Inhibitors of Asparagine-linked Oligosaccharide Processing Alter the Kinetics of the Nicotinic Acetylcholine Receptor

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ABSTRACT We used selective inhibitors of the asparagine-linked oligosaccharide processing pathway to study the effect of sugar trimming on the functional properties of the nicotinic acetylcholine (ACh) receptor expressed in clonal mammalian BC3H-1 cells. Inhibitors of initial steps of the processing pathway (1-deoxynojirimycin[DNJ] and castanospermine[CS]) reduced the density of ACh receptors on the cell surface (3- to 5-fold) but their responsiveness to ACh was more reduced (5- to 10-fold). These results suggest that the function of the ACh receptor was altered. When the ACh receptors were expressed in the presence of DNJ or CS, analysis of ACh-evoked single-channel currents (-100 mV and 11°C) revealed an approximate threefold reduction in the opening rate (control: 600-650 s⁻¹, treated: 130-250 s⁻¹) and an approximate twofold reduction in the rate of agonist dissociation (control: 900-1,000 s⁻¹, treated: 400-500 s⁻¹). In addition, the proportion of brief duration bursts (r = 50-100 μs) was increased (1.5- to 2-fold) by treatments with DNJ or CS. In contrast, an inhibitor of a late processing step (swainsonine) did not produce such alterations. The single-channel conductance was not altered by any of the three inhibitors, and the slopes of log-log dose-response curves at low concentrations and desensitization did not appear to be affected. Each inhibitor altered the electrophoretic mobility of the ACh receptor subunits. We conclude that early sugar trimming can influence the kinetics of the nicotinic ACh receptor in BC3H-1 cells.

INTRODUCTION

The nicotinic acetylcholine (ACh) receptor at the motor end plate and electroplax is a ligand-gated ion channel responsible for the primary response at the postsynaptic membrane (Adams, 1987). This receptor is an integral membrane glycoprotein consisting of four homologous subunits which are noncovalently associated in a stoichiometry of α₂βγδ (McCarthy et al., 1986; Merlie and Smith, 1986). Although all the subunits are N-glycosylated, they differ in the amount and type of carbohydrates.
The carbohydrate moiety of the ACh receptor is required for correct assembly (Merlie et al., 1982) and correct oligosaccharide processing is required to stabilize the ACh receptor subunits before assembly (Smith et al., 1986). However, there is no work that directly addresses the importance of the carbohydrate moiety in terms of receptor activation by ACh. This study was in part motivated by studies with other glycoproteins which suggest that the sugar moiety may play an important functional role (Olden et al., 1985; Sairam and Bhargavi, 1985). Further, it has been suggested that the two α subunits in the receptor oligomer are differentially glycosylated (Conti-Tronconi et al., 1984; Ratnam et al., 1986) and that glycosylation plays a role in receptor function.

We have studied the effects of oligosaccharide-trimming inhibition (see Fig. 1) on the function of the ACh receptors expressed by BC3H-1 cells (Sine and Steinbach, 1984, 1986a,b, 1987). Three trimming inhibitors were used: 1-deoxynojirimycin.
(DNJ), castanospermine (CS), and swainsonine (SW) (Elbein, 1984; Fuhrman et al., 1985; Kornfeld and Kornfeld, 1985). DNJ inhibits glucosidases I and II, enzymes that catalyze the first two steps of N-linked oligosaccharide processing in mammalian cells, whereas CS only inhibits glucosidase I (Fig. 1). These enzymes, mainly localized in the endoplasmic reticulum, remove three glucose molecules of the initial oligosaccharide. SW inhibits a later enzyme, α-mannosidase II (Fig. 1), which is mainly localized in the Golgi cisternae. We find that treatment of cells with DNJ or CS produces similar effects on ACh receptor kinetics, reducing both the channel opening rate and the agonist dissociation rate. Treatment with SW, on the other hand, does not affect ACh receptor function. Preliminary reports of this work have appeared (Covarrubias et al., 1986, 1987, 1988).

**METHODS**

**Cell Culture**

BC3H-1 cells (subclone 6M, originally provided by Dr. J. P. Merlie, Washington University School of Medicine) were maintained and grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% newborn bovine serum and 10% fetal bovine serum. They were plated in 35-mm culture dishes 2–5 × 10⁴ cells/ml. For electrophysiological experiments, cells were plated on laminin-coated glass coverslips. Under these conditions, the cultures reach confluence by the fifth or sixth day after seeding and express large quantities of ACh receptors on the cell surface (Patrick et al., 1977).

