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"Orphan" α6 Nicotinic AChR Subunit Can Form a Functional Heteromeric Acetylcholine Receptor

V. GERZANICH, A. KURYATOV, R. ANAND, and J. LINDSTROM

Departments of Neuroscience (V.G., A.K., J.L.) and Pharmacology (R.A., J.L.), University of Pennsylvania Medical School, Philadelphia, Pennsylvania, 19104-6074

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SUMMARY

Previously, a rat brain cDNA was reported that was designated α6 because of its homology with nicotinic acetylcholine receptor (AChR) α subunits, being especially similar to α3, but no acetylcholine-gated cation channels were detected when it was expressed in *Xenopus laevis* oocytes alone or in combination with other known rat AChR subunits. We cloned chicken α6 and human β4 AChR subunits and tested for acetylcholine-gated cation channels with α6 by expression in *X. laevis* oocytes alone or in pairwise combination with chicken α3, β2, or β4 or with human α3, β2, or β4 AChR subunits. Chicken α6 formed detectable functional AChRs only when expressed together with the human β4 subunit. The α6β4 AChR-mediated currents show strong inward rectification and dependence on extracellular Ca^{2+}. It exhibited a distinct pharmacological profile with an EC_{50} value of 28 μM for acetylcholine, 24 nM for (+)-epibatidine, 6.6 μM cytisine, and 15 μM 1,1-dimethyl-4-phenylpiperazinium. Both cytisine and 1,1-dimethyl-4-phenylpiperazinium behaved as partial (~30%) agonists. Remarkably, nicotine (EC_{50} = 22 μM) was an even weaker partial agonist (~18%) and had a relatively long-lasting inhibitory effect. Coexpression of the previously cloned rat α6 subunit with the human the β4 subunit also resulted in functional α6β4 AChRs with properties resembling those of the chicken/human α6β4 AChRs. Therefore, α6 can function as part of AChRs with unusual pharmacological properties.

The family of nicotinic AChRs consists of subunits termed α1–α9, β1–β4, γ, δ, and ε. All of these subunits, except the "orphans" α6 and β3, have been shown to function as components of ACh-gated cation channels either as homomers or in combination with one or more other AChR subunits (1–4).

Despite the fact that the cDNA sequence of the α6 AChR subunits has been known for several years (1), little data are available regarding the properties of this subunit. These data are limited to mentions in reviews of cDNA sequences of rat and chick subunits (4, 5), a recently submitted cDNA sequence of the human subunit (6), and published abstracts concerning α6 mRNA distribution in rat brain and cochlea (7, 8). High levels of sequence homology between α6 and α3 AChR subunits (>75%) and other features common to all functional nicotinic subunits (5) indicated that α6 subunits should form functional AChRs serving as a ligand-binding subunit. However, difficulties in obtaining functional AChRs formed exclusively or partially by this subunit have suggested that α6 may serve a structural role in combination with other α and β subunits as α5 does (9, 10), that it may require the presence of another subunit yet to be identified to function as an AChR, or that α6 may function as a receptor for some other ligand yet to be identified.

We show that α6 subunits can, in fact, act as ligand-binding subunits in functional AChRs. Here, we report cloning of cDNAs encoding α6 AChR subunits from a chicken cochlea library and a β4 AChR subunit from a human neuroblastoma SH-SY5Y library. When expressed in *Xenopus laevis* oocytes together with the human β4 subunit, chicken or rat α6 AChR subunits form nicotinic ligand-gated cation channels with novel pharmacological properties. Identification of this new subtype of AChR may prove important for understanding the pharmacological properties of centrally acting cholinergic ligands with possible therapeutic significance.

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Materials and Methods

Isolation of chicken α6 and human β4 cDNA clones. A lambda Zap II cDNA library (~9×10^6 plaques) using chick cochlear

ABBREVIATIONS: AChR, acetylcholine receptor; ACh, acetylcholine; DMPP, 1,1-dimethyl-4-phenylpiperazinium iodide; HEPES, 4-(2-hydroxyethyl)-1 piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate; SSPE, standard saline/phosphate/EDTA; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

mRNA, constructed by Stratagene (La Jolla, CA), was kindly provided by Dr. Paul Fuchs (Johns Hopkins University, Baltimore, MD). The chicken α6 cDNA was obtained by screening ~5 × 10^5 plaques from this library at low stringency using previously cloned chick α3, α4, α5, α7, α8, and β2, rat α2, β3, and β4, and human α1, β1, γ, and δ full-length or nearly full-length cDNA probes. The human β4 cDNA clone was obtained by screening a previously described lambda Zap II cDNA library (11) constructed using mRNA isolated from the human neuroblastoma cell line SH-SY5Y with human α3, α4, α5, α7, β2, and β4 and rat α2, β3, and α6 full-length or nearly full-length cDNA probes. All rat AChR subunit cDNAs used were kindly provided by Drs. Stephen Heinemann and Jim Boulter (Salk Institute, San Diego, CA). A human α5 cDNA clone and a partial β4 cDNA clone were kindly provided by Dr. Francesco Clementi (University Degli Studi di Milano, Milano, Italy). The low-stringency screens were performed by hybridizing the membranes overnight at 42° in 30% formamide, 5 × SSPE (1× = 0.18 M NaCl, 0.01 M sodium phosphate, pH 7.4, 1 mM EDTA), 1% SDS, 5 × Denhardt's solution (1 × Denhardt's = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 150 mg/ml sonicated salmon sperm DNA. The membranes were washed successively in 5 × SSPE and 0.1% SDS, at room temperature, and in 2 × SSPE and 0.1% SDS at 42° for 30 min each. Autoradiography was performed by exposing the membranes to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for 1–2 days. Clones thus isolated were purified and subjected to dideoxy sequencing using the Sequenase 2 Kit (United States Biochemical, Cleveland, OH). The identity of each clone was then determined by searching for their sequences against the sequences contained in the National Center for Biotechnology Information database using the Blast suite of programs (12). Alignment of the peptide sequences was performed using MacVector (Eastman Kodak) and The Wisconsin Package (Genetics Computer Group, Madison, WI).

