DNJ for 3 days. Cells were incubated in 0.45 ml with no drug (to define total binding), 1 μM BTX (to define nonspecific binding), or 1.1 times the indicated concentration of carbamylcholine (Carb, squares) or d-tubocurarine (dTC, circles) for 10 min at 20°C. Then 0.05 ml of 125I-BTX was added to make a final concentration of 10 nM and the incubation continued for 10 min. Cells were washed extensively, harvested, and the cell-associated 125I-BTX counted. In control cells, total binding was 30 fmol of 125I-BTX (5000 cpm), and nonspecific binding was less than 5% of the total. Data are plotted as the percent of specific 125I-BTX bound versus the concentration of carbamylcholine or d-tubocurarine. As has been shown by Sine and Taylor (1979), this is an initial rate binding assay rather than an equilibrium binding assay.

---

Fig. 3. Electrophoretic mobility of the α subunit synthesized in the presence of DNJ. Cultures were grown in the absence or presence of the indicated concentrations of DNJ for 2 days. During the last 2 h, [35S]methionine was added to the culture medium. Cells were harvested, solubilized, and immunoprecipitated with an anti-α antibody, mAb 61, and the immunoprecipitates subjected to SDS-polyacrylamide gel electrophoresis and fluorography. Incorporation of acid-precipitable 35S was not affected by the presence of DNJ, but in this experiment 1.6-fold more 35S was put into the immunoprecipitation for the leftmost lane. A shorter exposure of the fluorogram demonstrated that the leftmost lane was a single band.

---

![Diagram](image-url)
Time Course of Oligosaccharide Trimming—From the data in Fig. 4 we could not determine whether DNJ inhibited translation or stimulated degradation of the newly synthesized receptor or its subunits. To distinguish between these possibilities and to determine the time course of oligosaccharide trimming we performed pulse-chase experiments. Cells were pre-equilibrated with tunicamycin, or with DNJ, or without drug, and were then pulsed for 5 min and chased for 0–6 h in the continued absence or presence of drug. Cells were harvested and extracted, and the soluble detergent extracts immunoprecipitated with antibodies as described under "Experimental Procedures." Extracts immunoprecipitated with mAb 61 demonstrated the time course of oligosaccharide trimming of the α subunit in control cells and the inhibition of that trimming by DNJ (Fig. 6A). The difference in mobility between the α subunit immunoprecipitated from tunicamycin-treated versus control cells represents the mobility difference induced by addition of the core oligosaccharide to the α subunit (Fig. 6A). As was noted before (Merlie and Lindstrom, 1983), even in the absence of tunicamycin a very small amount of α subunit escapes core glycosylation and can be seen as a faint band at the same mobility as α subunit immunoprecipitated from tunicamycin-treated cells. This aglycosyl α subunit does not acquire the capacity to bind BTX with high affinity (Merlie and Lindstrom, 1983) and is degraded very rapidly as it is not detected after 10 min of chase (Fig. 6). The presence of 1 mM DNJ did not increase the appearance of this aglycosyl species.

DNJ-inhibited trimming of the core oligosaccharide occurred co-translationally, or immediately post-translationally, as evidenced by the increase in mobility of α subunit immunoprecipitated control cells as compared with that from DNJ-treated cells at time 0 of chase (Fig. 6A, C0 versus D0). This mobility difference, we assume, represents trimming of the glucose (and mannose) residues from the core oligosaccharide in the first 5 min after translation in control cells (provided the mobility of the α subunit from DNJ-treated cells is equated with the mobility of α subunit with an untrimmed core oligosaccharide). The difference in mobility of the α subunit from control cells versus that from DNJ-treated cells remained constant for 6 h after translation (Fig. 6A, C375 versus D375); however, the α subunit from both control and