**Treatment with the Inhibitors**

The same treatment protocol was used for the three drugs studied. When the cultures reached confluence, the culture medium was changed to DMEM supplemented with 0.5% fetal bovine serum and containing 40–50 nM α-bungarotoxin (α-BTX) to block all existing receptors. The cells were incubated with α-BTX for 30 min at 37°C. The culture dishes were then extensively washed (six to eight times) with α-BTX-free DMEM supplemented with 0.5% fetal bovine serum. The same medium containing the desired concentration of the inhibitors was used for the last washing step (1 mM DNJ [1,5-dideoxy-1,5-imino-l-glucitol, Genzyme Corp., Boston, MA]; 25 μM CS [8-α, β-indolizidine-1α, 6β, 7α, 8β-tetrol (1,6,7,8-tetrahydroxy-octahydro indolizine), Genzyme Corp.]; or 5.8 μM SW [8α, β-indolizidine-1α, 2α, 8β-troliol, Boehringer-Mannheim Biochemicals, Indianapolis, IN]). The cultures were then returned to the incubator and left in the presence of the inhibitor for 48–60 h before performing the experiments. Control dishes from the same culture were treated in parallel, but in the absence of the inhibitor. The number of ACh receptors on the cell surface and the whole-cell response to ACh from one set of control and inhibitor-treated cells were determined on the same day. For single-channel recordings, control and inhibitor-treated cells were, in general, recorded on consecutive days.

**Estimation of α-BTX Binding Sites on the Cell Surface**

The number of surface ACh receptors was determined by measuring the capacity of the intact cells to bind α-BTX. Triplicates of control and treated dishes were incubated for 1 h at room temperature with 20 nM 125I-α-BTX in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 6.4 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) containing 0.2% fetal bovine serum. Unspecific binding was estimated from binding in the presence of 1 mM D-tubocurarine. Labeled cells were scraped from the dishes and put over Durapore filters (Millipore Corp.,
Bedford, MA). The filters were rinsed four times, removed, and counted for the retained radioactivity. Triplicate samples did not differ by >20% and the unspecific binding was typically 10–15%. The results were expressed as number of ACh receptors per cell assuming two α-BTX binding sites per receptor. Cells were counted in a hemocytometer after dissociation (three to four aliquots from each of one or two dishes were averaged).

Treatment of cells with α-BTX completely blocked responses to puffer-applied ACh, for both control and treated cells.

Labeling and Immunoprecipitation of the ACh Receptors

Labeling of cells and immunoprecipitation of surface ACh receptors from solubilized cell extracts were performed generally as described by Smith et al. (1986). In brief, 5-d-old cultures were treated with inhibitors for 2 d as described above. Dishes were then rinsed twice with DMEM without leucine and incubated for 1 h at 37°C in DMEM with 50 μCi [3H]leucine and 12.5 μM unlabeled leucine. After the 1-h pulse the radioactive medium was removed, and the cells were incubated for 3 h in growth medium supplemented with 1 mM unlabeled leucine (chase). 10 nM α-BTX was added during the last 2 h of the chase to bind to surface ACh receptors. Inhibitors were included in both the pulse and chase media. After the chase all solubilization and immunoprecipitation steps were carried out at 4°C: the cultures were rinsed four times with 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM N-ethylmaleimide in PBS. The cells were then incubated for 5 min in extraction buffer (100 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 1 mM N-ethylmaleimide, 1 mM benzamidine, 0.2 mg/ml α2-macroglobulin, 0.2 mg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, 10 μg/ml antipain, 0.1% sodium dodecyl sulfate [SDS], 0.5% bovine serum albumin [BSA], 0.5% Triton X-100, 10 mM sodium phosphate, pH 7.5; SDS and BSA were preincubated together at 70°C for 30 min). The extract was centrifuged at 10,000 rpm for 5 min in a microfuge (model 12; Beckman Instruments, Inc., Palo Alto, CA). The supernatant was removed and incubated with rabbit anti-α-BTX antibodies (provided by Dr. J. P. Merlie) for 10 min, followed by a 20-min incubation with formalin-fixed Staph A cells (Immunoprecipitin, Bethesda Research Laboratories, Gaithersburg, MD). Washing of the Immunoprecipitin, elution from the pellet, SDS-polyacrylamide gel electrophoresis and fluorography were performed as previously described (Laskey and Mills, 1975; Smith et al., 1987a). Endoglycosidase H (endo H; Boehringer-Mannheim Biochemicals) digestion was performed on immunoprecipitated ACh receptors by briefly boiling the washed pellet in 0.3% SDS and centrifuging down the Immunoprecipitin, to release the subunits. The supernatant was diluted to final concentrations of 10 mM sodium phosphate, pH 5, 0.10% SDS, and 0.3 mM PMSF, and endo H digestion performed overnight following the data sheet provided by Boehringer-Mannheim Biochemicals.