Other cDNAs. Chicken β2 cDNA was described previously (13). Rat α6 was kindly provided by Drs. Stephen Heinemann and Jim Boulter. Human α3 was cloned from a human brain library. Chicken α3, β2, and β4 cDNAs were obtained through the generosity of Dr. Marc Ballivet (Department of Biochemistry, University of Geneva, Geneva, Switzerland).

Expression of AChR subunits in *X. laevis* oocytes. Chicken and human cDNAs were cloned into a modified SP64T expression vector (14) using standard DNA cloning procedures. cRNA was synthesized *in vitro* using the Megascript kit (Ambion, Austin, TX). Oocytes were defolliculated and injected with either 15 or 100 ng of cRNA per oocyte. Chicken α3, β2, and β4 subunits were expressed by nuclear injections of 2 ng of genomic DNAs per oocyte. The oocytes were incubated in semisterile conditions at 18° in saline solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.6) containing 50% Leibovitz-15 media (GIBCO/BRL, Gaithersburg, MD) buffered to pH 7.4 with 10 mM HEPES. Oocytes were incubated at 18° for 3–6 days before use.

Electrophysiological procedures and drug application. Currents in oocytes were measured using a standard two-microelectrode voltage clamp amplifier (oocyte clamp OC-725; Warner Instrument, Hamden, CT). Electrodes were filled with 3 M KCl and had resistances of 0.5–1.0 MΩ for the voltage electrode and 0.4–0.6 M½ for the current electrode. All records were digitized (MacLab/2*e* interface and Scope software; AD Instruments, Castle Hill, Australia), stored on a Macintosh IIcx computer (Apple Computer, Cupertino, CA) and analyzed using AXOGRAPH software (Axon Instruments, Burlingame, CA).

The recording chamber was continually perfused at a flow rate of 10 ml/min with a saline solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6. In most experiments, 1 μM atropine was added to the solution to suppress endogenous muscarinic responses in *X. laevis* oocytes. Application of the agonists was performed as described in detail previously (15). All agonists were applied by means of a set of 2-mm glass tubes directed on the animal pole of the oocyte. Application was achieved by manual unclamping and clamping of a flexible tube connected to the syringe with the test solution. Typical delay between beginning of the application and first deflection of the induced current was approximately 0.3 sec.

The Hill equation was fitted to the concentration-response relationships using a nonlinear least-squares error curve fit method (KaleidaGraph software; Abelbeck/Synergy, Reading, PA): $I(x) = I_{max}[x^n/(x^n + EC_{50}{}^n)]$, where $I(x)$ is current measured at the agonist concentration x, I_{max} is the maximal current response at the saturating agonist concentration, EC_{50} is the agonist concentration required for the half-maximal response, and n is the Hill coefficient.

Drugs used. Epibatidine (oxalate salt) was synthesized at Merck Sharp & Dohme Research Laboratories (Essex, UK) and was a gift from Stephen Fletcher (16). (−)-Nicotine tartrate, cytisine, DMPP, and ACh chloride were obtained from Sigma (St. Louis, IL).

Results

Isolation and primary structure of the chick α6 AChR subunit. A chicken cochlea cDNA library was screened at low-stringency hybridization conditions using a cocktail of AChR subunit cDNA probes. In addition to identifying cDNAs for α4, α7, β2, and β4 subunits, one 2233-bp cDNA was identified that closely resembled the previously cloned rat α6 AChR subunit (Genbank accession number L08277) as shown in Fig. 1A. The cDNA encodes a predicted mature protein of 464 amino acid residues, preceded by a leader peptide of 30 residues. The sequence contains a cysteine pair homologous to α1 128 and 142 and a second cysteine pair homologous to α1 192 and 193, which identify it as an α subunit. The sequence also contains the four putative transmembrane sequences typical of all AChR subunits. The predicted amino-acid sequence of mature chicken α6 is 86%, identical to that of rat α6 and 88% identical to human α6 (Fig. 1). Most sequence differences occur in the putative large cytoplasmic loop between transmembrane domains three and four, which is the region that typically shows the most variation between species of AChR subunits. Chicken and rat α6 subunits are identical both in parts of the sequence believed to contribute to the ACh binding site (e.g., amino acids of the mature protein 180–200) and in the lining of the cation channel (i.e., amino acids 200–250); therefore, α6 subunits from the two species would be expected to have both similar ligand binding and cation channel characteristics.

