

A Novel Human Nicotinic Receptor Subunit, $\alpha 10$, That Confers Functionality to the $\alpha 9$ -Subunit

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ABSTRACT

We present herein the cloning of the human nicotinic acetylcholine receptor $\alpha 9$ -ortholog and the identification of a new α -like subunit ($\alpha 10$) that shares 58% identity with $\alpha 9$. Whereas $\alpha 10$ fails to produce functional receptors alone, it promoted robust acetylcholine-evoked currents when coinjected with $\alpha 9$. The presence of $\alpha 10$ modifies the physiological and pharmacological properties of the $\alpha 9$ receptor indicating that the two subunits coassemble in a single functional receptor. Fusing the

N-terminal domain of $\alpha 9$ with the rest of the $\alpha 10$ -cDNA yielded a functional $\alpha 9$: $\alpha 10$ -chimera that displays the acetylcholine binding properties of $\alpha 9$ and ionic pore characteristics of $\alpha 10$ -containing receptors. In addition, $\alpha 9$ - and $\alpha 10$ -subunit mRNAs show limited similar tissue distribution patterns and are expressed in cochlea, pituitary gland, and keratinocytes. These data suggest that, in vivo, $\alpha 9$ -containing receptors coassemble with $\alpha 10$ -subunit.

Nicotinic acetylcholine receptors are members of the ligand-gated ion channel superfamily that are formed by the pentameric association of multiple subunits (Galzi and Changeux, 1995). In vertebrates, neuronal nAChR subunits are encoded by a large family of genes and many of them have already been identified in humans (Boyd, 1997). Special interest has been devoted to the $\alpha 7$ - to $\alpha 9$ -subunits that have the unique capacity of forming functional homomeric receptors (Couturier et al., 1990; Anand et al., 1993; Elgoyhen et al., 1994; Gotti et al., 1994). Expression of the most recently cloned subunit in this subfamily, $\alpha 9$, has been described in only very restricted areas such as the pituitary pars tuberalis, the olfactory epithelium and in the cochlea (Elgoyhen et al., 1994). In particular, this subunit has been shown to be expressed on the cochlear outer hair cells (OHCs), where it is supposed to mediate the cholinergic efferent transmission (Puel, 1995), which activates hyperpolarizing current mediated by small conductance calcium-activated potassium channels (Oliver et al., 2000). Functional properties of the receptors obtained by expression of the $\alpha 9$ -subunit closely resemble those of native nAChRs from OHC that display very original pharmacological features (Erstegui et al., 1994; Guth and Norris, 1996). However, the amplitude of the acetylcholine-evoked currents generated by the expression of the

$\alpha 9$ -subunit in *Xenopus laevis* oocyte remains unusually small and the fraction of positive cells very low. These data suggest that although able to reconstitute homomeric receptors, the $\alpha 9$ -subunit may require another subunit to be fully functional, although attempts to coexpress $\alpha 9$ with other known α nAChR subunits failed to generate functional receptors (Elgoyhen et al., 1994). Because other genes coding for neuronal nAChRs could well exist in the human genome, we have sought to clone the human $\alpha 9$ -subunit and examined the possibility of identifying the missing $\alpha 9$ -partner.

Materials and Methods

Cloning of the Human $\alpha 9$ - and $\alpha 10$ -cDNAs. The rat $\alpha 9$ -nAChR amino acid sequence (Swissprot accession number P43144) was used to perform a TblastN search against an expressed sequence tag (EST) database. A single EST was identified which presented a high degree of homology with the rat $\alpha 9$ -sequence and the corresponding cDNA clone originating from a human whole embryo (8-week-old) cDNA library was retrieved. This cDNA clone was found to contain a nearly complete open reading frame (ORF) coding for the human $\alpha 9$ nAChR subunit. An unspliced intron was present which was removed by PCR. The sequence corresponding to the missing 5' end of the ORF and a portion of the 5'-untranslated region was obtained from human genomic DNA using the Genome Walker system (CLONTECH, Palo Alto, CA). An oligonucleotide containing 16 nucleotides of the 5'-untranslated region and the first 18 nucleotides of the ORF was then synthesized to complete the original cDNA by

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; EST, expressed sequence tag; OHC, outer hair cells; ORF, open reading frame; bp, base pair(s); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; ACh, acetylcholine; α -bgt, α -bungarotoxin; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester.

PCR. The resulting full-length cDNA clone (GenBank accession number AJ243342) was sequenced and inserted into the pTracer-EF eukaryotic expression vector (Invitrogen, Carlsbad, CA) for further use.

