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Alternate Stoichiometries of $\alpha4\beta2$ Nicotinic Acetylcholine Receptors

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ABSTRACT

Two functional types of nicotinic acetylcholine receptors (nAChRs) are expressed when human embryonic kidney cells are permanently transfected with equal amounts of human $\alpha4$ and $\beta2$ subunit cDNAs. Most (82%) of these nAChRs exhibit an EC_{50} of 74 ± 6 μM for ACh, a much lower sensitivity than the remaining fraction (EC_{50} of 0.7 ± 0.4 μM) or than expected from expression of equal amounts of $\alpha4$ and $\beta2$ mRNAs in *Xenopus laevis* oocytes. We have found three conditions that can increase the number of nAChRs with high sensitivity to activation. These are: 1) transient transfection with additional $\beta2$ subunits, 2) overnight incubation in nicotine, or 3) overnight culture at 29°C. Using metabolic labeling with [^{35}S]methionine to measure subunit stoichiometry, we found that the majority of nAChRs had a stoichiometry of $(\alpha4)_3(\beta2)_2$. Overnight treatment with nicotine increased the number of nAChRs and increased the proportion of the $(\alpha4)_2(\beta2)_3$ stoichiometry. Alternate $\alpha4\beta2$ nAChR stoichiometries with distinct functional properties raise the possibility for an interesting mode of synaptic regulation for nicotinic signaling in the mammalian brain.

The $\alpha4\beta2$ nAChR is the predominant nAChR subtype in the mammalian brain that has high affinity for nicotine. nAChRs composed of $\alpha4$ and $\beta2$ subunits modulate neurotransmitter release (Dani, 2001) and play a direct role in addiction to nicotine (Picciotto et al., 1998; Marubio et al., 1999). Mutations in $\alpha4\beta2$ nAChRs have been linked to autosomal-dominant nocturnal frontal lobe epilepsy (Weiland et al., 2000). They also are thought to be involved in Alzheimer's and Parkinson's diseases (Rusted et al., 2000).

Two different approaches showed independently that chick $\alpha4\beta2$ nAChRs have a stoichiometry of $(\alpha4)_2(\beta2)_3$ when expressed in *Xenopus laevis* oocytes from cRNAs or cDNAs injected at a 1:1 (α/β) ratio (Anand et al., 1991; Cooper et al., 1991). A more recent study showed that when the rat $\alpha4/\beta2$ subunit ratio is varied, nAChRs of two functional classes are formed in oocytes (Zwart and Vijverberg, 1998). When the $\alpha4/\beta2$ ratio was 1:9, nAChRs were formed that were more sensitive to activation and desensitized more slowly. However, when the ratios were 1:1 or 9:1, nAChRs appeared that were less sensitive to activation and desensitized more rapidly. These findings raised the possibility that $\alpha4\beta2$ nAChRs can also exist in a stoichiometry that differs from $(\alpha4)_2(\beta2)_3$.

Here, we report that the majority (82%) of $\alpha4\beta2$ nAChRs expressed in a stable HEK cell line exhibit sensitivity to activation by ACh that is much lower (EC_{50} = 74 ± 6 μM) than when $\alpha4\beta2$ nAChRs are expressed in *Xenopus laevis* oocytes [EC_{50} = 2.2 ± 0.1 μM (Kuryatov et al., 1997)]. This confirms the observations by Buisson and Bertrand (2001) in another independently derived line. They also reported that nicotine and other nicotinic agents increased the proportion of high-sensitivity nAChRs and speculated that the increase was caused by slow conversion of existing low-sensitivity nAChRs to high-sensitivity nAChRs. However, it is well known that nicotine, other nicotinic agonists, and some nicotinic antagonists cause an increase in both total and surface nAChRs (Benwell et al., 1988; Wonnacott, 1990; Marks et al., 1992; Peng et al., 1994; Gopalakrishnan et al., 1996; Whiteaker et al., 1998). Also, the properties of the low-sensitivity nAChRs are very much like those reported by Zwart and Vijverberg (1998) when nAChRs are formed from an $\alpha4$:$\beta2$ ratio of 9:1. Consistent with the hypothesis that our $\alpha4\beta2$ cell line might express high $\alpha4/\beta2$ subunit ratio nAChRs, we found that boosting $\beta2$ subunit levels by transient transfection with additional $\beta2$ cDNA increased the number of more sensitive nAChRs. A similar effect was found for incubation in nicotine and by culturing at 29°C. Most significantly, we show, using metabolic labeling with [^{35}S]methionine, that the majority of nAChRs produced by our cell line have a stoichiometry of $(\alpha4)_3(\beta2)_2$. Long-term exposure to nicotine resulted in a large increase in assembled nAChRs accompanied by an increase in the proportion of those having greater sensitivity to activation and a stoichiometry of $(\alpha4)_2(\beta2)_3$. We speculate that two $\alpha4\beta2$ stoichiometries exist in mammalian brain. Functional studies with mouse thalamic synaptosomes

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; HEK, human embryonic kidney; mAb, monoclonal antibody; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; DMPP, 1,1-dimethyl-4-phenylpiperazinium.

support this hypothesis because they reveal the existence of equal proportions of low- and high-sensitivity $\alpha4\beta2$ nAChRs (Marks et al., 1999; Butt et al., 2002). Only $\alpha4$ and $\beta2$ subunits were detected in significant numbers in thalamus by in situ hybridization, and knockout of either subunit eliminated essentially all binding of epibatidine in this area (Picciotto et al., 2001). The thalamus is the largest contiguous area of $\alpha4\beta2$ nAChR expression in rodent brain. Therefore, a strong case can be made for a functional role for each of the two $\alpha4\beta2$ nAChRs stoichiometries in mammalian brain. The shift in assembly toward the $(\alpha4)_2(\beta2)_3$ form caused by long-term nicotine exposure could be important in understanding nicotine addiction.

Materials and Methods

Cloning and Tissue Culture. The details of the cloning of the stably transfected cell line expressing the human $\alpha4\beta2$ nAChR will be described elsewhere (A. Kuryatov and J. Lindstrom, in preparation). Briefly, human $\alpha4$ cDNA in the pcDNA3.1/Zeo(+) vector (Invitrogen, Carlsbad, CA) and human $\beta2$ cDNA in the pRc/CMV vector (Invitrogen), in equal amounts, were transfected into human embryonic kidney (HEK) tsA201 cells using Fugene 6 (Roche Diagnostics, Indianapolis, IN) transfection reagent. Cloning rings were used to isolate individual clones that were subsequently screened for high, stable expression by radioimmune assays using [^{3}H]epibatidine binding to nAChRs isolated from Triton X-100 cell extracts on mAb 295-coated Immulon 4 microwells (Dynatech Laboratories, Chantilly, VA). Transfected cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen), and 10% fetal bovine serum (Hyclone, Logan, UT) as described previously (Wang et al., 1998). Zeocin (0.5 mg/ml; Invitrogen) was used for selection of $\alpha4$ expression, and G-418 (0.6 mg/ml; Invitrogen) was used for selection of $\beta2$ subunit expression. For some experiments, cells were transiently transfected with additional $\beta2$ or $\beta4$ subunit cDNA by mixing 1 μg of DNA in a 1:6 ratio (w/v) with Fugene 6, per the manufacturer's directions, 1 day after plating in 35-mm plastic tissue culture dishes that contained five glass coverslips (12-mm diameter).