Isolation and primary structure of the human β4 AChR subunit. The deduced amino acid sequence of the human β4 AChR subunit obtained by low-stringency screening of a SH-SY5Y cDNA library is compared with the rat and chicken β4 subunits in Fig. 1B. Protein encoded by the human β4 cDNA has substantial identity with sequences of the chick (75%) and rat (85%) β4 AChRs. A leader peptide, four hydrophobic putative transmembrane domains, and two highly conserved cysteine residues at positions 153 and 167 are characteristic of all β-type subunits. Designation of this clone as a β subunit was confirmed by functional tests in which it was shown to form functional AChRs when expressed in combination with human α3 subunits (Fig. 2). An incomplete, nonfunctional human β4 AChR cDNA was published earlier (17).

Functional expression of the chicken α6 AChR subunit. Multiple attempts to detect functional nicotinic AChRs in *X. laevis* oocytes injected with *in vitro* synthesized chicken α6 transcripts either alone or after prior nuclear injection of

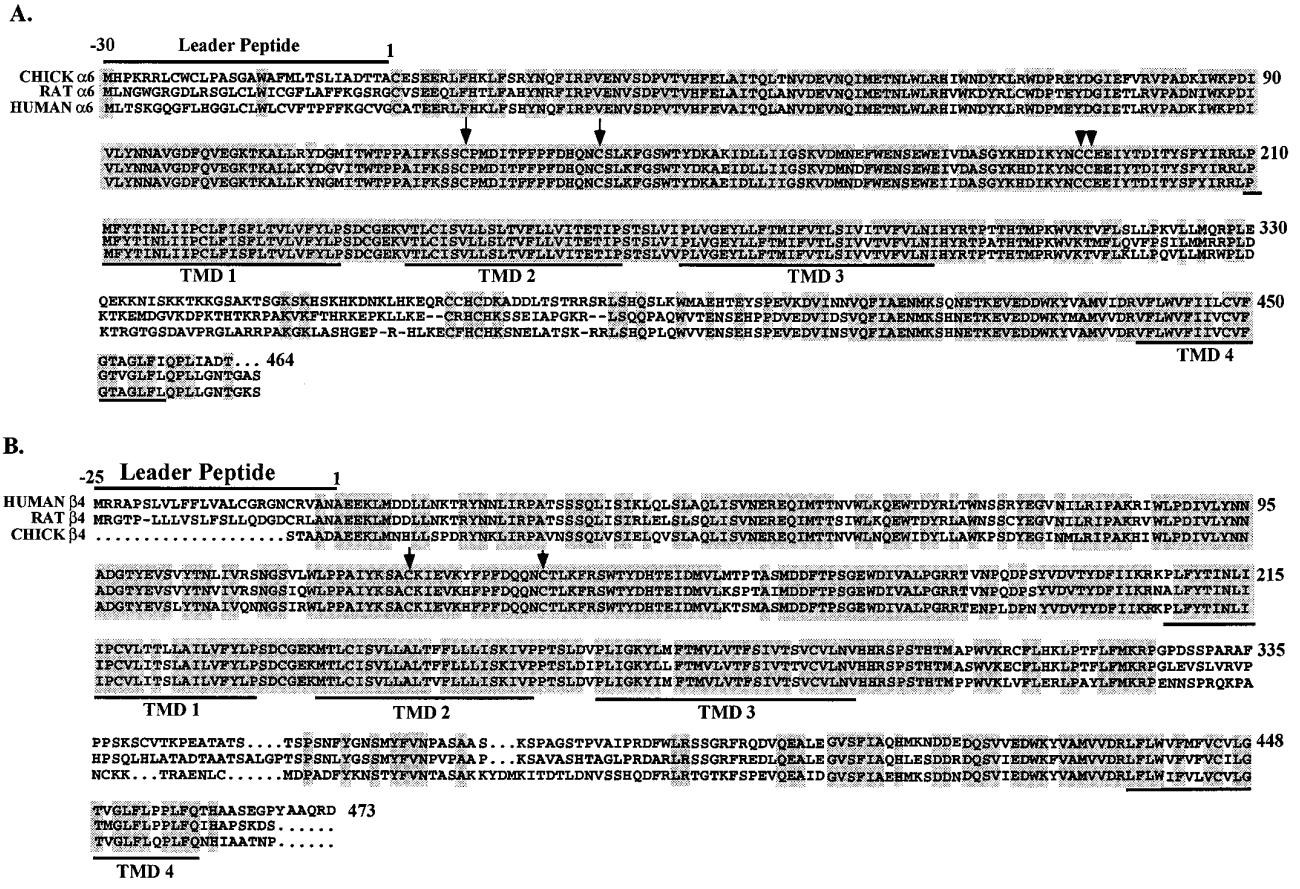

Fig. 1. A, amino acid sequence alignment of chicken, rat, and human AChR α6 subunits. B, amino acid sequence alignment of the human, rat, and chicken β4 AChR subunits. *Shading*, identical residues. Gaps were introduced in the sequences to maximize homologies. *Arrows*, positions of the predicted leader peptide, cysteine residues conserved in all AChR subunits; *arrowheads*, cysteine residues conserved in all α subunits; *TMD1–TMD4*, putative transmembrane domains.

chick β2, β3, or β4 cRNAs were unsuccessful (Fig. 2). Parallel control experiments with pair-wise coexpression of β2 or β4 together with α3 confirmed the functionality of these cDNAs. Additionally, no functional AChRs were detected when oocytes were injected with mixtures containing 15–100 ng per oocyte of both *in vitro* synthesized α6 and β4 transcripts (these were obtained by linearizing the chicken α3 cDNA recloned into the *Not*I site of the pBS SK(−) vector). Functionality of β4 cRNA was confirmed by successful coexpression with the chicken α4 AChR subunit. In general, maximal currents resulting from expression of the chicken α3β2 (Fig. 3), α3β4, and α4β4 subunit combinations in oocytes clamped at −70 mV did not exceed 500 nA.