The ACh receptor subunits were identified by comparison with previous studies (Merlie et al., 1982; Smith et al., 1986; Smith et al., 1987b) and by immunoprecipitations with specific monoclonal antibodies directed against the ACh receptor subunits. The α and β subunits immunoprecipitate with monoclonal antibodies 61 and 148, respectively (Tzartos et al., 1981; Wan and Lindstrom, 1985; antibodies provided by Dr. J. Merlie). The γ and δ subunits were similarly identified with monoclonal antibodies G10E11 (provided by Dr. S. Pedersen, Washington University School of Medicine) and 88B (Froehner et al., 1988, provided by Dr. Froehner, Dartmouth Medical School, Hanover, NH). Densitometer tracings of the gel shown in Fig. 2 gave estimated subunit stoichiometries (corrected for leucine content in the subunits, and assuming equal specific activities of labeling) of 2/1.2/0.8/0.8 (control; α/β/γ/δ, normalized assuming two α subunits), 2/1.2/1.0/0.4 (DNJ), 2/1.5/0.9/1.0 (CS), and 2/1.3/0.7/0.7 (SW). The ratio of specific to nonspecific radioactivity was lower in the CS and DNJ-treated preparations, owing to the lower number of ACh receptors per cell (see Table I). These data are consistent with the idea that the subunit stoichiometry is 2/1/1/1 for both
control and treated BC3H-1 cells. In addition, when surface ACh receptors were labeled with \(^{125}\text{I}-\alpha\text{-BTX}\) and extracted, the majority of the bound \(^{125}\text{I}-\alpha\text{-BTX}\) could be precipitated from both control and treated cells using monoclonal antibodies to the \(\alpha\), \(\beta\), and \(\delta\) subunits. In one experiment using control cells and treated sister cultures, for control cells 78%, 55%, and 75% of the bound toxin was precipitated, respectively, for DNJ-treated cells 82%, 65%, and 81%. These data are consistent with the idea that each \(\alpha\text{-BTX}\)-binding site includes \(\alpha\), \(\beta\), and \(\delta\) subunits in both normal and treated cells.

**Patch Clamp Methods and Data Analysis**

Coverslips were transferred to the recording chamber and washed with 10–12 ml of extracellular solution to remove inhibitor. The extracellular bath solution contained 140 mM KCl, 5.3 mM NaCl, 1.8 mM CaCl\(_2\), 1.7 mM MgCl\(_2\), and 25 mM HEPES (~310 mosM). Single-channel records were obtained cell attached at 10–11°C as described by Sine and Steinbach (1986a, b, 1987). Data reduction and analysis were performed as described (Sine and Steinbach, 1986a, 1987). Briefly, the records were filtered at 8,000 Hz (low-pass Bessel, –3 dB point) and sampled at 25-\(\mu\)s intervals. Channel opening and channel closing transitions were detected using a threshold-crossing procedure with the detection threshold set at one-half of the mean channel open current. Open and closed duration histograms were constructed for events exceeding a specified dead time (75 or 100 \(\mu\)s), and were fitted by a sum of exponentials. In some cases (in particular when the cells were treated with DNJ or CS) the number of entries from individual patches was limited, because of the very low frequencies of unitary currents. In these cases, the compiled open and closed dwell times from three to four patches were pooled. When the comparison was possible, we observed no significant differences between the parameters obtained from single patches or from pooled data (see also Sine and Steinbach, 1986a). Burst duration histograms at low ACh concentrations were made from concatenated open intervals which were separated by a closed period that was equal or less...
than a critical time (2-5 ms) equal to five times the mean intermediate closed time duration (see Results).

Single-channel conductance was estimated using inside-out patches at room temperature (22°C). For these experiments the bath and the pipette solutions were symmetrical and had the following composition: 150 mM KCl, 2 mM MgCl₂ and 10 mM HEPES (the pipette solution also contained 2.5 or 10 μM ACh). All solutions were adjusted to pH 7.4 with KOH.

Whole-cell currents were elicited by the puffer method (Choi and Fischbach, 1981). The pipette solution (intracellular) was: 140 mM KCl, 5.3 mM NaCl, 2 mM EGTA, 2 mM MgCl₂ and 10 mM HEPES (~290 mosM). In these experiments ACh (0.1-0.25 μM) was also included in the pipette solution in order to monitor the single-channel frequency in the patch before the formation of the whole-cell configuration. Puffer pipettes had a tip diameter of 2-3 μm. The pipette was initially positioned near the middle of the cell (the majority of the cells studied were spindle-shaped). ACh was rapidly delivered onto an isolated cell by a 10-psi, 500-ms pneumatic pulse controlled by a solenoid-driven valve (General Valve Co., Fairfield, NJ). The onset of the response began ~25 ms after the initiation of the pulse. The relative position and the distance between the pipette tip and the surface of the cell (5-15 μm) were adjusted by maximizing the amplitude of the response. Allowing proper recovery between the pulses (15-30 s), 5-10 pulses of ACh (0.25-1 μM) could be applied onto a cell without a change in the amplitude of the response. The current traces were recorded on FM analog tape and replayed for analysis. These traces were low pass filtered at 500 or 1,000 Hz and digitized at 10-ms intervals. The peak amplitude of the responses was estimated by eye. At a given ACh concentration two or more consecutive responses from a single cell were averaged (their peak amplitudes did not differ by >5%).

RESULTS

Fig. 2 demonstrates that a 2-d treatment with 1 mM DNJ (see also Smith et al., 1986) or 25 μM CS decreased the mobility of the α and β subunits (lanes D and C). The mobilities of the γ and δ subunits, on the other hand, were virtually unaltered. Treatment of detergent solubilized, and immunoprecipitated ACh receptors with endoglycosidase H (which selectively removes high-mannose carbohydrate chains from detergent-denatured glycoproteins, Trimble and Maley, 1984) increased the mobility of the α and β subunits from normal and treated cells and resulted in identical mobilities on electrophoresis (data not shown). This observation suggests that the differences in mobilities for α and β chains resulted from altered carbohydrate moieties. In contrast, SW, an inhibitor of α-mannosidase II (a late enzyme of the sugar-trimming pathway; see Fig. 1), produced a change in the electrophoretic mobilities of the γ and δ subunits without affecting the mobilities of α and β (lane S).