Whole-Cell Electrophysiology. At least 2 days before recording, HEK cells were plated onto glass coverslips coated with rat tail collagen (type 1; Collaborative Biomedical Products, Bedford, MA). Agonist-containing solutions were applied to the cells by gravity through fused glass tubing that was connected to multiple reservoirs mounted above the recording chamber. The recording solution contained 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 5 mM HEPES and was adjusted to pH 7.3 with NaOH. Electrodes (5–8 MΩ) were formed from borosilicate glass and were filled with a solution containing 150 mM Cs-gluconate, 10 mM Cs-EGTA, and 10 mM HEPES and was adjusted to pH 7.2 with CsOH. Currents were acquired and analyzed as described previously (Nelson et al., 2001). Currents for each cell were normalized to the response of that cell to the application of 300 μM ACh. Concentration-response curves were constructed and fitted to the following equation in Origin (Ver. 4.1; Microcal Software, Inc., Northampton, MA): $y = A_{high}/[1 + ([Agonist]/EC_{50,\ high})^{phigh}] + A_{low}/[1 + ([Agonist]/EC_{50,\ low})^{plow}]$, where y is the normalized response amplitude, high and low refer to relative agonist sensitivity, A_x is the amplitude of that component of the fitted curve, and p_x is the steepness for that component of the fitted curve. To calculate the fractions of the nAChRs that corresponded to the higher sensitivity population of nAChRs, the peak currents that were measured in response to the application of 10 μM ACh were used. This concentration was at the plateau phase of the high sensitivity portion of the curve and minimized contributions of the lower sensitivity population of nAChRs to the current. The response to 300 μM ACh was used to represent all functional nAChRs so that the relative amounts of the low- and high-sensitivity population of nAChRs could be estimated. Desensitization time constants were determined by fitting exponential equations to the data. Representative traces were constructed by opening data files in Axograph 3.55 (Axon Instruments, Union City, CA) and exporting data to Canvas 7.0 (Deneba Software, Inc., Miami, FL).

Single-Channel Analyses. Single-channel currents were recorded and analyzed as described previously (Nelson and Lindstrom, 1999). Briefly, channel activity was recorded in outside-out configuration patches by isolating the patch in a stream of ACh (0.05–5 μM). To allow data to be compared with data obtained using oocyte expression (Kuryatov et al., 1997), recordings were performed as described previously (Nelson and Lindstrom, 1999) in ND-96 solution consisting of 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.6, that also contained 50 mM dextrose. The recording electrodes were filled with a solution consisting of 80 mM CsF, 20 mM CsCl, 10 mM Cs-EGTA, 10 mM HEPES, and 3 mM MgATP, pH 7.2. Data were sampled off-line at 15 kHz (Axoscope 2.0, Axon) and filtered at 3 kHz (model 902; Frequency Devices, Inc., Haverhill, MA) for analysis. All single-channel analyses and fitting were performed with pClamp 6.0.3 (Axon).

nAChR Isolation. Detergent extraction of nAChRs was performed as described previously (Nelson et al., 2001) with slight modification. Briefly, cells were removed from 100-mm tissue culture dishes using 5 mM EDTA in phosphate-buffered saline (PBS) and pelleted by centrifugation. The pellet was then suspended in 1 ml buffer A (50 mM Na_2HPO_4-NaH_2PO_4, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide, and 2 mM phenylmethylsulfonyl fluoride) and the cells were lysed by sonication. Membrane fragments were collected by ultracentrifugation (20 min, 100,000g) in an XL-90 ultracentrifuge with a 50.2 Ti rotor and 6.5-ml thick-walled polycarbonate tubes with adapters (all from Beckman Coulter, Fullerton, CA) and the pellets extracted with 0.5 ml of 2% Triton X-100 in buffer A for 1 h at room temperature or 2 h at 4°C. The nAChR extracts were then cleared of insoluble material by ultracentrifugation (30 min, 170,000g). Crude extracts were subjected to radioimmunoassays, sucrose gradient sedimentation, or direct immunopurification. Radioimmunoassays were performed to access the number of nAChRs in extracts, as described previously (Nelson et al., 2001), using [^{3}H]epibatidine (2 nM).

Subunit Stoichiometry Using [^{35}S]methionine Incorporation. For stoichiometry measurements, cells were plated in 100-mm plates and grown under normal conditions until 80% confluent. To metabolically label $\alpha4$ and $\beta2$ subunits with [^{35}S]methionine, the cells were first depleted of methionine by removing the normal tissue culture medium, followed by a rinse with serum-free, methionine-deficient, high-glucose DMEM (Invitrogen) and then incubated for 1 h in methionine-free medium. After 1 h, the medium was replaced with fresh methionine-free media (5 ml for 100 mm plate) that also contained 1 mCi L-[^{35}S]methionine (1175 Ci/mM; PerkinElmer Life Sciences, Inc., Boston, MA) and 10% normal (containing methionine) DMEM. Labeling was continued overnight under otherwise normal conditions. To test the effect of nicotine treatment, nicotine (0.2 μM) was added at the same time as [^{35}S]methionine. Control dishes without [^{35}S]methionine were handled in parallel. When labeling was complete, the labeling medium was removed and the cells were washed twice with 5 ml of ice-cold PBS and then removed from dishes with 5 mM EDTA in PBS. Detergent extracts were prepared as described above. Selection of fully assembled nAChRs was achieved by sucrose gradient sedimentation as described below. Immunopurification of $\alpha4\beta2$ nAChRs from sucrose gradient peak fractions was achieved by incubating pooled fractions at 4°C overnight with mAb 295-(binds to $\beta2$ subunits) coupled Sepharose (30 μl of 2 mg/ml; Activated CH Sepharose 4B resin, Amersham Biosciences AB, Uppsala, Sweden). The resin was then collected in compact reaction columns on 90-μm filters (United States Biochemical Corp, Cleveland, OH) and washed six times with 400 μl of 0.5% Triton X-100/PBS. Bound nAChRs were then eluted with 3% SDS sample

buffer (75 μl) without reducing agent. SDS-PAGE was then used to separate α4 and β2 subunits, as described below under *Western Blotting*. Control and [^{35}S]methionine-labeled samples were run on the same gel. The gel was divided and the [^{35}S]methionine-labeled side dried in 20% glycerol (diluted in a solution of 40% methanol and 10% acetic acid), whereas the control side was transferred to polyvinylidene difluoride membrane for Western blotting. After autoradiography of the dried gel using a Kodak Biomax Transcreen LE intensifying screen (Eastman Kodak, Rochester, NY), the bands corresponding to α4 or β2 subunits were cut from the gel, minced with a sharp blade, and then transferred to scintillation tubes for measurement of incorporated [^{35}S]methionine activity. Because human α4 and β2 subunits contain equal numbers of methionine residues, their relative numbers can be calculated directly from the measured radioactivity after subtracting background (section of an unused lane from the same gel).

Sucrose Gradients. Total membrane extracts were prepared, as described above with 2% Triton X-100 in buffer A, and 150 μl was layered on 5-ml sucrose gradients (5–20%, linear) at 4°C. Centrifugation (390,000*g*) was conducted for 1 h at 4°C (using rotor NVT90 in the XL-90 ultracentrifuge. Fractions from the gradients were collected on bovine serum albumin-coated microwells (11 drops per fraction; approximately 110 μl) using a Gilson 203 microfraction collector (Gilson Inc., Middleton, WI). nAChRs were quantitated using radioimmune assays on mAb 295-coated wells using 10 nM [^{3}H]epibatidine at room temperature. After 2 h, unbound material was removed from the wells followed by three washes with ice-cold 0.5% Triton X-100 in PBS. Bound material was eluted with 100 μl of 2% SDS that contained 2.5% β-mercaptoethanol and then subjected to scintillation counting. These data were used to determine which four fractions from the sucrose gradients of the [^{35}S]methionine-labeled nAChRs form the peak corresponding to fully assembled nAChRs that would be used for further immunopurification. *Torpedo californica* nAChRs were sedimented simultaneously on the gradients as an internal standard. These were isolated on wells coated with mAb 210 (to α1 subunits) and labeled with ^{125}I-α-bungarotoxin (20 nM) overnight at 4°C. Wells were then washed three times with ice-cold 0.5% Triton X-100 in PBS and then subjected to gamma counting.