We continued to search for α6 function by coexpressing this subunit with human α3, β2, or β4 subunits (Fig. 2). Only oocytes injected with both α6 and β4 subunits produced detectable responses to ACh. These responses could be detected only more than 72 hr after cRNA injection and only in about 50% of the injected oocytes. Expression typically reached a plateau on day 5 or 6 after cRNA injection. Peak amplitudes of the currents in oocytes clamped at −100 mV ranged from 5 to 250 nA; most responses were lower than 100 nA. By contrast, currents mediated by human α3β2 and α3β4 AChRs were much larger (3–5 μA). α6β4 AChR currents usually did not show significant rundown, even after 2 hr of recording.

Oocytes expressing α6β4 AChRs responded to ACh in a concentration-dependent manner (Fig. 3) with a EC_{50} value of 28 μM and a Hill coefficient greater than 1. Maximal responses were obtained using 300 μM ACh. Further increase of the concentration resulted in decreased peak amplitude. Presence of "rebound" currents after termination of the application of high ACh concentrations (>100 μM) indicated a possible channel block effect of this agonist. At all concentrations, responses exhibited relatively slow activation and desensitization kinetics.

α6β4 AChR-mediated responses exhibited a nonlinear voltage dependence typical of neuronal nicotinic AChRs. Currents reversed at -17 ± 3 mV ($n = 5$). Strong inward rectification was observed not only at positive potentials but also at negative potentials at which the current/voltage dependence significantly deviated from linearity (Fig. 3). "Rebound" current upon agonist removal (Fig. 3) was attributed to recovery from agonist-mediated channel blockage. It correlated with holding potential, being more prominent at more negative potentials.

Amplitude of the α6β4-mediated currents was dramatically attenuated [to 33 + 5% ($n = 5$)] upon removal of Ca^{2+} ions from the external solution (Fig. 3). Voltage dependence of the resulting responses showed less inward rectification at both positive and negative potentials (Fig. 3). Reversal potential in low Ca^{2+} had a tendency to shift to the more positive potentials. More precise estimation of this shift was

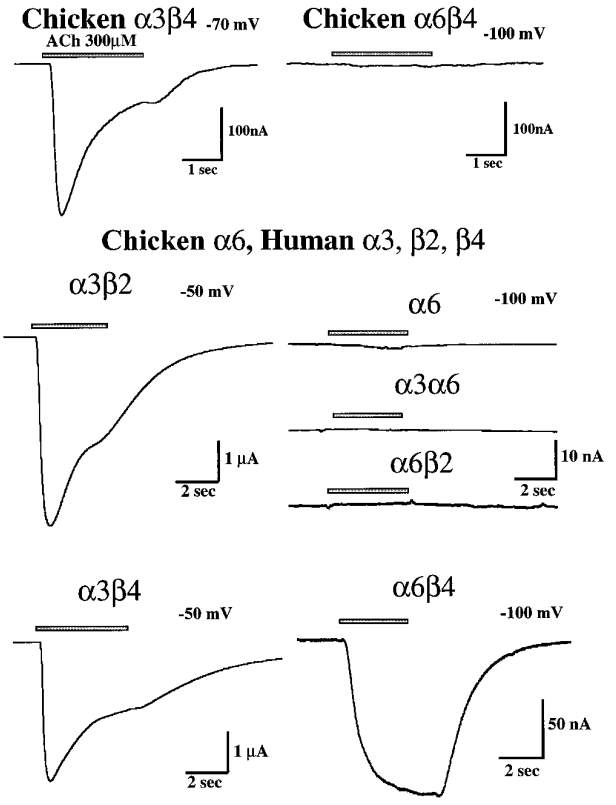

Fig. 2. Chicken α6 AChR subunit mediates detectable function only in combination with the human β4 AChR subunit. *Top*, currents induced by 300 μM ACh applied as indicated by the bars to oocytes expressing chicken AChRs: *left*, α3β4; *right*, α6β4. *Middle and bottom*, responses were recorded from oocytes expressing human α3 and β2 (*middle left*) or α3 and β4 (*bottom left*) AChR subunits. Chicken α6 subunits expressed alone or with human α3, β2 (*middle right*) AChR subunits did not result in detectable responses. However, chicken α6 coexpressed with human β4 (*bottom right*) consistently produced responses. Currents were obtained 3–5 days after nuclear injection of the cDNAs (chicken α3 and β4) and cRNAs (chicken α6 and human α3, β2, β4). Holding potentials are indicated above the traces in each panel.

precluded by the low signal-to-noise ratio at potentials close to 0 mV.