The TblastN search also resulted in the identification of an EST from the GenBank database (accession number AA243627) that had been identified as a putative homolog of the rat $\alpha 9$ -subunit but whose sequence identity was lower than that expected for the $\alpha 9$ -ortholog. The clone was obtained from the IMAGE Consortium (685357; <http://image.llnl.gov/>) and its sequencing showed that it contained a partial ORF corresponding to a novel nAChR α -subunit. A 700-bp fragment of the $\alpha 10$ -cDNA was used as a probe to analyze $\alpha 10$ -mRNA expression in human tissues to obtain the missing coding sequence. Strong hybridization was observed in skeletal muscle and human Marathon (CLONTECH) skeletal muscle cDNA was used to clone the 5' coding region of the $\alpha 10$ -cDNA by 5' RACE. Those experiments showed the presence of two unspliced introns in this 5' region. Total coding sequence (GenBank accession number AJ278118) was obtained from several RACE-PCR products and a full-length cDNA clone containing the entire coding sequence was then obtained by RT-PCR from human pituitary mRNA and inserted in pTracer-EF vector. Detailed intron-exon boundaries were analyzed by sequencing PCR products obtained from human genomic DNA.

Chromosomal Localization of $\alpha 10$ -Gene. A PAC containing the sequence of $\alpha 10$ was isolated by PCR using oligonucleotide primers flanking an intron. It was then used to localize the $\alpha 10$ gene to 11p15.5 using fluorescence in situ hybridization (Incyte Genomics, Palo Alto, CA).

Northern Blot Analysis. Multiple human tissue Northern blots (2 μ g/lane; CLONTECH) were hybridized with a *Bsp*HI 360-bp fragment of the $\alpha 10$ -cDNA. The probe was radiolabeled with [³²P]dCTP (PerkinElmer Life Sciences, Boston, MA) using the random priming technique (Megaprime labeling kit; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). Stringent washing conditions (65°C; 0.1 \times standard saline citrate/0.1% SDS) were used. The blots were scanned using an STORM 860 Imager (Molecular Dynamics, Sunnyvale, CA).

Analysis of $\alpha 9$ - and $\alpha 10$ -mRNA Expression by RT-PCR. Human pituitary gland mRNA was obtained from CLONTECH. Rat total RNA from pituitary gland, tongue, nasal epithelium, and cochlea was isolated from frozen tissues dissected from adult Sprague-Dawley rats using RNeasy silica-gel membrane spin columns (QIAGEN, Hilden, Germany). First strand cDNA synthesis was carried out using 50 ng of mRNA or about 1 μ g of total RNA with the SuperScript reverse transcriptase (Invitrogen). Specific human and mouse $\alpha 9$ - and $\alpha 10$ - and rat $\alpha 10$ -primers were designed for PCR amplification: human $\alpha 9$, ctacaatggcaatcaggtgg and atgatgtcaacg-cagtgg (predicted amplified fragment length, 425 bp); human $\alpha 10$, tctcaagctgttcctgacc and aagctgtctatccacgc (predicted amplified fragment length, 391 bp); mouse $\alpha 9$, ccttaccagatgtcacctcactc and aacaccatagcaaagaaatccaca (predicted amplified fragment length, 177 bp); mouse $\alpha 10$, aatgtgacctggaggtgac and gtaggcattgttccacacgtg (predicted amplified fragment length, 108 bp); and rat $\alpha 10$, ttagac-cagtggcagatacag and ccattcaacgttctccacg (predicted amplified fragment length, 472 bp). The predicted amplified fragments contain either one or two intron positions, those in $\alpha 10$ -segments being known to correspond to unspliced introns in human skeletal muscle. PCRs were performed on 5 μ l of the 20 μ l of cDNA synthesis volume using the Expand long template polymerase mix (Roche Diagnostics, Mannheim, Germany) and buffer, 0.5 mM dNTP, 0.5 μ M each primer in the following cycling conditions: 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 58°C (rat $\alpha 10$ primers) or 64°C (human $\alpha 9$ and $\alpha 10$ primers), and 1 min at 68°C, followed by 5 to 10 cycles of 30 s at 94°C, 30 s at 58 or 64°C, and 1 min at 68°C with an auto-extension step of 20 s per cycle, followed by 4 min at 68°C. All PCR products were subcloned into pCR-II Topo (Invitrogen) vector and sequenced.