Western Blotting. nAChRs were eluted from mAb 295-coupled Sepharose using 3% SDS sample buffer (75 μl) without reducing agents with a 10-min room temperature incubation. The samples were prepared and electrophoresed on 10% polyacrylamide gels containing SDS as described previously (Nelson et al., 2001). After transfer to polyvinylidene difluoride membranes (Trans-Blot; Bio-Rad, Hercules, CA), the blots were blocked with 5% (w/v) dried milk (Nestlé USA, Solon, OH) in PBS with 0.05% Tween 20 and 10 mM NaN_3 (PBS/Tween), for 1 h at room temperature and then labeled overnight at 4°C with rat antisera against α4 or β2 subunits (Kuryatov et al., 2000) at 1:200 dilutions. After three washes with PBS/Tween, labeling was done by the addition of ^{125}I-labeled goat anti-rat IgG antibodies (2 nM, ~0.8 Ci/mmol) for 3 h at room temperature. After a brief rinse and 3 washes (10 min each) with PBS/Tween, autoradiography was performed at −80°C with exposures between 10 min and overnight using Kodak Biomax MS film and a Biomax intensifying screen (Eastman Kodak).

Results

Properties of Macroscopic α4β2 Currents in Permanently Transfected HEK Cells. HEK (tsA201) cells stably expressing human α4 and β2 subunits exhibited robust whole cell currents in response to agonist exposure. The concentration/response relationship to various agonists revealed a rank order of agonist potency of nicotine ≥ 1,1-dimethyl-4-phenylpiperazinium (DMPP) > ACh (Fig. 1) for the major component of the curves. The data for both ACh and nicotine exhibited a two-component relationship, indicating the presence of two functionally distinct classes of agonist-nAChR interactions. DMPP exhibited a monophasic concentration/response relationship, but a second component to the curve could have been obscured by its reduced efficacy. The EC_{50} determined for DMPP was 20 ± 2 μM, whereas nicotine had EC_{50} values of 0.3 ± 0.1 and 18 ± 2 μM and ACh had EC_{50} values of 0.7 ± 0.4 and 74 ± 6 μM. Cytisine's efficacy was too low to determine its potency. For ACh, the higher sensitivity component represented about 18 ± 2% of the nAChRs contributing to the current. This minor population of nAChRs in the cell line exhibited sensitivities similar to those of oocyte-expressed nAChRs activated by ACh (2.2 ± 0.1 μM) or nicotine [0.3 ± 0.04 μM (Kuryatov et al., 1997)]. Relative to ACh, the efficacies of the other agonists were: nicotine, 80%; DMPP, 50%; and cytisine, ~5%. Judging from the rebound in current amplitude upon washout of both nicotine and DMPP, the mechanism of the partial efficacy probably was caused by channel block by agonist. The duration of the rebound currents during washout of high concentrations of nicotine was particularly striking (Fig. 1D).

Upon activation with relatively high concentrations of agonist, the currents exhibited rapid activation followed by a significant decay phase that reflected nAChR desensitization (Fig. 2A). At 300 μM ACh, the desensitizing component rep-

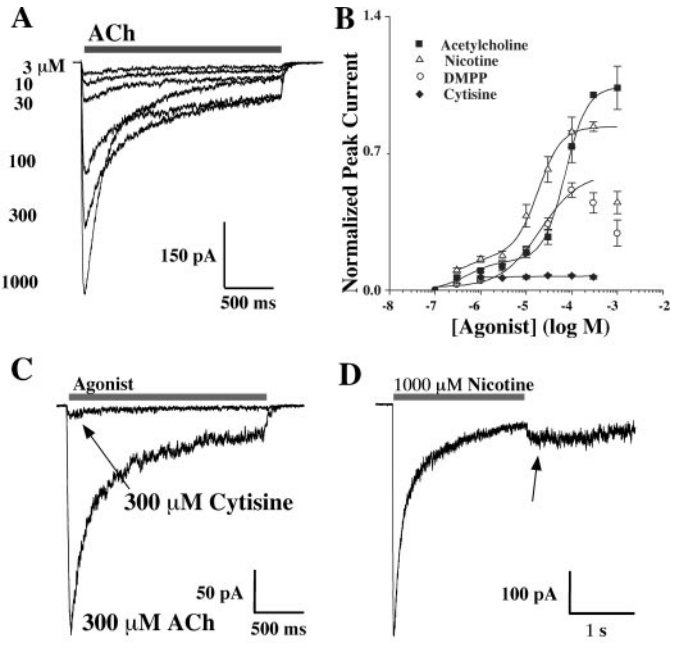

Fig. 1. Pharmacological properties of human α4β2 nAChRs expressed in HEK cells. A, family of currents recorded in response to increasing concentrations of ACh. B, the concentration/response relationship for α4β2 nAChRs in HEK cells for ACh, nicotine, DMPP, and cytisine are shown. ACh and nicotine were full agonists, whereas DMPP had about 50% efficacy, and cytisine had 5% efficacy compared with ACh. The EC_{50} values were as follows: DMPP, 20 ± 2 μM (n = 4); nicotine, 0.3 ± 0.1 and 18 ± 2 μM (n = 4); and ACh, 0.7 ± 0.4 and 74 ± 6 μM (n = 7). The actual potency of cytisine was obscured by its poor efficacy but seems to be similar to that of nicotine. The responses for nicotine and DMPP that were significantly reduced by secondary properties of the drugs (presumed channel blockade) were excluded from the fits (nicotine at 1 mM and DMPP at 300 μM and 1 mM). C, extremely poor efficacy of cytisine is common to β2-containing nAChRs (Nelson et al., 2001) and is a hallmark characteristic of α4β2 nAChRs (Papke and Heinemann, 1994). D, application of high concentrations of nicotine to α4β2 cell line reveals a sustained rebound current (*arrow*) that is probably caused by recovery from agonist-mediated channel block. All data were recorded at −60 mV.

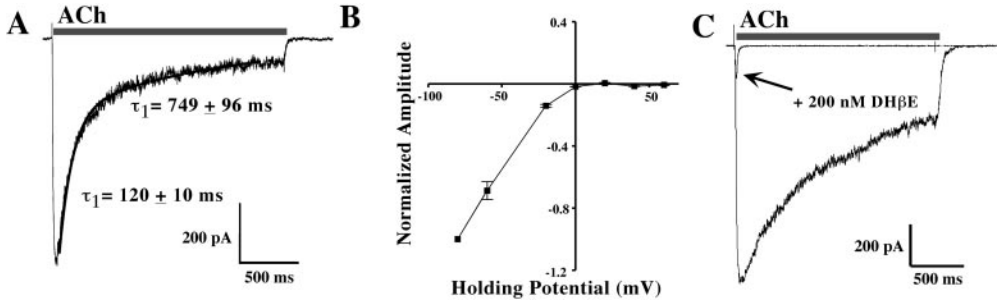

Fig. 2. The functional properties of human $\alpha4\beta2$ nAChRs expressed in stably transfected HEK cells. A, currents evoked with saturating concentrations of ACh exhibited double exponential decay that could be fit with equations having time constants of 120 ± 10 ms and 750 ± 100 ms, representing about 80 and 20%, respectively, of the current amplitude. The solid line superimposed on the current trace corresponds to the fit for that trace (B). The current voltage relationship for $\alpha4\beta2$ nAChRs in HEK cells exhibited the complete inward rectification that is typical of neuronal nicotinic AChRs with the apparent reversal potential occurring near 0 mV. C, the current evoked by 300 μM ACh was almost completely blocked by coapplication of dihydro-β-erythroidine (200 nM) after a 1-min pre-equilibration.

resented about 80% of the peak current. The current/voltage relationship for the currents activated by 100 μM ACh exhibited the strong inward rectification that typifies neuronal nAChRs (Fig. 2B). The apparent reversal potential for these ACh-activated currents occurred at approximately 0 mV. Currents that were activated by 300 μM ACh were nearly completely inhibited by 200 nM dihydro-β-erythroidine (Fig. 2C).