![Fig. 6. Time course of oligosaccharide trimming of the α and β subunits in the presence of DNJ. Cells were preincubated in the absence of drugs (C), in the presence of DNJ for 2 h (D), or in the presence of tunicamycin for 8 h (T). At the end of this time cells were pulsed with [35S]methionine for 5 min and then chased for the indicated minutes in the continued presence of the indicated drugs, as described under "Experimental Procedures." Cells were harvested, extracted, and the extracts immunoprecipitated with an anti-α antibody, mAb 61 (A), by the Tx-anti-Tx immunoprecipitation protocol (B), or with an anti-β antibody, mAb 148 (B), as described under "Experimental Procedures." The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. The lanes, from left to right, are, respectively, cells treated with 1 μg/ml tunicamycin and pulsed (T0), control cells pulsed and then chased for 6 h (C375*), paired lanes of control and DNJ-treated cells chased for from 0 to 6 h, and another lane of control cells at 0 chase (C0). Panel C is from another experiment in which cells were treated with or without DNJ for 2 days, pulsed with [35S]methionine for 10 min, and then chased for 2 or 6 h in the presence of 0.1 μM BTX. At the end of the chase, cells were incubated with mAb 210 in phosphate-buffered saline for 5 min on ice, washed extensively, harvested, and extracted. The soluble extracts were immunoprecipitated with mAb 61, with BTX and anti-BTX antibodies (total Tx-anti-Tx), with anti-BTX antibodies (surface Tx-anti-Tx), with mAb 210 added after solubilization (total mAb 210), or without added mAb 210 (surface mAb 210), all with Immunoprecipitin as described under "Experimental Procedures."
DNJ-treated cells increased in mobility slightly with the time of chase; most of this change in mobility occurred in the first 40 min of chase. This DNJ-independent change in mobility of the α subunit can be detected best by aligning a straight edge from C375 on the left to C375 on the right. Aligning the tops of the bands in these duplicate lanes shows that the α subunit at early times of chase have a slightly lower mobility than at later times. Thus, we found 2 mobility changes during the post-translational processing of the α subunit: an early phase of oligosaccharide trimming inhibited by DNJ and a later mobility change not altered by DNJ.

Similar2similar amounts of α subunit were immunoprecipitated from control and DNJ-treated cells at 0 chase; however, from the fluorograms it is apparent that α subunit from DNJ-treated cells disappeared more quickly. These fluorograms were scanned and quantitated (Fig. 7). In control cells, immunoprecipitable α subunit was translated in 2.5-fold excess over that which is incorporated into receptor (Fig. 7); the excess α subunit disappeared by 40 min. In DNJ-treated cells, nearly the same amount of α subunit was detected after a 5-min pulse; however, this α subunit disappeared to a lower amount than in control cells (Fig. 7). Furthermore, the initial rate of disappearance was twice as fast as the rate of disappearance of α subunit from control cells.

The effects of DNJ on the trimming of the α subunit were found with the β subunit as well (Fig. 6B). The difference in mobility between the β subunit immunoprecipitated by mAb 148 from control and DNJ-treated cells was less than the difference seen with the α subunit. The β subunit was core-glycosylated (control versus tunicamycin) and was rapidly trimmed in a DNJ-inhibitable manner (control versus DNJ treated at 0 chase).

The α subunit species which binds BTX, αTx, was formed with some delay after translation (Fig. 6B). At 0 chase, in the absence or presence of DNJ, less than 5% of the total α subunit immunoprecipitated by mAb 61 bound BTX with high affinity and was immunoprecipitated by anti-TX antibodies. With increasing times of chase, more α subunit bound BTX with high affinity (Figs. 6B and 7). In cells treated with DNJ, the time course of formation of αTx was the same as in control cells, but the amount of αTx produced was less (Figs. 6B and 7). The αTx formed in DNJ-treated cells migrated more slowly than αTx formed in control cells indicating that DNJ-inhibitable trimming was not necessary for the α to αTx transition. Similar observations were made with respect to formation of the assembled receptor. Although the anti-β antibody, mAb 148, immunoprecipitated β subunit immediately after translation, significant quantities of α subunit were not co-immunoprecipitated by mAb 148 until 40 min of chase (Figs. 6B and 7). The time course of formation of assembled receptor was not changed by DNJ; only the final amount of assembled receptor decreased (Figs. 6B and 7). As with the formation of αTx, the decrease in relative mobility of both the α and β subunits was evident in the assembled receptor formed in DNJ-treated cells indicating that assembly did not require DNJ-inhibitable trimming of the oligosaccharide. There was a significant delay between assembly and the appearance of 35S-labeled α subunit on the cell surface (Figs. 6C and 7). 35S-Labeled surface receptor was defined in 2 ways. Intact cells, before being detached from the plate, were incubated with BTX and with an anti-α subunit antibody that binds to an extracellular epitope, mAb 210. Cells were washed extensively, detergent extracted, and the soluble extracts exposed to anti-BTX antibodies and Immunoprecipitin (surface Tx-anti-Tx) or to Immunoprecipitin preabsorbed with rabbit-anti-rat IgG antibodies (surface mAb 210). Thus, only those receptors which had bound the BTX or mAb 210 in the intact cell would be immunoprecipitated by this protocol. 35S-Labeled cell surface receptors did not accumulate in significant quantities until 80 min of chase (Fig. 7). The α and β subunits in surface receptor from DNJ-treated cells showed the same decreased mobility relative to control (Fig. 6C) as had the intracellular forms, indicating that surface expression of this protein did not require DNJ-inhibitable trimming of the oligosaccharide.