Fig. 3 shows two typical whole-cell current traces obtained from (A) a control cell and (B) a DNJ-treated cell in response to 0.5 μM ACh. Panels C (control cell) and D (DNJ-treated cell) show continuous records of single-channel traces obtained from cell-attached patches in the presence of 0.1 μM ACh. The response of the DNJ-treated cells to ACh was reduced in both types of experiments. To determine whether the reduced responsiveness resulted merely from a reduction in the number of ACh receptors on the cell surface, we conducted experiments in which the α-BTX binding sites, the amplitude of the whole-cell current, and the frequency of unitary currents were monitored in parallel from the same set of control and inhibitor-treated cells.
Table I shows that although treatment with 1 mM DNJ reduced the number of surface ACh receptors to ~35% of control, the amplitude of the macroscopic currents and the frequency of unitary currents were more depressed (to 18% and 20% of control, respectively). Similar results were obtained with 25 μM CS (Table I). This finding suggests that the treatment with inhibitors of early oligosaccharide trimming affected the functional properties of the ACh receptor. In contrast, treatment of cells with SW did not seem to affect the frequency of single channel currents or the density of surface ACh receptors (Table I).

Fig. 4 shows the relationship between single-channel burst frequency from cell-attached patches and the amplitude of the macroscopic currents recorded from the
same cell (controls and CS- and DNJ-treated). It can be seen that both parameters reflect the sensitivity of a cell to ACh. The correlation coefficient was 0.83, a value reasonably close to 1, which indicates that the receptor activity seen in individual patches is a random sample of the ACh receptors of the entire cell surface (i.e., the ACh receptors are uniformly distributed).

Responses from DNJ-treated cells were depressed, but the slopes of log(dose)–log(response) curves at low ACh concentrations were not different from control (1.5 and 1.3 for controls and DNJ-treated, respectively; Fig. 5), and consistent with

| Table 1 |
| --- |
| **Density of Surface Receptors and the Responsiveness to ACh** |
|                | AChR per cell* | Whole-cell response | Burst frequency* |
| Controls       | 111,300 ± 19,000 (12) | 900 ± 300 (12) | 7.5 ± 7.2 (13) |
| + DNJ          | 42,000 ± 16,000 (12) | 170 ± 150 (9) | 1.2 ± 1.2 (14) |
| DNJ per controls† | 0.35 ± 0.10 (2) | 0.18 ± 0.10 (3) | 0.20 ± 0.14 (3) |
| Controls       | 105,000 ± 14,000 (6) | 730 ± 280 (9) | 3 ± 9 (10) |
| + CS           | 24,000 ± 7,000 (6) | 81 ± 110 (10) | 0.5 ± 0.6 (6) |
| CS per controls† | 0.23 ± 0.02 (2) | 0.11 ± 0.09 (2) | 0.04 ± 0.04 (2) |
| Controls       | 115,000 ± 32,000 (6) | — | 0.4 ± 0.2 (5) |
| + SW           | 107,000 ± 16,000 (6) | — | 0.7 ± 0.4 (6) |
| SW per controls† | 0.98 ± 0.19 (2) | — | 1.7 ± 0.37 (2) |

*The number of surface ACh receptors (AChR) was determined by measuring the number of α-BTX binding sites on intact cells as described under Methods. The values shown are the mean ± SD (number of dishes assayed).
†The whole-cell response is the peak current elicited by a 500-ms pneumatic pulse of 0.25 μM ACh, holding the membrane potential at −100 mV (see Methods). Mean ± SD (number of cells assayed).
‡The burst frequency was measured by counting the number of resolved single channel currents over 10–20 periods of 1 or 10 s. A bandwidth of 500–1,000 Hz was used, so the events are more likely to represent long-duration bursts. Burst frequency was measured before the formation of the whole-cell configuration, with 0.25 μM ACh and V_{mem} = +100 mV and using a low-calcium intracellular solution in the pipette (see Methods). For the experiments with SW-treated cells and their matched controls, the burst frequency was determined with 0.1 μM ACh in normal bath medium containing Ca, V_{mem} = +100 mV. Mean ± SD (number of patches).
†The ratios given are mean ± SD of ratios computed for paired sets of control and treated cultures (not the ratios of overall means). The number of paired experiments is given in parentheses.

the requirement of two agonist molecules for receptor activation (Colquhoun, 1979). Additional experiments were also done in a lower concentration range between 0.05 and 0.2 μM ACh at −100 mV (data not shown). Under these conditions as well, the slopes for both control and DNJ-treated cells were the same (1.8–2.0).