Single Channel Properties of $\alpha4\beta2$ nAChRs in Permanently Transfected HEK Cells. Single channel recordings were performed in the outside-out configuration using recording conditions that would mimic those that were used previously to characterize human $\alpha4\beta2$ nAChRs expressed in *X. laevis* oocytes (Fig. 3; Kuryatov et al., 1997). $\alpha4\beta2$ nAChRs from transfected HEK cells exhibited two channel amplitudes of 1.7 ± 0.1 and 2.3 ± 0.1 pA at −80 mV (Fig. 3). These amplitudes were similar to those found for oocyte-expressed nAChRs [1.4 ± 0.1 and 2.3 ± 0.1 pA at −80 mV (Kuryatov et al., 1997)]. The main difference observed for nAChR activity recorded from the cell line was that the smaller amplitude channel was observed at very low frequency regardless of agonist concentration (not shown). The larger conductance channels appeared at sufficient levels to perform kinetic analysis. Histograms of the mean open times revealed two kinetic components that were best fit with double exponential functions having mean time constants of 1.6 ± 0.2 ms and 8.5 ± 1 ms (n = 4; Fig. 3). These values were very similar to those obtained previously for the larger amplitude channel recorded from oocyte-expressed $\alpha4\beta2$ nAChRs, 1.9 ± 0.2 and 8.1 ± 0.6 ms (Kuryatov et al., 1997). The lower conductance channel from the cell line appeared at frequencies too low to fit in histograms, but 220 events pooled from eight patches had an arithmetic mean duration of 3.9 ± 0.5 ms. In oocytes, the lower amplitude channel had time constants of 3.6 ± 0.5 and 23 ± 5 ms (Kuryatov et al., 1997). The predominance of the larger conductance channel in the transfected cell line paralleled the predominance of the low sensitivity population of nAChRs. The existence of two functional types of nAChRs, with domination of both the macroscopic and microscopic functional data by one, raised the question of what mechanism was responsible for the difference between the two forms.

Transient Transfection of the $\alpha4\beta2$ Cell Line with Additional $\beta2$ cDNA Increased the Proportion of nAChRs that Exhibited High Agonist Sensitivity. The existence of the higher sensitivity component in the concentration/response studies validates the capability of the HEK cells to assemble and/or process the mature protein 'properly', but their scarcity indicates that HEK cells are predisposed to express an alternate form of $\alpha4\beta2$ nAChRs. Such an alternate form could result from different possible mechanisms (e.g., 1) nAChRs formed in HEK cells are differentially modified post-translationally or 2) HEK cells can assemble nAChRs with different subunit stoichiometries. There is pre-

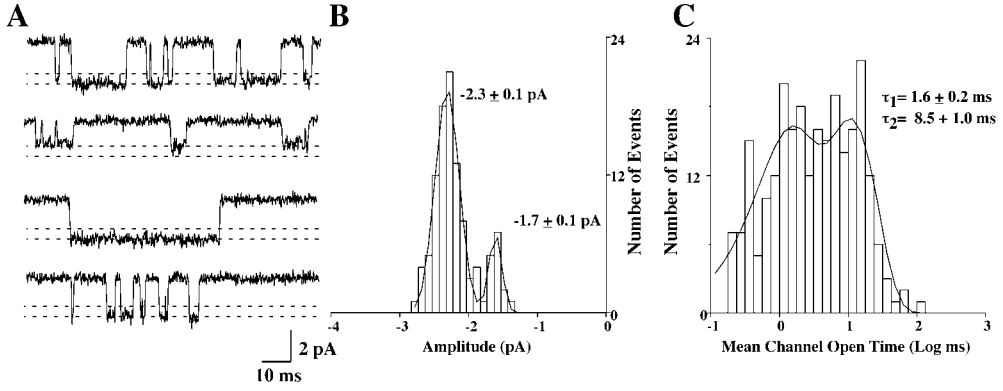

Fig. 3. Single channel properties of human $\alpha4\beta2$ nAChRs in HEK cells. A, single channel currents were activated by continuous application of 100 nM ACh in outside-out patches at −80 mV. Useable data were achieved with concentrations of ACh up to 3 μM. Higher concentrations only caused faster desensitization of the channel activity and revealed no preference for a conductance. B, two different conductance types were observed; the vast majority of channel activity corresponded to the larger conductance channel. The channel amplitudes were −1.7 ± 0.1 and −2.3 ± 0.1 pA at −80 mV. C, histograms of the channel open durations were best fitted by double exponential functions having mean time constants of 1.6 ± 0.2 and 8.5 ± 1.0 ms (n = 7).

cedent for both of these mechanisms to affect the functional properties of nicotinic AChRs. Various studies have reported that phosphorylation alters the functional properties of nAChRs (Huganir et al., 1986; Margiotta et al., 1987; Fenster et al., 1999). Alternatively, one study suggested that different functional properties of $\alpha4\beta2$ nAChRs might be attributed to different subunit stoichiometries (Zwart and Vijverberg, 1998). In this case, the functional properties correlated with the particular ratio of $\alpha4$:$\beta2$ subunit cDNAs that were expressed. When the α:β ratio was high, the resulting nAChRs had low agonist sensitivity and exhibited significant desensitization at saturating concentrations of agonist. Alternately, when the $\alpha4/\beta2$ cDNA ratio was lower, the resulting nAChRs exhibited higher sensitivity to activation by agonist and desensitized little at saturating agonist concentrations. To investigate the molecular explanation for the existence of two functionally distinct populations of $\alpha4\beta2$ nAChRs in the permanently transfected HEK cells, we designed several experiments that attempted to alter the functional properties of the nAChRs expressed by permanently transfected HEK cells. Because the functional properties of the majority of the nAChRs expressed by the $\alpha4\beta2$ cell line resembled the properties of high α/β nAChR subunit ratios expressed in oocytes, we considered that the HEK cells were also expressing a high α/β subunit ratio, even though they were transfected with equal amounts of cDNA. For example, unassembled $\beta2$ subunits might be degraded more rapidly than unassembled $\alpha4$ subunits. This might result in formation of nAChRs with more α than β subunits. Alternatively, $\alpha4$ subunits might be assembled preferentially into mature nAChRs. In either case, increased expression of $\beta2$ subunit might alter the stoichiometry. To test this hypothesis, we transiently transfected our stable $\alpha4\beta2$ cell line with additional $\beta2$ subunit cDNA to further promote the production of $\beta2$ protein. The rationale was based on the observation that expression of exogenous proteins is higher within days after transfection and falls during subsequent cloning of permanently transfected cells. The result of transient transfections of permanently transfected $\alpha4\beta2$ cells with more $\beta2$ cDNA was an increase in the higher sensitivity component of the concentration/response relationship (Fig. 4) to about $43 \pm 4\%$ of the total population of nAChRs. The EC_{50} values were 2 ± 0.4 and 90 ± 17 μM. This result is consistent with the idea that increased expression of $\beta2$ protein resulted in its in-