The nicotinic nature of the responses observed in oocytes expressing α6β4 AChRs was confirmed through showing blockage by classical nicotinic antagonists. Curare at 20 μM completely inhibited responses when coapplied with ACh (Fig. 4). Inhibition by curare showed relatively fast on and off rates. Half-time for inhibition of the response by coapplication of curare with ACh was approximately 2 sec ($n = 4$), whereas recovery of the ACh response followed the more rapid time course of the solution exchange in the perfusion system used (half-time approximately 0.2 sec). Mecamylamine (10 μM; Fig. 4) effectively inhibited α6β4 AChR-mediated currents with much slower washout than curare. When either mecamylamine (at 10 μM) or hexamethonium (at 30 μM) were coapplied with ACh, it took more than 30 sec to reach steady-state inhibition and the response recovered fully only after 8–10 min of washing with test ACh responses every 2 min. No attenuation of the responses was observed after 1-hr incubation in 200 nM α-bungarotoxin.

A novel pharmacological profile of α6β4 AChRs was revealed by using various nicotinic agonists. Nicotine seemed to behave as a very poor partial agonist with maximal currents at 18% of the current induced by a saturating concentration of ACh (Fig. 4). Moreover, at high concentrations (>100 μM), nicotine behaved as a long-lasting antagonist, inhibiting currents induced by subsequent applications of ACh (Fig. 4). Partial recovery of the response from the inhibition induced by a 4-sec application of nicotine was observed only after 10–15 min. Both cytisine and DMPP also behaved as partial agonists, with 36% and 27% efficacy, respectively, relative to ACh (Fig. 4). The (+)-enantiomer of the synthetic alkaloid epibatidine behaved as a full agonist and exhibited extremely high potency for α6β4 AChRs with an EC_{50} of 24 nM (Fig. 4). EC_{50} values for the agonists tested are listed in Table 1. The rank order of potency of nicotinic agonists for the activation of the α6β4 AChRs was epibatidine≫cytisine>DMPP≥nicotine>ACh.

Functional expression of the rat α6 AChR subunit. Coexpression of the rat α6 subunit, along with the human β4 subunit, resulted in appearance of ACh-induced inward currents that resembled those observed for chick α6 human β4 AChRs (Fig. 5), as expected from the sequence identities of α6 subunits from the two species in both the regions believed to govern ACh binding and channel function (Fig. 1). Currents reversed around −15 mV, and the current/voltage showed strong inward rectification. Rat α6 human β4 AChR-mediated currents were inhibited by curare ($n = 4$) (Fig. 5). ACh was slightly less potent on rat α6 AChRs ($EC_{50} = 37$ μM) compared with chicken α6 AChRs. Nicotine, cytisine, and DMPP behaved as partial agonists with 52%, 30%, and 19% efficacy, respectively, compared with ACh (Fig. 5).

Discussion

α6 subunits are most closely related in sequence to α3 subunits (~75% amino acid sequence identity) (4, 5). In contrast to α3 subunits, coexpression of the chick α6 subunit with either chick or human β2 subunits did not yield detectable AChR function. It is not clear whether this is an intrinsic attribute of the α6 subunit that discriminates between assembly properties of the α3 and α6 subunit or if it is due to technical shortcomings. Maximal currents detected for the α3β4 combination were almost two orders of magnitude higher compared with the α6β4 combination. This might indicate that α6 and α3 subunits differ in assembly affinity for the β4 subunit and/or, possibly, additional subunits are required for more effective functional expression of the α6 subunit. On the other hand, the relatively small amplitudes of the α6β4-mediated currents and failure to detect function on coexpression with the β2 subunit could reflect levels of α6 expression insufficient for functional detection. We have no independent measure of the quality of the α6 cRNA or the amount of α6 protein produced. The low overall levels of α6β4 AChR function detected also may reflect inefficient processing or assembly of α6 in *X. laevis* oocytes compared with the neurons in which α6 might normally be found. There is, as yet, no characterization of α6 protein or function in neurons with which to compare the properties of these cDNAs expressed in *X. laevis* oocytes.

Despite the very high level of homology between α6 and α3 AChR subunits, they exhibit significant differences in their functional properties. As discussed above, in addition to differences in expression levels and coexpression with β2 and β4 subunits, α6β4 AChRs exhibit significantly different phar-