Prolonged ACh applications (1 μM, ~60 s) were used to explore the time course of desensitization in control and DNJ-treated cells. There was no difference in the rate of decline of the responses obtained in the continuous presence of ACh (data
Cells Treated with CS or DNJ Express an ACh Receptor with Altered Activation Kinetics

We next examined the kinetics of ACh receptor activation using single-channel records (cf. Sine and Steinbach, 1986, 1987). Our kinetic analysis was based on the scheme most generally applied to explain the major features of ACh receptor activation (e.g., Colquhoun and Sakmann, 1985; Sine and Steinbach, 1987):

\[
2A + R \xrightleftharpoons[\beta]{\alpha} A_2R \xrightleftharpoons[\kappa_{-2}]{\kappa_{+2}} AR + A
\]

(Scheme 1)

where \( A \) is the free agonist, \( \kappa_{+1}, \kappa_{+2}, \) and \( \kappa_{-1}, \kappa_{-2} \) are the rate constants of agonist association and dissociation, respectively, and \( R, AR, \) and \( A_2R \) are closed conforma-
FIGURE 5. Double logarithmic dose-response curves for (○) normal and (O) DNJ-treated cells at -70 mV. Whole-cell currents were elicited and recorded as described under Methods. Each symbol represents the mean ± SEM of n cells (indicated by the number next to each symbol). The solid lines through the symbols represent linear least-squares estimations of the slopes (nH: 1.5 and 1.3 for normal and DNJ-treated cells, respectively). For comparison, slopes of 2 and 1 are shown (dashed lines).

FIGURE 6. Consecutive single-channel currents recorded from cell-attached patches in the presence of 0.1 μM ACh at -100 mV (11°C), from (A) a normal cell or (B) a DNJ-treated cell. Low pass filtered at 8,000 Hz. The horizontal lines indicate the mean baseline current amplitude.
tions of the ion channel. $A_2R$ isomerizes with a rate constant $\beta$ to the open conformation $A_2R^*$, and returns to $A_2R$ with a rate constant $\alpha$.

Fig. 6 shows consecutive single channel currents elicited by 0.1 $\mu$M ACh (at $-100$ mV), from a control cell (Fig. 6 A) and a DNJ-treated cell (Fig. 6 B). At low concentrations of ACh there are two qualitatively different patterns of single channel currents: bursts of long-duration opening intervals which are commonly interrupted by brief gaps (e.g., second trace in Fig. 6 A), and isolated brief duration openings (e.g., fifth and ninth traces in Fig. 6 A). Most charge transfer occurs during bursts of long openings, which has been called the main activation mode (Sine and Steinbach, 1987). However, a given ACh receptor can produce both long and brief duration openings, apparently by switching between two “modes” of operation (Sine and Steinbach, 1984, 1986a, b). The following analysis of receptor function focuses on the brief closed periods, which occur in bursts of long openings, and provide information on the activation kinetics of the main activation mode. The closed time distributions at a low concentration of ACh (Fig. 7) were best described by the sum of three exponential components ($C_0$, $C_1$, $C_2$). The three exponentials (thinner lines) and their sum (thicker line) are shown superimposed in the main panels. The complete histogram under both conditions was best described by the sum of three exponential components ($C_0$, $C_1$, $C_2$). The parameters of the fit in A were: $\tau_0 = 662$ ms, $f_0 = 0.46$; $\tau_1 = 0.44$ ms, $f_1 = 0.10$; $\tau_2 = 20$ $\mu$s, $f_2 = 0.44$. The parameters of the fit in B were: $\tau_0 = 2.73$ s, $f_0 = 0.70$; $\tau_1 = 1.06$ ms, $f_1 = 0.05$; $\tau_2 = 40$ $\mu$s, $f_2 = 0.25$.

![Figure 7](https://jgp.rupress.org/content/17/7/775/F7)

**FIGURE 7.** Closed time histograms from single-channel records obtained in the presence of 0.1 $\mu$M ACh at $-100$ mV (11°C) from (A) a normal cell (1,535 entries) and (B) a CS-treated cell (1,166 entries). Long-duration gaps are illustrated in the insets, whereas short-duration gaps appear in the main panels. The complete histogram under both conditions was best described by the sum of three exponential components ($C_0$, $C_1$, $C_2$). The three exponentials (thinner lines) and their sum (thicker line) are shown superimposed in the main panels. The activation related component $C_1$ has been highlighted with filled circles. The parameters of the fit in A were: (time constant $\tau$, and fractional area $f$): $\tau_0 = 662$ ms, $f_0 = 0.46$; $\tau_1 = 0.44$ ms, $f_1 = 0.10$; $\tau_2 = 20$ $\mu$s, $f_2 = 0.44$. The parameters of the fit in B were: $\tau_0 = 2.73$ s, $f_0 = 0.70$; $\tau_1 = 1.06$ ms, $f_1 = 0.05$; $\tau_2 = 40$ $\mu$s, $f_2 = 0.25$. 
1986a, b, 1987). The time constant of this component was increased 2 to 4-fold on
cells treated with DNJ and CS (Fig. 7 and Table II). Using the approach developed
by Colquhoun and Hawkes (1981), we estimated the rate constants for channel
opening ($\beta$), channel closing ($\alpha$), and agonist dissociation ($k_{-2}$) (Table II B). This
analysis showed that treatment with DNJ or CS reduced both $\beta$ and $k_{-2}$ (Table II B).