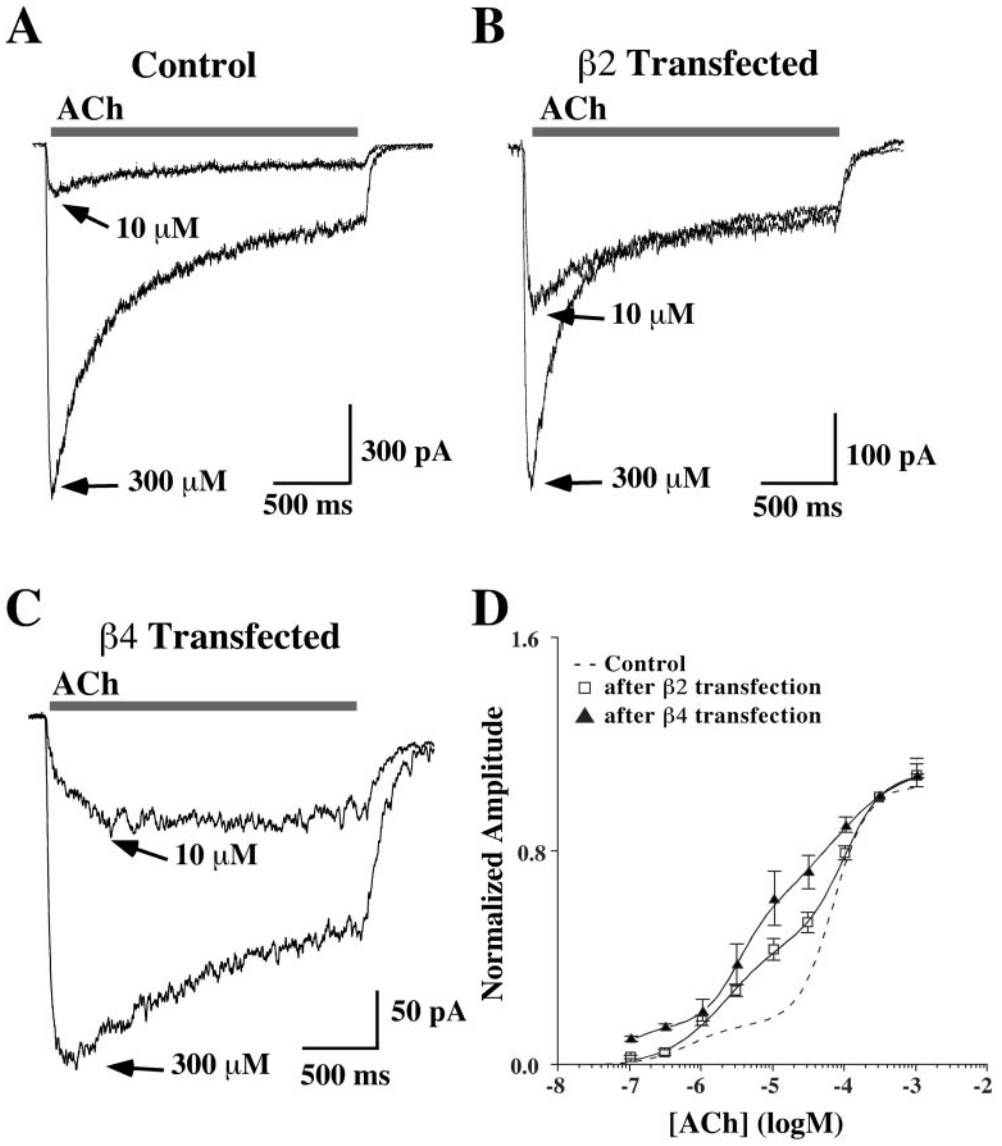

Fig. 4. Transient transfection of the human $\alpha4\beta2$ cell line with additional $\beta2$ subunit alters sensitivity to activation by ACh. Representative traces are shown for control $\alpha4\beta2$ cell line (A), for the $\alpha4\beta2$ cell line transiently transfected with either additional $\beta2$ (B) or additional $\beta4$ (C) subunit cDNA. D, the ACh concentration/response relationship for $\beta2$- or $\beta4$-transfected $\alpha4\beta2$ cells compared with the ACh concentration/response relationship for naive cells (dashed line). The EC_{50} values for $\beta2$-transfected cells were 2 ± 0.4 and 90 ± 17 μM ($n = 9$). For $\beta4$-transfected cells, the EC_{50} values were 0.1 ± 0.04, 3 ± 0.4, and 84 ± 20 μM ($n = 4$).

creased assembly into new nAChRs, which were more sensitive to activation by ACh (i.e., nAChR $\alpha4/\beta2$ stoichiometry preference was shifted).

To demonstrate that the altered functional properties were the direct result of incorporation of more $\beta2$ subunit after transfection, the $\alpha4\beta2$ cell line was transiently transfected with $\beta4$. $\beta4$ was chosen because it was an obvious substitute for $\beta2$ and because $\alpha4\beta4$ nAChRs have been shown to desensitize significantly more slowly than $\alpha4\beta2$ nAChRs, allowing for unambiguous detection of its incorporation (e.g., Chavez-Noriega et al., 1997). After $\beta4$ transfection, the currents exhibited slower activation and less desensitization than naive $\alpha4\beta2$ cells or $\beta2$-transfected $\alpha4\beta2$ cells (Fig. 4). The concentration/response relationship for ACh on $\beta4$-containing nAChRs revealed a three-component curve that reflected a complex mixture of nAChRs. The EC_{50} values were 0.1 ± 0.04, 3 ± 0.4, and 84 ± 20 μM. Thus, additional, transient transfection of the stable $\alpha4\beta2$ cell line with either $\beta2$ or $\beta4$ subunit cDNA resulted in nAChRs that reflect incorporation of the additional subunit.

Long-term Treatment with Nicotine or Culture at 29°C Increased the Relative Proportion of nAChRs That Had Higher Agonist Sensitivity. Recently, it was reported that long-term exposure of $\alpha4\beta2$ expressing HEK cells to low concentrations of nicotine increased the higher sensitivity fraction of nAChRs (Buisson and Bertrand, 2001). We tested this effect of nicotine on our $\alpha4\beta2$ cell line to compare it with that of overexpression of $\beta2$ subunit. The cells were incubated overnight with nicotine (0.5 or 5 μM) and then tested for their sensitivity to activation by ACh. In agreement with Buisson and Bertrand (2001), overnight treatment with nicotine increased the population of the nAChRs more sensitive to activation by ACh to about $32 \pm 5\%$ of the total. The EC_{50} values were 0.6 ± 0.4 μM and 84 ± 9 μM (Fig. 5). The effect of nicotine on these cells was similar to the result of transient transfection with additional $\beta2$ subunit. The similarity in effect between these two treatments suggested that both led to increased production of nAChRs with higher $\beta2$ content and that these nAChRs exhibited higher sensitivity to activation.

The effect of nicotine treatment on the single channel behavior of $\alpha4\beta2$ nAChRs was investigated also. Surprisingly, nicotine treatment reduced the complexity of channel activity recorded from these cells. The appearance of the lower conductance channel was infrequent (Fig. 6) but when present, it exhibited the same amplitude as the naive nAChRs. Additionally, the gating kinetics were more uniform, with open duration distributions that reflected a single kinetic species with a mean time constant of 2.2 ± 0.1 ms ($n = 7$; one patch required an additional time constant of 12 ms). One explanation for the channel behavior after nicotine treatment could be that the more sensitive nAChRs (i.e., lower conductance channels) are more susceptible to the phenomenon of rundown after long-term exposure to nicotine.

When cultured at reduced temperature, HEK cells transfected with neuronal nicotinic AChRs increased the number of nAChRs expressed (Cooper et al., 1999; Nelson et al., 2001), particularly on the cell surface (Nelson et al., 2001). We tested whether culture at 29°C altered the functional properties $\alpha4\beta2$ nAChRs. The effect of this treatment was much the same as both transiently transfecting the $\alpha4\beta2$ cells with additional $\beta2$ subunit or overnight exposure to nicotine. The cells had a larger fraction of nAChRs that exhibited greater sensitivity to activation by ACh. The EC_{50} values were 1.7 ± 0.3 μM and 58 ± 4 μM, accounting for $34 \pm 6\%$ and $67 \pm 6\%$ of the distribution, respectively. Thus, overnight culture at reduced temperature seems to favor formation of nAChRs that consist of more β subunits than nAChRs formed under normal culture conditions. This temperature-dependent effect might reflect reduced destruction of unassembled $\beta2$ subunits and/or increased assembly of $\beta2$ with $\alpha4$ subunits. Because the standard protocol for *X. laevis* oocytes is to maintain them at 16 to 19°C, oocytes that are injected with equal amounts of $\alpha4$ and $\beta2$ cDNAs or cRNAs would, presumably, tend to assemble nAChRs in a manner more like the HEK cells that were incubated overnight at 29°C. This also might explain the requirement for the high $\alpha4/\beta2$ injection ratios that are needed to form recombinant nAChRs with low sensitivity to activation (Zwart and Vijverberg, 1998).