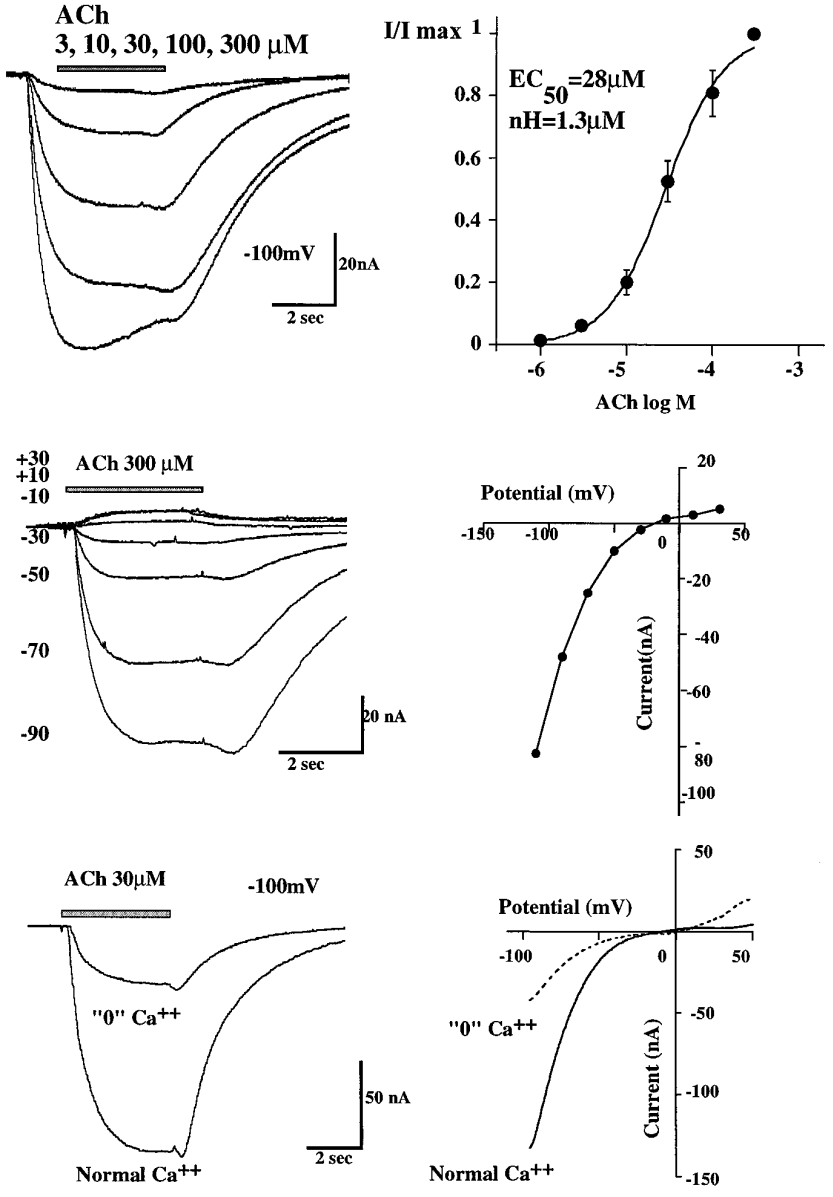

Fig. 3. Chicken α6 human β4 AChRs, activation by acetylcholine and channel properties. *Top left*, typical currents induced by application of increasing concentrations of ACh on oocytes coinjected with chick α6 and human β4 cRNAs. *Top right*, a plot of the dose-response curve for ACh obtained from five oocytes held at −100 mV. Currents were normalized to the maximal current (at 300 μM ACh). Further increase of the ACh concentration induced currents with lower amplitude, suggesting the occurrence of channel blockage by the ACh. Recordings were performed 5 days after injection on an oocyte voltage clamped at −100 mV. Data (mean ± standard error) were fitted with the Hill equation. *Middle*, potential dependence of chick α6 human β4 AChRs; *left*, the family of currents induced by application of 300 μM ACh to oocytes expressing α6β4 held at different potentials from −90 to +30 mV is shown at 20-mV increments; *right*, a plot of the peak current versus holding potential. The current reverses at −18 mV. *Bottom*, dependence of the α6β4 AChR-mediated current on extracellular Ca^{2+} concentration; *left*, currents induced at normal Ca^{2+} concentration (1.8 mM) and after removal of the Ca^{2+} ions from the perfusion solution (5 mM EGTA was added to chelate possible Ca^{2+} contamination); *right*, voltage dependence of the α6β4 AChR-mediated current in control and in the "0" Ca^{2+} solution. Voltage ramps were obtained by continuously changing the holding potential from −100 to +50 mV more than 2 sec. *Traces* are the result of the subtraction of the currents obtained in the absence of the drug from currents during application of 30 μM ACh.

macological properties compared with either chicken or human recombinant α3β4 AChRs (Table 1) (16, 18). Therefore, both chicken α6 human β4 AChRs and rat α6 human β4 AChRs retain a unique agonist profile with nicotine, cytisine, and DMPP as poor partial agonists. In contrast, nicotine was shown to be a full agonist for chicken, rat, and human α3β4 AChRs (Table 1) (16, 18, 19). Nicotine behaves virtually as an antagonist on α6β4 AChRs. The time course of the nicotine-induced current does not indicate either accelerated desensitization or channel block of the AChRs. Inhibition of α6β4 AChRs by nicotine strongly resembles the action of nicotine previously described for chicken α3β2 AChRs but not for α3β4 AChRs (18). After extensive studies of this phenomenon, these authors concluded that nicotine behaves as a competitive antagonist at low concentrations, but as a partial agonist at higher concentrations, and that its inhibitory action is at least in part contributed by the β2 subunit. Nicotine also behaves as an antagonist for rat homomeric α9 AChRs (20). Epibatidine exhibits extremely high potency for α6β4 AChRs. High potency of this alkaloid also was described recently for all recombinant and native, chicken, and human α3-containing AChRs (16). Therefore, sensitivity to ACh and epibatidine is similar for α3β4 AChRs and α6β4 AChRs, whereas actions of cytisine, DMPP, and especially nicotine clearly distinguish between these two subtypes of AChR. These features could be extremely useful for potential future functional identification of native α6-containing AChRs.