Full-length $\alpha 10$ -cDNA containing the entire open reading frame

used for expression analysis was obtained using the following primers: tcacatccagagacctgcc and tgagagctccaataaccagc. PCR conditions were as described above with the following cycling conditions; 3 min at 94°C followed by 25 cycles of 30 s at 94°C, 30 s at 61°C, and 1.5 min at 68°C, followed by 10 cycles of 30 s at 94°C, 30 s at 58 or 64°C, and 1.5 min at 68°C with an autoextension step of 10 s per cycle, followed by 4 min at 68°C.

Western Blot Experiments. Human epidermal keratinocytes were obtained from BioWhittaker Inc. (Walkersville, MD) and grown as recommended by the supplier. COS cells were transfected using FuGene (Roche Diagnostics) according to the manufacturer's instructions using 2 μ g of vector. Protein extracts were obtained by recovering cell monolayers in lysis buffer [100 μ l of Laemmli buffer (Bio-Rad, Hercules, CA)/5% (v/v) β -mercaptoethanol and 50 μ l of PBS were used for 4 $\times 10^5$ cells]. Twenty microliters of cell lysate per lane was loaded onto SDS-polyacrylamide gel electrophoresis 4 to 15% gradient acrylamide gel (Bio-Rad) and proteins separated by electrophoresis. The gels were electroblotted onto a nitrocellulose membrane (Amersham Biosciences). The membranes were blocked with 5% nonfat milk/0.1% Tween 20 (Sigma, St Louis, MO) in PBS buffer for 1 h. Each membrane was incubated with a primary anti- $\alpha 10$ antibody (Eurogentech, Seraing, Belgium) diluted to 10 μ g/ml in PBS supplemented with 0.1% Tween 20, 5% nonfat milk, washed three times in PBS-Tween 20 0.1%, and incubated 1 h with a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma) diluted 1:10,000. Binding was visualized with the ECL Western blot detection system (Amersham Biosciences).

For control experiments in which the specificity of binding was tested, the primary anti- $\alpha 10$ serum was preincubated for 1 h with 200 μ g/ml of the immunizing peptide (CGQSRPPELSPSPQSPE) in PBS-0.5% Tween 20.

In Situ Hybridization. RT-PCR was used to amplify a 494-base pair cDNA (corresponding to nucleotide 180–674 of the GenBank sequence AF196344) from rat pituitary mRNA. The T7 promoter was added by a second PCR and then the cDNA was purified after gel electrophoresis and sequenced. The riboprobe was transcribed with ³⁵S-labeled UTP, purified by phenol/chloroform extraction, and precipitated.

Brains from Sprague-Dawley male rats (180–200 g) and E18 rat embryos were frozen. Fifteen-micrometer-thick cryostat sections were defrosted, rehydrated, and fixed with 4% paraformaldehyde. After proteinase K treatment, acetylation, and prehybridization, the slides were hybridized overnight at 55°C with 5 $\times 10^4$ cpm/ μ l of probe in a hybridization solution (60% formamide, 300 mM NaCl, 20 mM Tris, pH 7.4, 5 mM EDTA, pH 8, 10% dextran sulfate, 0.4 ng/ μ l tRNA, 1 \times Denhardt's solution, and 200 mM dithiothreitol). After high stringency washes, slides were dehydrated and dipped in Kodak NBT2 emulsion and stored for 1 month in the dark at 4°C. After development the slides were lightly colored with Hemalun, mounted, and examined with light- and dark-phase microscopy.

Oocyte Preparation and Injection. *X. laevis* oocytes were isolated and prepared as described previously (Bertrand et al., 1991). The oocytes were intranuclearly injected with 2 ng of expression vector cDNA. They were kept in a separate well of a 96-well microtiter plate at 18°C. OR2 control medium consisted of 88 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 2 mM CaCl₂, pH 7.4, adjusted with NaOH.

Electrophysiology. Throughout each experiment, oocytes were continuously superfused with control medium and fluid exchanges were controlled by electromagnetic valves. Gravity-feed solution was flowing at an approximate rate of 6 ml/min. Oocytes were measured 2 to 4 days after cDNA injections. Electrophysiological recordings were performed using a two-electrode voltage-clamp (GeneClamp amplifier; Axon Instruments, Union City, CA). Electrodes were made of borosilicate glass, pulled with a BB-CH-PC puller (Mecanex, Nyon, Switzerland), and filled with a filtered 3 M KCl. Unless specified, the holding potential was –75 mV. Oocytes were continuously maintained at 18°C during preparation and experiments. Calcium

permeability measurements were effectuated using *N*-methyl-D-glucamine and oocytes were incubated for at least 6 h with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) to prevent activation of the endogenously expressed calcium activated chloride currents (Boton et al., 1989). Data from the reversal potential were fitted using a Hodgkin-Goldman-Katz constant field equation appropriately adapted (Jagger et al., 2000; Katz et al., 2000). Calcium blockade was simulated using the empirical Hill equation (see Katz et al., 2000).