Most $\alpha4\beta2$ nAChRs Expressed in Permanently Transfected HEK Cells Have an $(\alpha4)_3(\beta2)_2$ Subunit Stoichiometry. Although the preceding experiments were consistent with the existence of two nAChR stoichiometries

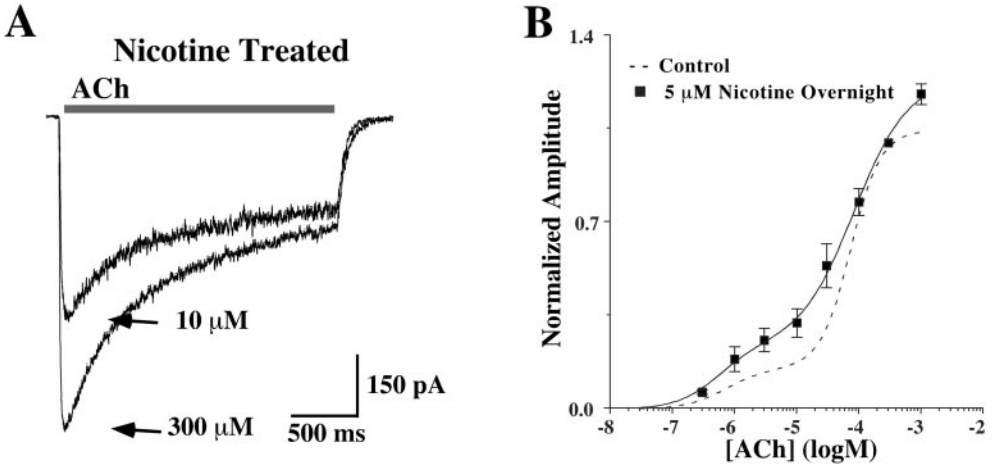

Fig. 5. Overnight incubation in nicotine causes an increase in the high-sensitivity population of nAChRs. A, representative traces illustrating the increased number of high sensitivity nAChRs activated by 10 μM ACh relative to the total nAChRs activated by 300 μM ACh. B, concentration/response relationship of the $\alpha4\beta2$ cell line treated overnight with nicotine (5 μM) compared with that of naive cells (dashed line). The EC_{50} values for the nicotine-treated cells were 0.6 ± 0.4 and 84 ± 9 μM ($n = 5$), which were much like the values determined for untreated cells (0.7 ± 0.4 and 74 ± 6 μM).

that have different functional properties, they were not definitive. As final confirmation, a direct measure of the number of α and β subunits present in assembled nAChRs was necessary. We chose to perform these measurements using an approach similar to that used to establish the subunit stoichiometry of chick $\alpha4\beta2$ nAChRs (Anand et al., 1991). This was achieved by metabolically labeling the proteins in the $\alpha4\beta2$ cell line with [^{35}S]methionine and then selecting for fully assembled nAChRs using sucrose gradient sedimentation. Fully assembled nAChRs were then collected from the gradients and purified further using mAb 295 (to the $\beta2$ subunit) coupled to Sepharose beads. Immunopurified nAChRs were denatured into individual subunit proteins and then resolved by SDS-PAGE. After autoradiography of the dried gel, the bands corresponding to $\alpha4$ and $\beta2$ subunits were cut from the gels and the amount of [^{35}S]methionine in each subunit was determined by liquid scintillation counting (Fig. 8). Because human $\alpha4$ and $\beta2$ subunits have the same number of methionine residues, the relative amount of each subunit was determined by direct comparison of the measured activity after subtraction of background. For $\alpha4\beta2$ cells that were grown under normal conditions, the $\alpha4/\beta2$ subunit ratio of fully assembled nAChRs was 1.5:1 (Table 1). Addition of nicotine (0.2 μM) along with [^{35}S]methionine increased the amount of [^{35}S]methionine that was incorporated into newly assembled nAChRs compared with untreated cells. This was similar to the increase in [^{3}H]epibatidine binding measured by radioimmunoassays for cells handled in parallel: binding increased 3.8-fold (from 1258 $\pm$ 81 fmol/mg of protein to 4790 $\pm$ 548 fmol/mg of protein) after nicotine (0.2 μM) incubation. Nicotine incubation also shifted the ratio of $\alpha4/\beta2$ subunits to 1:1, seemingly reflecting a nearly even mixture of the two populations of nAChRs after nicotine treatment. These results illustrated three important points: 1) in HEK cells, the predominant nAChRs formed had an $(\alpha4)_3(\beta2)_2$ stoichiometry, 2) long-term nicotine treatment increased the number of nAChRs measured in terms of both assembled protein and ligand binding, and 3) nicotine treatment lowered $\alpha4/\beta2$ ratio in fully assembled nAChRs, consistent with an increase in the proportion of those with $(\alpha4)_2(\beta2)_3$ stoichiometry. These findings paralleled the functional studies that showed the existence of a dual population of nAChRs. If for the functional studies, the more sensitive nAChR is assumed to be $(\alpha4)_2(\beta2)_3$, and the less sensitive nAChR is $(\alpha4)_3(\beta2)_2$, the α/β ratio predicted from the relative contributions of each species to the total current would be 1.4 for control and 1.2 for nicotine-treated cells. From the [^{35}S]methionine incorporation measurements (Table 1), the average α/β ratios were 1.5 and 1.0, respectively. The control values compared well between the two measurements, but the nicotine-treated values were less similar. The discrepancy in the value for nicotine-treated cells probably arose from the fact that functional nAChRs included pre-existing nAChRs as well as those formed during nicotine exposure, whereas metabolic labeling measured only nAChRs formed in the presence of nicotine. Thus the α/β ratio calculated from the functional studies overestimated the $(\alpha4)_3(\beta2)_2$ levels after nicotine treatment. Both functional and biochemical measures indicated that cells normally produced predominantly an $(\alpha4)_3(\beta2)_2$ nAChR, which was less sensitive to activation, whereas long-term exposure to nicotine favored formation of the $(\alpha4)_2(\beta2)_3$ nAChR, which was more sensitive.

TABLE 1
Subunit stoichiometry determined from [^{35}S]methionine incorporation

Experiment	Subunit Incorporated [^{35}S]Methionine Activity		Ratio (α/b)[a]
	$\alpha4$	$\beta2$	
	dpm		
1			
Control	2435	1524	1.6
Cultured in nicotine[b]	5230	5326	1.0
2			
Control	682	503	1.4
Cultured in nicotine	4061	3758	1.1

[a] human $\alpha4$ and $\beta2$ subunits contain the same number of methionine residues.
[b] Nicotine (0.2 μM) was added along with [^{35}S]methionine.