Depletion of Ca^{2+} ions from the extracellular solution resulted in a dramatic decrease of α6β4 AChR-mediated currents. Similar phenomena were characterized originally for oocyte-expressed and native rat α3 AChRs (21, 22). It was concluded that physiological concentrations of extracellular Ca^{2+} ions enhance neuronal AChR-mediated currents by direct binding on the extracellular side of these AChRs. Alternatively, decrease of the α6β4 AChR-mediated currents in low Ca^{2+} could be the result of the prevention of activation of a secondary endogenous Ca^{2+}-dependent Cl^- current. This current is known to accompany currents mediated by recombinant AChRs or *N*-methyl-D-aspartate receptors with rela-

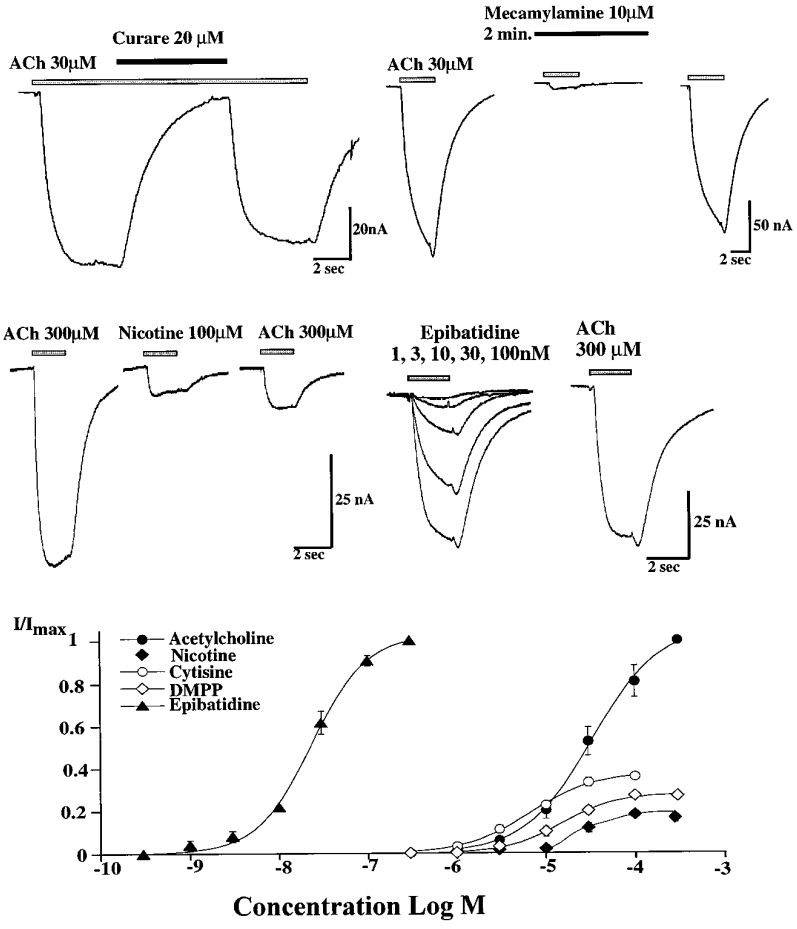

Fig. 4. Pharmacological properties of chick α6 human β4 AChRs. *Top*, inhibition of α6β4-mediated currents by D-tubocurarine (*left*) and mecamylamine (*right*). Curare was coapplied for 6 sec after 4 sec of perfusion of 30 μM ACh. Currents induced by 30 μM ACh before, after 2-min perfusion with 10 μM mecamylamine, and after 10-min washout. *Middle left*, currents induced by 300 μM ACh before and after 4-sec application of 100 μM nicotine (*middle trace*) to illustrate the long-lasting antagonism produced by exposure to nicotine. *Middle right*, currents induced by application of increasing concentrations of (+)-epibatidine are compared with the control current induced by 300 μM ACh. *Bottom*, a family of concentration-response curves obtained for ACh, nicotine, cytisine, DMPP, and (+)-epibatidine. Averaged data from three to five experiments are presented.

TABLE 1

Comparison of effectiveness of nicotinic agonists on chick α6 human β4, chick α3β4, and human α3β4 AChRs

	Chick α6 Human β4[a]			Chick α3β4[b]		Human α3β4[b]	
	EC_{50}	Efficacy	nH	EC_{50}	Efficacy	EC_{50}	Efficacy
	μM			μM		μM	
Acetylcholine	28 ± 4	100%	1.3	53	100%	163	100%
Nicotine	22 ± 3	18%[c]	2	410	100%	106	100%
Cytisine	6.6 ± 0.6	36%[c]	1.2			76	47%[c]
DMPP	15 ± 1	27%[c]	1.4			10	100%
Epibatidine	0.024 ± 0.002	100%	1.3	0.021	100%	0.073	100%

[a] Data from Fig. 4.
[b] Data from Ref. 15.
[c] Partial agonist.

tively high Ca^{2+} permeability expressed in *X. laevis* oocytes (11, 15, 22). However, the time course of the currents mediated through the oocyte-expressed α6β4 AChRs suggests that the contribution of the Ca^{2+}-dependent Cl^- current is minimal or nonexistent. A Ca^{2+}-dependent Cl^- current usually is observed as a peak current with a relatively fast inactivation at the beginning of the agonist application and sometimes is misinterpreted as a fast component of desensitization (15, 22, 23).