Binding Experiments. Measure of receptor expression on the oocyte surface was carried out using ^{125}I - α -bgt (2000 Ci/mmol, Amersham). Oocytes were incubated for 2 h in 200 μl of a 50 nM solution of ^{125}I - α -bgt in OR2 buffer and briefly washed four times with OR2, and the amount of radioactivity determined by γ -counting. Electrophysiological recordings were carried out to verify proper expression of α 9- or α 9- α 10-expression.

Results

Cloning of the Human α 9-Subunit. A search by homology was performed against human EST databases using the rat α 9 nAChR amino acid sequence. A single EST was found with very high homology and the relevant cDNA clone was fully sequenced. The results showed that this clone encoded a protein displaying more than 90% identity to the rat α 9 sequence. A full-length cDNA clone was then constructed and inserted into an expression vector (see *Materials and Methods*).

The resulting clone encodes a 479-amino acid polypeptide with a predicted molecular mass of 54.7 kDa. The human α 9-deduced amino acid sequence exhibits 90.8% identity with its rat homolog (Fig. 1A).

Identification and Isolation of a Novel Human nAChR α -Subunit. The homology search carried out in the human databases with the rat α 9-nAChR amino acid sequence also identified a different EST that showed relatively high homology to the α 9-subunit sequence. The missing 5' extremity of the corresponding partial cDNA clone was then obtained by 5' RACE and a continuous cDNA containing the entire open reading frame was generated by RT-PCR from human pituitary mRNA. This ORF encoded for a 450-amino acid polypeptide (Fig. 1A) and classified as the nAChR α 10-subunit. Amino acid comparison with the other known nAChR subunit (Fig. 1B) indicates that this novel α 10-subunit is more closely related to the subunits that are able to form functional homomeric receptors (α 7, α 8, and α 9) rather than to those requiring a β -subunit for functional expression.

Using fluorescence in situ hybridization to human chromosomes, the α 10 gene was mapped to 11p15.5. This region contains a number of genetic diseases loci and most noticeably a locus linked to a deficit in inhibitory gating phenotype related to the brain's response to auditory stimuli (Freedman et al., 1994), although more precise genetic mapping would be required to link α 10 to this locus.

Analysis of α 10-Expression. Because α 10 presents relatively high amino acid sequence similarity with α 9, mRNA expression of α 10 was investigated in tissues known to express the α 9-subunit. The presence of both α 9- and α 10-transcripts was detected by RT-PCR in human pituitary gland (Fig. 2A) from which a cDNA encoding the complete coding sequence was isolated and in keratinocytes (not shown). In addition, the presence of partially spliced α 10-transcript was also detected in these tissues. Northern blot

analysis showed a strong expression of a single 6.4-kilobase transcript in skeletal muscle and a faint signal in heart (not shown). However, RT-PCR experiments showed that this transcript is not completely spliced in these tissues, and as such would not be translated into a full-length α 10-protein. Further analysis showed that the position of introns within the gene structure (Fig. 1A) is similar to that described for the rat α 9-gene (Elgoyhen et al., 1994).

To verify that correctly processed α 10-mRNA leads to the expression of α 10-protein, a Western blot analysis was carried out on human keratinocyte protein extract. The results (Fig. 2B) show that an affinity-purified anti- α 10 antibody recognized a major protein band of about 55 kDa. The specificity of the antibody was confirmed in control experiments showing that the staining of this band could be eliminated by preincubating the antibody solution with the α 10-peptide