Discussion

We have found that human $\alpha4\beta2$ nAChRs in an HEK cell line coexist in two functional forms. The minority of the

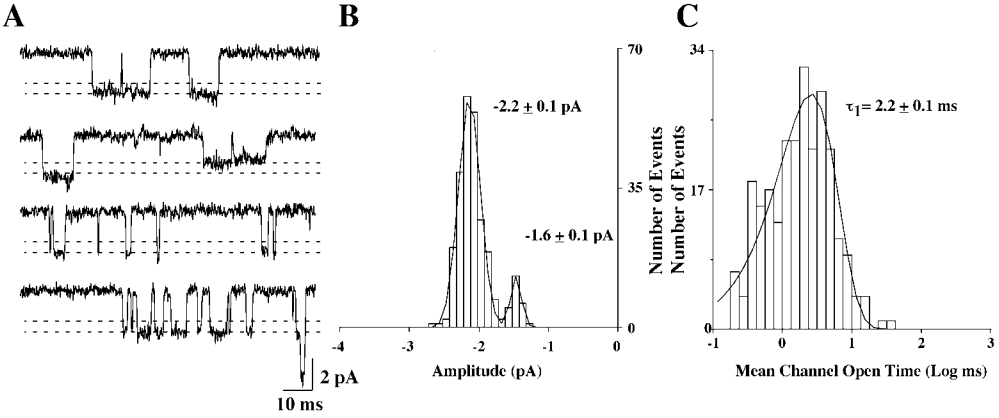

Fig. 6. Single-channel properties of $\alpha4\beta2$ nAChRs after overnight treatment with nicotine. $\alpha4\beta2$ expressing cells were treated overnight with nicotine (5 μM) before single channel recording. Outside out patches were exposed to equilibrium concentrations of ACh (0.05–10 μM). A, the representative channel activity was recorded in 500 nM ACh at -80 mV. B, a single conductance-type channel was overwhelmingly present under all conditions. It had a mean amplitude of -2.2 ± 0.3 pA. Very few openings were observed to a lower conductance level (the amplitude was -1.6 ± 0.1 pA) regardless of agonist concentration. C, the kinetic behavior of channels recorded after nicotine-treatment was very uniform. Single exponential functions having mean time constants of 2.2 $\pm$ 0.1 ms (n = 7) were most representative of the data. A single patch required an additional component having a time constant of 12 ms.

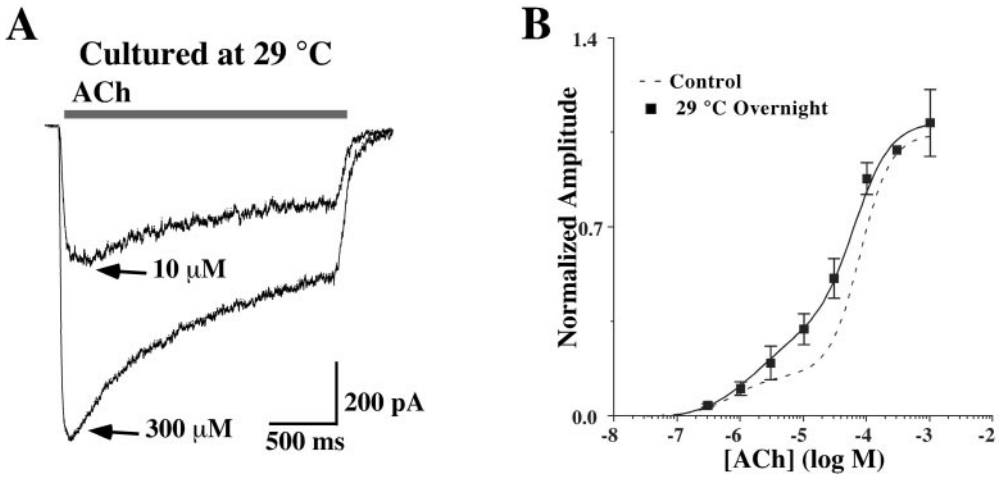

Fig. 7. Overnight culture at 29°C causes an increase in the high-sensitivity population of nAChRs. A, representative traces illustrating the increased number of high-sensitivity nAChRs activated by 10 μM ACh relative to the total nAChRs activated by 300 μM ACh. B, concentration/response relationship of the $\alpha4\beta2$ cell line cultured overnight at 29°C compared with that of naive cells (dashed line). The EC_{50} values for 29°C-treated cells were 1.7 ± 0.3 and 58 ± 4 μM (n = 5), which were very similar to the control values of 0.7 ± 0.4 and 74 ± 6 μM.

nAChRs exhibited properties that resembled nAChRs expressed in *X. laevis* oocytes (Kuryatov et al., 1997), whereas the majority were considerably less sensitive to activation and desensitized more quickly. Similar findings were reported for another human $\alpha4\beta2$ cell line (Buisson and Bertrand, 2001). More significantly, we found that $\alpha4\beta2$ nAChRs in the cell line preferentially exist in a $(\alpha4)_3(\beta2)_2$ stoichiometry. By increasing the amount of $\beta2$ subunit, by incubation time in nicotine, or by culture at reduced temperature, the magnitude of the minority fraction of functional nAChRs was increased. The properties of this fraction of nAChRs resembled those of $\alpha4\beta2$ nAChRs in *X. laevis* oocytes (Kuryatov et al., 1997). $\alpha4\beta2$ nAChRs with these properties, were shown to have an $(\alpha4)_2(\beta2)_3$ stoichiometry (Anand et al., 1991; Cooper et al., 1991; Whiting et al., 1991). Thus, human $\alpha4\beta2$ nAChRs assemble with two different stoichiometries, each exhibiting distinct functional properties. The fraction of $(\alpha4)_2(\beta2)_3$ stoichiometry formed was increased by factors that promoted the availability of $\beta2$ subunit for assembly.

Electrophysiological studies have been reported on several stably transfected HEK cell lines expressing rat and human $\alpha4\beta2$ nAChRs (Sabey et al., 1999; Buisson et al., 2000; Chavez-Noriega et al., 2000). In all cases, the predominant nAChRs exhibited low sensitivity to activation by ACh. In oocytes, it was reported that $\alpha4\beta2$ nAChRs could be manipulated to express high- and low-sensitivity forms by varying the relative amounts of each subunit (Zwart and Vijverberg, 1998). It was suggested that nAChR stoichiometry was altered, but no direct evidence was provided. Our $\alpha4\beta2$ cell line exhibited properties that were remarkably similar to those reported for $\alpha4\beta2$ nAChRs formed from $9\alpha/1\beta$ subunit ratios. By direct measurement, we have found that the preponderance of the less sensitive form of $\alpha4\beta2$ nAChRs corresponds to a novel $(\alpha4)_3(\beta2)_2$ stoichiometry in stably transfected HEK cells.

Buisson and Bertrand (2001) reported that an increase in the fraction of high-sensitivity $\alpha4\beta2$ nAChRs occurred after incubating transfected cells in nicotine. They speculated that the shift was caused by isomerization between existing pools of surface nAChRs (Buisson and Bertrand, 2001). Such a mechanism does not account for nicotine-induced increases

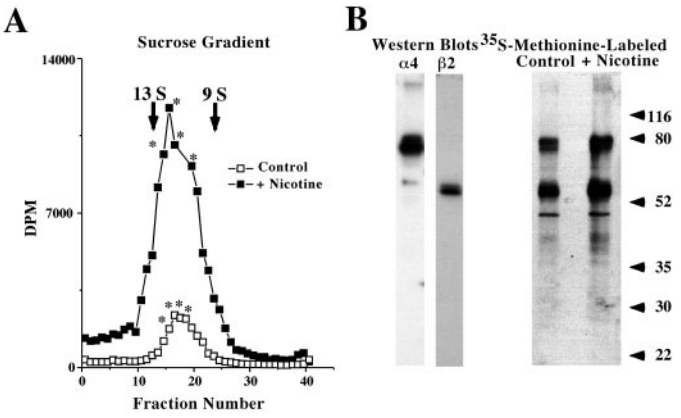

Fig. 8. Purification and separation of fully assembled, [^{35}S]methionine-labeled $\alpha4\beta2$ nAChRs expressed by stably transfected HEK cells. A, sucrose gradients of $\alpha4\beta2$ nAChRs in 2% Triton X-100 extracts from the $\alpha4\beta2$ cell line without and with overnight treatment with nicotine (0.2 μM) were used to isolate fully assembled nAChRs. Extracts for each condition were loaded on 5 to 20% sucrose gradients as described under *Materials and Methods*. [^{35}S]Methionine-labeled nAChRs were run in parallel. Fractions were collected and then tested on mAb 295-coated microwells with [^{3}H]epibatidine to quantitate nAChRs. The size of *T. californica* nAChR dimers and monomers are indicated. The location of the peak of the [^{3}H]epibatitine binding was consistent with fully assembled pentameric $\alpha4\beta2$ nAChRs. The four fractions corresponding to the peak (denoted by asterisks) of the gradients were chosen for further immunopurification. These fractions were pooled and nAChRs were isolated with mAb 295-coupled Sepharose. After thorough washing, the isolated nAChRs were eluted with 3% SDS sample buffer and then separated by SDS-PAGE on 10% gels. B, Western blots for $\alpha4$ or $\beta2$ subunits are shown for the same gel that was used for separation of [^{35}S]methionine-labeled control or nicotine–up-regulated nAChRs. The part of the gel with the [^{35}S]methionine-labeled samples was dried for autoradiography. The bands corresponding to $\alpha4$ and $\beta2$ subunits were cut from the gels and the amount of ^{35}S activity was measured by liquid scintillation counting. The values are shown in Table 1.