**DISCUSSION**

DNJ inhibits the trimming of glucose residues from the asparagine-linked oligosaccharide GlcαMan3GlcNAc2 core oligosaccharide (Saunier et al., 1982; Peyrieras et al., 1983; Gross et al., 1983). Our conclusion that DNJ inhibited the trimming of the oligosaccharides on the acetylcholine receptor α and β subunits was based on the increase in the M, of the subunits synthesized in the presence of the drug. As these subunits have single oligosaccharide chains, the difference in mobility was not as great as that detected with proteins of lower M, or proteins with multiple oligosaccharide chains. As noted in the introduction, DNJ has been reported to have different effects on different proteins. We found that DNJ did not alter the time course of formation of cell surface receptor, assembled receptor, or αTx (Fig. 7). Although the same amount of α subunit was translated in control and DNJ-treated cells, in DNJ-treated cells less αTx was formed. In contrast to the lack of effect of DNJ on the rate of formation of αTx, in the presence of DNJ the rate of degradation of the precursor α subunit was increased. We observed a DNJ-inhibited change in the mobility of the α subunit as well as a DNJ-independent change in the mobility of the α subunit (Fig. 6A). Finally, DNJ treatment did not alter the binding of the cholinergic agonist, carbamylcholine, or the antagonist, d-tubocurarine to the receptor, as evidenced by the capacity of these compounds to inhibit the binding of [125I]-BTX to the receptor (Fig. 5).

N-Linked oligosaccharide processing follows a defined
pathway (reviewed in Kornfeld and Kornfeld, 1985). A Glc$_3$Man$_2$GlcNAc$_2$ core oligosaccharide is transferred to the asparagine residue of the nascent polypeptide, with subsequent trimming of the glucose and mannose residues. Co-translational trimming of the glucose residues has been detected by Atkinson and Lee (1984), who reported that the glucose residues had been removed from nascent chains of vesicular stomatitius virus G protein isolated from membrane-bound polysomes. The carbohydrates from DNJ-treated cells have been found to contain glucose residues not found on the carbohydrate from control cells (Saumier et al., 1982; Gross et al., 1983). Several groups have documented that, in the presence of DNJ or other inhibitors of glucosidase I and/or II, although glucose residue trimming is inhibited, trimming of mannoses on the other branches of the core oligosaccharide occurs (Datema et al., 1982; Gross et al., 1983). Romero et al. (1985) reported data consistent with the hypothesis that DNJ-treated cells produce an oligosaccharide Glc$_3$Man$_2$GlcNAc$_2$ instead of Glc$_2$Man$_2$GlcNAc$_2$, as would be expected from an absence of mannose trimming and a complete inhibition of glucose trimming. We found DNJ-sensitive trimming after a 5-min pulse with no chase; thus glucose (and mannose) trimming in control cells of the oligosaccharide must occur cotranslationally or immediately post-translationally; we did not detect any α subunit from control cells with the same mobility as α subunit from DNJ-treated cells. We have not determined the cause for the DNJ-insensitive change in the mobility of the α subunit between 0 and 40 min; various possibilities exist. Potentially, the change in mobility could be due to a change in the conformation of the translated receptor subunit with time (Covarrubias et al., 1984). Furthermore, a change in mobility might be expected due to trimming of several mannose residues from the core oligosaccharide by the mannosidases. A less likely explanation is that, as the inhibition by DNJ is competitive, some trimming of some of the glucose residues does occur. However, the carbohydrate tree on the α subunit synthesized in the presence of DNJ is obviously altered in comparison to control (Figs. 3 and 6). The time course of formation of α$_{|2,3|}$, the assembled receptor, or surface receptor was not altered by DNJ; only the amounts were decreased (Fig. 7). Thus, we conclude that formation of α$_{|2,3|}$, the assembled receptor, or surface receptor does not require a trimmed oligosaccharide tree.