### TABLE II

| Part A: Parameters of Activation-related Gaps within Bursts |
|-------------|

| $n$ | $t_0$ | $t_g$ | Gaps per burst |
|-----|-------|-------|----------------|
| Controls | 3 | 30.7 ± 5.8 | 0.39 ± 0.09 | 0.32 ± 0.08 |
| +SW | 1 | 36.3 | 0.43 | 0.47 |
| +DNJ | 3 | 29.0 ± 2.0 | 0.82 ± 0.13* | 0.50 ± 0.10 |
| Controls | 3 | 21.3 ± 4.0 | 0.42 ± 0.02 | 0.32 ± 0.10 |
| +CS | 2 | 22.0 ± 4.2 | 1.05 ± 0.04* | 0.16 ± 0.04 |

| Part B: Derived Activation Parameters |
|-------------|

| $k_{-2}$ | $\beta$ | $\alpha$ |
|----------|----------|----------|
| Controls | 1051 ± 232 | 633 ± 186 | 45.9 ± 7.1 |
| +SW | 790 | 740 | 41 |
| +DNJ | 485 ± 124 | 278 ± 35* | 42.7 ± 11.3 |
| Controls | 915 ± 91 | 570 ± 131 | 54.0 ± 6.5 |
| +CS | 418 ± 3* | 158 ± 39 | 58 ± 11 |

Part A: The values presented in the first three rows are the mean ± SD obtained from the
analysis of $n$ histograms, where each histogram is made of the summed data of three to
two patches obtained at one concentration of ACh between 0.1-0.4 μM. The values pre-
sented in the fourth and fifth rows are the mean ± SD obtained from the analysis of $n$
histograms (n patches in the presence of 0.1 μM ACh). $t_0$ is the fitted time constant for
the slow component of the burst duration histogram, $t_g$ is the fitted time constant of the
activation related component (C) of the closed time histogram (Fig. 7). Gaps per burst is
the ratio of the number of activation related gaps to the number of long duration
bursts.

Part B: $k_{-2}$ and $\beta$ were derived from $t_0$ and the number of gaps per burst. $\alpha$ was derived
by dividing the number of openings per burst by the difference between $t_g$ and the pro-
duct of $t_0$ multiplied by the number of gaps per burst. The values in italics are independent
estimates of $\beta$ and $\alpha$ derived from the analysis of a closed time histogram generated from
single-channel data obtained with 250 μM ACh (see text). All determinations were done at
-100 mV and 11°C.

*P < 0.01 (two-tail Student’s t test applied throughout).

In contrast $\beta$ and $k_{-2}$ estimates from SW-treated cells were indistinguishable from
controls (Table II). The closing rate ($\alpha$) did not appear to be affected by any of the
treatments.

To obtain independent estimates for $\beta$ and $\alpha$, we performed experiments using a
concentration of ACh (250 μM) that should saturate binding to these receptors
Histograms of closed times obtained with 250 μM ACh were qualitatively identical for control and DNJ-treated cells (data not shown) and agreed with previous work (Sine and Steinbach, 1987). However, for DNJ-treated cells the mean duration of the activation-related component was 3.98 ms in comparison to 1.56 ms for control cells. Previous work has shown that the mean duration of this component is close to $1/\beta$ at this concentration of ACh (Sine and Steinbach, 1987; closed time component $G_3$). The rate of occurrence of these closures per second of open time provides an estimate of the channel closing rate, $\alpha$. The high concentration estimates of $\alpha$ and $\beta$ are shown in italics in Table IIb, and it can be seen that they are in excellent agreement with estimates from low concentration experiments.

To check for kinetic homogeneity, we implemented the tests of Sine and Steinbach (1986b, 1987), who demonstrated that under normal conditions the ACh receptors in BC3H-1 cells are kinetically homogenous. We analyzed the properties of bursts at low ACh concentrations and clusters and groups at a high ACh concentration. There was no indication that bursts or clusters reflected the presence of a

### Table III

Parameters of the Distributions of Burst Durations

| n | $\tau_0$ | $\tau_1$ | $\tau_2$ |
|---|---|---|---|
| Controls | 3 | 30.7 ± 5.8 | 0.39 ± 0.16 | 0.06 ± 0.02 |
| + SW | 1 | 36.3 | 0.38 | 0.06 |
| + DNJ | 3 | 29.0 ± 2.0 | 0.74 ± 0.19 | 0.10 ± 0.03 |

Note some variation in the mean burst duration $\tau_0$ (see also Table II) and the fractional areas. Nevertheless, within the same set, the treatments with CS or DNJ increased $a_2$, while the treatment with SW did not produce major changes in any of the parameters. All data recorded at -100 mV and 11°C.

*P < 0.001 (two-tail Student t test applied throughout).

1P < 0.02.