in nAChR numbers observed in cell culture and in brain (Benwell et al., 1988; Wonnacott, 1990; Marks et al., 1992; Peng et al., 1994; Gopalakrishnan et al., 1996; Whiteaker et al., 1998). It has been well documented that nicotine-induced up-regulation of nAChRs occurs independently of protein synthesis (Peng et al., 1994; Wang et al., 1998) and that up-regulation results from increased assembly (Wang et al.,

1998) in combination with reduced turnover of existing surface nAChRs (Peng et al., 1994). Nicotine-induced change in stoichiometry is compatible with these observations. Additionally, different stoichiometries are more likely to account for different conductance properties of each nAChR type that we and others (Buisson and Bertrand, 2001) observed, because the change in subunit composition would change the amino acid residues lining the lumen of the channel. Our data showed that the larger conductance, shorter gating channel correlated better with the less sensitive nAChR form, because it was the predominant channel type observed in patches from our $\alpha4\beta2$ cell line. This was opposite the conclusion of Buisson and Bertrand (2001). By our account, nAChRs that contained three $\alpha4$ subunits had higher conductance than those with three $\beta2$ subunits. This observation correlated nicely with the single channel study that helped to establish the $(\alpha4)_2(\beta2)_3$ stoichiometry for chick nAChRs (Cooper et al., 1991). The channel forming M2 transmembrane regions of human and chick $\alpha4$ and $\beta2$ subunits are identical. Single channel currents for chick $\alpha4\beta2$ nAChRs [with the $(\alpha4)_2(\beta2)_3$ stoichiometry] had a homogeneous population of channels with a conductance [20 pS (Cooper et al., 1991)] that was similar to the lower conductance infrequently observed from our cell line (21-pS chord conductance). A residue that differed between $\alpha4$ and $\beta2$ in the channel region was found to alter channel conductance. When this residue was mutated in $\beta2$ to match the residue at the same position in $\alpha4$, the channel conductance increased. When mutant nAChRs contained three '$\alpha4$-like' residues, the channel chord conductance was 27 pS (Cooper et al., 1991). In our $\alpha4\beta2$ cell line, the predominant channel type had a chord conductance of 29 pS that reflected three '$\alpha4$-like' residues in $(\alpha4)_3(\beta2)_2$ nAChRs. Three other residues differ in M2 between $\alpha4$ and $\beta2$, but $\beta2$ to $\alpha4$ substitutions would probably not alter channel conductance at these positions (Karlin and Akabas, 1995). The similarities between human and chick nAChR channel conductances provided evidence to support our belief that the larger conductance channel was the $(\alpha4)_3(\beta2)_2$ nAChR that had low sensitivity to activation.

Consider the possibility that in brain, each of the $\alpha4\beta2$ nAChR stoichiometries might serve a different functional role. Muscle nAChRs provide an example of different nAChR subunit compositions serving different functional roles. With innervation at the neuromuscular junction, the functional properties of nAChRs are changed by a switch from nAChRs that contain γ subunits to those that contain ϵ subunits (Sanes and Lichtman, 2001). Nonjunctional nAChRs in immature muscle exist at low density and are optimized to respond to diffuse transmitter release with long channel openings during development. Postsynaptic nAChRs in mature muscle are optimized to respond to high ACh concentrations with relatively low affinity and brief channel openings to accurately transmit rapid impulse patterns. In the brain, most $\alpha4\beta2$ nAChRs act presynaptically or preterminally to modulate transmitter release (Wonnacott, 1997). In some cases, this modulation might occur by 'volume transmission,' whereby diffusion of transmitter from adjacent synapses activates these nAChRs (Zoli et al., 1999). In such cases, more sensitive $(\alpha4)_2(\beta2)_3$ nAChRs would better serve such a role. However, where $\alpha4\beta2$ nAChRs serve a traditional postsynaptic role in which complex signals must be rapidly and faithfully transmitted, less sensitive $(\alpha4)_3(\beta2)_2$ nAChRs would be optimal.

Long-term nicotine exposure favors formation of more sensitive $\alpha4\beta2$ nAChRs. By increasing both nAChR numbers and their sensitivity to activation, the response to ACh and nicotine should be increased. These changes could reflect a form of plasticity that is activated in response to the desensitizing effects of nicotine in an attempt to normalize nicotinic signaling. Because over-expression of $\beta2$ causes a shift in functional properties that resembles the effect of nicotine, this subunit might be key to the process. $\beta2$ might be disfavored from assembling with intermediates perhaps through limiting its availability by targeted degradation. $\alpha4$-$\beta2$ dimers must form to create agonist-binding sites. These might assemble into $\alpha4\beta2\alpha4\beta2$ tetramers. At this point, the fifth subunit to assemble would occupy the position analogous to the $\beta1$ position of muscle-type nAChRs. The subunit at this position does not contribute to an agonist binding site or change nAChR ligand binding affinity, but influences gating kinetics and channel conductance (Gerzanich et al., 1998). When $\beta2$ subunit is limiting, we propose that $\alpha4$ assembles in this position to produce the $(\alpha4)_3(\beta2)_2$ pentamer. With sufficient $\beta2$ subunit present, as when additional subunit was transiently transfected, we propose that more $\beta2$ assembles to complete the $(\alpha4)_2(\beta2)_3$ pentamer in this position. This position might be occupied by $\alpha5$, as in 26% of rat brain nAChRs (Gerzanich et al., 1998), suggesting that changes in both nAChR composition and stoichiometry at this site can be used to regulate $\alpha4\beta2$ nAChR function. Long-term exposure of the nAChR to nicotine or culturing at 29°C acts to shift the relative amounts of each population of nAChRs, also. These conditions might provide a signal to reduce constitutive degradation of $\beta2$ subunits. Constitutive degradation of muscle nAChR $\alpha1$ subunits has been demonstrated in which most are degraded before assembly (Merlie and Lindstrom, 1983). Another trafficking molecule might also be involved that shields or exposes subunit regions that are important in assembly.

What are the implications of these finding on long-term exposure of $\alpha4\beta2$ nAChRs to nicotine in the brain? It is well known that exposure to nicotine (at concentrations that are readily achieved by smokers) causes the number of nAChRs in brain to increase (Benwell et al., 1988; Wonnacott, 1990; Marks et al., 1992). Here, we show that nicotine exposure also favors the formation of nAChRs with increased sensitivity because of a stoichiometry with higher $\beta2$ content. In cells coexpressing $\beta2$ and $\beta4$ containing nAChRs (IMR-32 and SH-SY5Y), nicotine-induced up-regulation can be directly attributed to an increase in $\beta2$-containing nAChRs (Wang et al., 1998; Nelson et al., 2001). In HEK cells expressing $\alpha3\beta2$ nAChRs, limited numbers of nAChRs are expressed on the surface, but culturing in nicotine or at 29°C results in large numbers of surface nAChR (Nelson et al., 2001). All of these observations implicate tight regulation of the $\beta2$ subunit in nAChR expression. An additional signal might be required for $\beta2$ to be made available to associate with nAChR assembly intermediates. Its identity and any other players involved in the process remain to be determined. Because nicotine influences this process, the control of stoichiometric preference could be important in addiction.

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