Preliminary *in situ* hybridization studies of mRNA expression for the α6 AChR subunit revealed the pattern of distribution of this subunit in developing rat brain (8). Message for the α6 subunit was localized within the medial habenula, locus ceruleus, ventral tegmental area, and substantia nigra compacta. This restricted pattern distribution of α6 message in brain contrasts with the more diverse and diffuse distribution of the α4 (see Refs. 1 and 2 for review) and α7 (24, 25) subunits and rather parallels the distribution of the α3 AChR subunit (1, 26). Now that we have demonstrated that α6 can participate in functional AChRs in combination with β4, the significance of α6 localization in brain will have more effect on our understanding the central effects of ACh, nicotine, and nicotinic drugs. Message for the β4 subunit is colocalized in, but not limited to, the brain areas that contain α6 mRNA (27). Message for α6 RNA is localized in parts of the brain traditionally believed to participate in the rewarding properties of drugs of abuse, with one of the putative mechanisms of addiction involving dopamine release in the neurons of these areas (28). Functional, unidentified, neuronal AChRs were shown to be present in these areas (29–31). The substantia

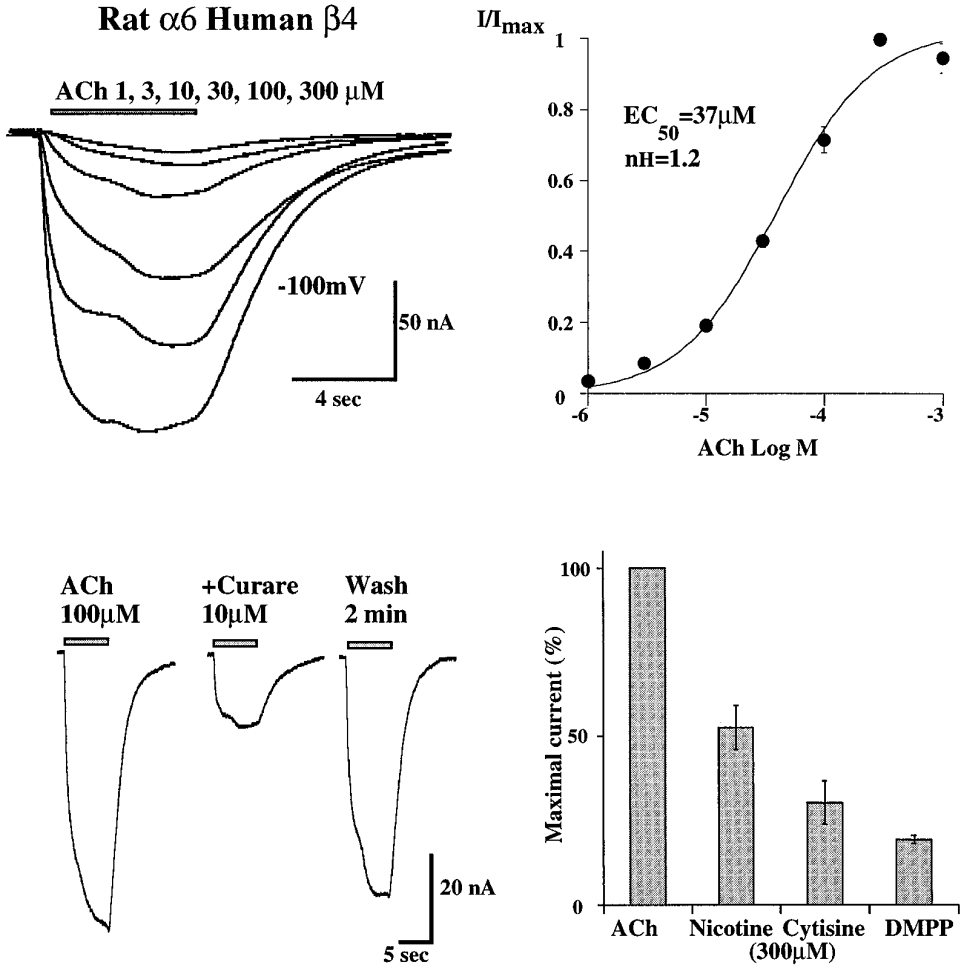

Fig. 5. Rat α6 subunits form functional AChRs with human β4 subunits. *Top left,* a family of currents induced by application of increasing concentrations of ACh to oocytes 5 days after cytoplasmic injection of rat α6 and human β4 AChRs cRNAs. Oocytes were voltage clamped at −100 mV. *Top right*, a plot of the dose-response curve for ACh obtained from 5 oocytes. *Bottom left*, inhibition of the rat α6 human β4 AChR-mediated current by the nicotinic antagonist curare. ACh-induced currents before, during, and after, coapplication with 10 μM curare. *Bottom right*, the maximal currents induced by ACh, nicotine, cytisine, and DMPP are compared. Data are from four oocytes. In each experiment, currents induced by 300 μM agonist were normalized to the current induced by 300 μM ACh (a saturating concentration).

nigra, which degenerates in Parkinson's disease, expresses α6 (8). Nicotinic AChRs are lost in Parkinson's disease (32, 33), and smoking seems to be protective in this disease (34); therefore, AChR subtypes with a limited distribution, including this nucleus, might be useful drug targets for subtype-specific AChR agonists intended for therapy of Parkinson's disease.

With our initial demonstration that α6 can function as part of AChRs formed from subunit cDNAs expressed in *X. laevis* oocytes, α6 leaves the ranks of orphan subunits and joins the company of numerous potential AChR subtypes that are much better characterized as expressed cDNAs in oocytes than they are in any native neurons. Now the challenge is to detect α6 AChR proteins in neurons, determine their subunit composition, and relate their functional properties to those we have observed in oocytes.

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Send reprint requests to: Dr. Jon Lindstrom, University of Pennsylvania, Department of Neuroscience, 36th & Hamilton Walk, 217 Stemmler Hall, Philadelphia, PA 19104-6074.