2P < 0.05.
heterogeneous population of ACh receptors in either control or treated cells (data not shown), and the results were essentially identical to those reported earlier. The single-channel records shown in Fig. 6 suggest that the relative frequency of isolated brief duration openings was increased by the treatment with DNJ. To quantify this observation, we analyzed the distributions of burst durations (see Methods). Application of the Kolmogorov-Smirnov nonparametric test (Siegel, 1956) to summed burst-duration histograms showed that distributions differed significantly between control cells and cells treated with DNJ or with CS (\( P < 0.005 \)), but not between control cells and cells treated with SW (\( P > 0.1 \)). The burst-duration histograms were described by the sums of three exponentials (data not shown; cf. Sine and Steinbach, 1986a, b). The time constants of the long-duration component was not affected by any treatment, but the time constants of the intermediate duration component was prolonged by DNJ or CS. The consistent effect of treatment with DNJ or CS, however, was that the fraction of total bursts in the brief-duration component was increased (Table III).

These results indicate that the frequency of long bursts is decreased to a greater extent than the frequency of brief bursts by DNJ or CS treatment. Indeed, the frequency of brief bursts seemed to be depressed only as much as the number of surface ACh receptors. In two sets of experiments with DNJ-treated cultures and paired control cultures, the total frequencies of bursts were depressed to 14% and 29% of the controls but the frequencies of brief duration bursts were reduced to only 23% and 50%, respectively. Similarly, for one comparison of CS-treated and control cells, the total frequency and the frequency of brief duration bursts were reduced to 8% and 39% of controls, respectively. For these comparisons, the frequency of brief-duration bursts was estimated from the corrected area of the brief component of burst duration histograms divided by the total record length.

DISCUSSION

These observations show that treatment of BC3H-1 cells with CS or DNJ has four effects on the ACh receptors expressed. First, ACh receptor kinetics are altered, as seen in the single-channel current records. Secondly, the steady-state response is decreased, as shown by the whole-cell responses and the burst frequencies. Thirdly, the number of surface ACh receptors is decreased, as shown by the surface binding of \( \alpha \)-BTX. Fourthly, the mobilities of subunits on SDS electrophoresis was affected.

The three inhibitors produced discrete alterations in the electrophoretic mobility of the receptor subunits: CS and DNJ decreased the mobility of the \( \alpha \) and \( \beta \) subunits (see also Smith et al., 1986 for DNJ), whereas SW increased the mobility of \( \gamma \) and \( \delta \) (Fig. 2). These observations are in qualitative agreement with the expected change in apparent molecular mass produced by the structural modifications of the sugar moiety (Fig. 1). By blocking glucosidase I with CS or glucosidase I and II with DNJ, the core oligosaccharide will not lose three glucose molecules (a total of 540 daltons) which, under normal conditions, are removed to form high-mannose oligosaccharides in the \( \alpha \) and \( \beta \) subunits. The shift in the electrophoretic mobilities of the \( \gamma \) and \( \delta \) subunits observed with SW is consistent with the idea that mannose trimming catalyzed by \( \alpha \)-mannosidase II is necessary for further elongation of the sugar chains on these subunits.
It is important to note that CS and DNJ are structurally dissimilar, whereas CS and SW are structurally related (Fuhrmann et al., 1985). However, CS and DNJ affect the same step(s) in carbohydrate processing and produce identical effects on subunit mobility. The changes in ACh receptor kinetics correlate with the known effects of the drugs on carbohydrate structure and with the observed changes in subunit mobility rather than with drug structure. This correlation suggests that the functional changes are not the result of an effect of the drugs which is unrelated to their specific effects on carbohydrate processing. Further, the fact that SW did not cause the same alterations in ACh receptor function but did alter subunit mobilities suggests that the effects are relatively specific—not all changes in carbohydrate processing produce effects on ACh receptor kinetics.

Three sets of observations indicate that the function of the ACh receptors was altered by CS or DNJ. First, two rate constants, the opening rate $\beta$ and the rate of agonist dissociation $k_{-2}$ were reduced. Secondly, the distributions of burst durations were altered. Thirdly, the responsiveness of the cells to ACh was reduced more than expected from the observed reduction in the number of surface ACh receptors. It would be attractive to have a single hypothesis to account for all of these changes. At first glance, the observed reduction in the channel opening rate ($\beta$) could explain the reduction in steady-state parameters (whole-cell current and burst frequency). However, this cannot be the case, since $k_{-2}$ was also reduced by a similar factor (twofold). This is more clearly seen in the following equations:

$$I = N \gamma (V_r - V) \left( \beta [A]^2 k_{+1} k_{+2} / \alpha k_{-1} k_{-2} \right) \tag{2}$$

$$f = N \left( [A]^2 k_{+1} k_{+2} / k_{-1} \right) \left( \beta / (\beta + k_{-2}) \right) \tag{3}$$

In the low limit of agonist concentration these equations define the steady state macroscopic current ($I$) and the single channel burst frequency ($f$). $N$ is the number of ACh receptor channels on the cell surface, $\gamma$ is the single-channel conductance, $V_r$ and $V$ are the reversal and holding potentials, respectively, and other terms are described in Scheme 1. From our measurements $\gamma$, $V_r$, and $\alpha$ are not altered by any of the treatments, but $N$, $\beta$, and $k_{-2}$ were reduced by the treatments with CS or DNJ. Since in both equations $\beta$ and $k_{-2}$ appear as ratios, parallel and comparable changes in these rates will cancel. In other words, the responses would only be depressed as much as $N$, in contrast to what was observed (Table I). Thus, to account for the reduced response other alterations must have occurred.