As we have previously described, at least 60% of the α subunits from control cells are proteolyzed very rapidly after translation (Merlie and Lindstrom, 1983). In DNJ-treated cells, the precursor α subunit is translated at the control rate but is proteolyzed more rapidly; this more rapid degradation (Fig. 7) may be the primary cause for the decrease in the number of cell surface receptors (Fig. 2). We cannot rule out the possibility that the DNJ inhibition of processing of oligosaccharides is having a more profound effect on the γ or δ subunits. However, we do not consider that possibility very likely as the time course of the degradation of the α subunit is much faster than formation of the assembled receptor (Fig. 7). We, through the use of tunicamycin (Merlie et al., 1982), and Numa's group, by site-directed mutagenesis (Mishina et al., 1985), have described the pronounced effects of the lack of carbohydrate trimming on receptor synthesis. Our data shows that trimming plays a very important role in stabilizing the primary translation product as well.

The 2 α subunits in each receptor are similar but not identical. The 2 α subunits are identical by electrophoretic migration (reviewed in Karlin, 1980; however, see Nathanson and Hall, 1979) and by N-terminal sequencing of the first 54 amino acids of the electrophoretically purified α subunit (Raferty et al., 1980). Further, SI endonuclease digestion mapping, which can detect very similar but nonidentical species of mRNA, has detected only 1 coding sequence for the mouse α subunit (Goldman et al., 1985). In contrast, Hall and coworkers have described a serum from a myasthenia gravis patient which inhibits only 50% of BTX binding to the α subunits (Gu et al., 1985). Since the 5 subunits are all transportable and arranged in a rosette around a central ion pore (reviewed in Popot and Changeux, 1984), the 2 α subunits cannot have identical contacts with neighboring subunits; thus, the 2 α subunits must be different in their quaternary structure. The binding of many competitive cholinergic antagonists can be modeled as if these ligands were binding to the 2 α subunits with different affinities (Sine and Taylor, 1979, 1981; however, see Walker et al., 1984); these differences have been postulated to be due to either the differences in the quaternary structure of the 2 α subunits or to differences in the carbohydrates on the 2 α subunits (Gu et al., 1986). We wished to determine whether major alterations in the N-linked oligosaccharide on the α subunits would significantly alter the affinity of the receptor for cholinergic ligands. The receptor expressed in the presence of DNJ, although altered in its oligosaccharides (Figs. 3 and 6), does not have significantly altered affinity for a cholinergic agonist, carbamylcholine, or a cholinergic antagonist, d-tubocurarine, as evidenced by the capacity of these drugs to inhibit the binding of $^{125}$I-BTX (Fig. 5). Differences in the affinities of the 2 α subunits in each receptor for antagonists were preserved in the presence of alterations in oligosaccharide structure, and, therefore, DNJ-inhibitable differences in the trimming of the N-linked oligosaccharides are not the basis for the difference in affinities. As further evidence that the receptor synthesized in the presence of DNJ binds ligand normally, single channel recording from the DNJ-treated cells has demonstrated that in response to agonist a trans-membrane ion channel is opened (Covarrubias et al., 1986).

Acknowledgments—We wish to acknowledge the assistance of J. Kornhauser for tissue culture and Drs. B. Carlin, C. Cotton, and K. Isenberg for critical reading of the manuscript.

REFERENCES


Effects of Deoxynojirimycin on Nicotinic ACh Receptor