Two immediate possible explanations come to mind, which we will term the “one population” and the “two population” hypotheses. The one population hypothesis is that cells treated with DNJ or CS express only one functional class of ACh receptors, which have the altered kinetic properties determined in the analysis of single channel data. To account for the steady-state activity, it is postulated that another rate constant is altered in addition to the changes observed in $\beta$ and $k_{-2}$. The two population hypothesis postulates that treated cells express two classes of receptor, one whose function is altered as found in the analysis of the single-channel data. The second class is essentially nonfunctional (“dead”). From the ratios in Table I, about one-half of the surface receptors on treated cells would be “dead.” We favor the first alternative, as will be discussed, but cannot eliminate the second.
Smith et al. (1986) studied the capacity of D-tubocurarine and carbamylcholine to reduce the initial rate of α-BTX binding, with control and DNJ-treated cells. They found that the competition isotherms with both cholinergic ligands were not altered by the treatment with DNJ. These results demonstrated that the equilibrium binding of D-tubocurarine (a nondesensitizing antagonist in BC3H-1 cells; Sine and Taylor, 1979) to the resting ACh receptor was not affected. Similarly, the equilibrium binding of carbamylcholine and the accompanying desensitization were also not affected. Based on these observations we assumed that the forward rate of binding $k_{+2}$ was reduced as much as $k_{-2}$, and the overall affinity $(K_1K_2 = k_{-1}k_{-2}/k_{+1}k_{+2})$ was constant. Using this assumption, we calculated the predicted magnitude of the response to ACh ($\ell$ and $f$), based on our estimations of $N$ and the kinetic constants (Table IV). Table IV shows a good agreement between the observed and predicted values for whole-cell response and burst frequency, indicating that a parallel reduc-

**TABLE IV**

Observed and Predicted Ratios for Whole-Cell Responses ($\ell$) and Burst Frequencies ($f$)

|        | DNJ       | CS        |
|--------|-----------|-----------|
| $\ell/\ell$ | 0.18      | 0.11      |
| $f/f$   | 0.20      | 0.04      |
| $N'/N$  | 0.35      | 0.23      |

$I'/I$, $f'/f$, and $N'/N$ are the ratios of treated to normal for the whole-cell responses, burst frequencies, and surface ACh receptors, respectively. Observed values were taken from Table I. The predicted values were calculated from the following equations, simplified from Eqs. 2 and 3:

$$I'/I = \frac{(N'\beta)}{(NB)}$$

$$f'/f = \frac{(N'\beta k'_{+1}/k_{+1} + \beta)}{(NBk'_{+1}/(k_{-1} + \beta))}$$

The primed parameters indicate values for treated cells. As explained in the text, the ratios were calculated assuming that $k'_{+1}/k_{+1} = k'_{-1}/k_{+1}$ and that $k'_{+1} = k_{+1}$. Values for parameters were taken from Tables I and II.

In $k_{+2}$ and $k_{-2}$, and the reduced $N$ and $\beta$ are sufficient to explain the reduced response to ACh observed with DNJ- and CS-treated cells. Similarly, the larger relative frequency of brief openings can be accounted for if receptors produce a normal frequency of brief bursts.

The second hypothesis considered is that two classes of altered ACh receptors are expressed on the surface of cells treated with CS or DNJ. The binding experiments discussed above (Smith et al., 1986) suggest that all surface ACh receptors on cells treated with DNJ bind D-tubocurarine and carbamylcholine in the same fashion. Hence, "dead" receptors would be characterized by a low channel-opening rate (few openings), a high channel-closing rate (brief openings), or a low single-channel conductance (no current flow). Also, to account for the increased proportion of brief openings, either dead receptors must be able to produce brief openings or the functional but altered receptors must make a larger proportion than normal of brief

**TABLE IV**

|        | DNJ       | CS        |
|--------|-----------|-----------|
| $\ell/\ell$ | 0.18      | 0.11      |
| $f/f$   | 0.20      | 0.04      |
| $N'/N$  | 0.35      | 0.23      |
openings. We cannot eliminate the two population hypothesis, but it requires an even larger number of functional consequences resulting from treatment with DNJ or CS than the one population hypothesis.

Several possible mechanisms could explain the altered function of ACh receptors expressed by BC3H-1 cells treated with CS or DNJ. It might be a direct result of the altered oligosaccharide chains in the ACh receptor subunits (e.g., steric effects), which affected agonist binding and/or intersubunit interactions. The observed functional alterations might result from the action of a putative modulatory protein which had been affected by the glucose trimming inhibitors, and which could affect the ACh receptor by noncovalent interactions, or covalent posttranslational modifications. It is also possible that the altered function is the result of the assembly and surface expression of conformationally immature ACh receptors. Our data do not allow us to distinguish among these mechanisms.

In conclusion, we have shown that glucose trimming in BC3H-1 cells can influence the channel kinetics of the ACh receptors expressed on their surface. Although the exact mechanism by which this occurs is unknown, we have provided physiological evidence which suggests that the carbohydrate moiety of the ACh receptor can affect its functional properties.

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