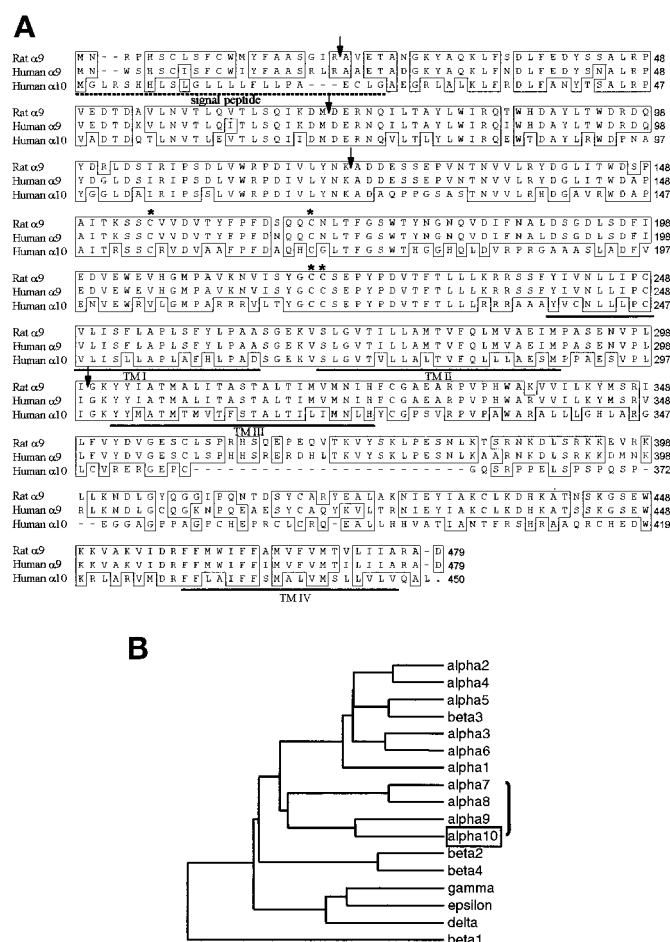


Fig. 1. Comparison of the deduced amino acid sequences from the human α 9- and α 10-subunits together with sequences of other nAChR subunits. A, alignment of the deduced human α 9- and α 10-subunit with the rat α 9-subunit. Identical residues between the three subunit sequences are boxed. The predicted signal peptides are underlined in dotted line while predicted transmembrane domains (TM) are underlined in bold line. *, characteristic cysteine residues found in all nAChR α -subunits. Arrows indicate the positions of the four introns detected within the coding sequences of both α 9- and α 10-genes. B, dendrogram illustrating the relationship between the novel α 10-subunit (boxed) and the other known nAChR subunits. This tree was generated from the alignment (Clustal W software) of all known human nAChR subunit amino acid sequences together with the chick α 8-subunit. Square bracket highlights the closer similarity between α 10 and the other subunits known to form functional homo-oligomeric receptors.

used for immunization and that a band of similar size could be labeled with protein extract from $\alpha 10$ -transfected but not from $\alpha 9$ -transfected COS cells (Fig. 2B).

RT-PCR analysis was also carried out on rat for tissues that were not available from human, the rat $\alpha 10$ ortholog sequence having recently been deposited in GenBank (accession number AF196344). A PCR product corresponding to $\alpha 10$ -mRNA with properly spliced introns 2 and 3 was also detected in the rat pituitary gland and in the cochlea, although, in contrast, no $\alpha 10$ -signal was detected in rat tongue or whole brain (Fig. 2A). Similarly, both $\alpha 9$ - and $\alpha 10$ -transcripts were found (not shown) in the UB/OC-2 mouse cochlear cell line known to express $\alpha 9$ -containing nAChRs (Jagger et al., 2000). A cRNA rat $\alpha 10$ -probe was also hybridized onto sections of adult rat brain. $\alpha 10$ -mRNA expression was found in the pars tuberalis region of the pituitary gland (Fig. 3), exactly as described previously for the rat $\alpha 9$ -transcript (Elgoyhen et al., 1994).

Functional Expression of $\alpha 9$ and $\alpha 10$ in *X. laevis* Oocytes. Reconstitution experiments of the human $\alpha 9$ - and $\alpha 10$ -subunits were carried out by intranuclear cDNA injections in *X. laevis* oocytes. Expression of the human $\alpha 9$ -cDNA yielded functional receptors that can be activated by acetylcholine with an EC_{50} of $30 \pm 6 \mu M$ (Fig. 4A; Table 1). However, the amplitude of this acetylcholine-evoked current was small by comparison with current recorded in sibling oocytes expressing the homomeric human $\alpha 7$ receptor (not

shown). The human $\alpha 9$ -receptor also exhibited a peculiar pharmacological profile similar to that displayed by the rat ortholog. For example, nicotine evoked no detectable currents but acted as an antagonist with an IC_{50} of $41.2 \pm 5.4 \mu M$ (not shown).

Surprisingly, despite its homology with $\alpha 9$, $\alpha 10$ -cDNA failed to reconstitute functional homomeric receptors in *X. laevis* oocytes. Application of acetylcholine concentrations up to 1 mM elicited no currents in cells injected with this sub-

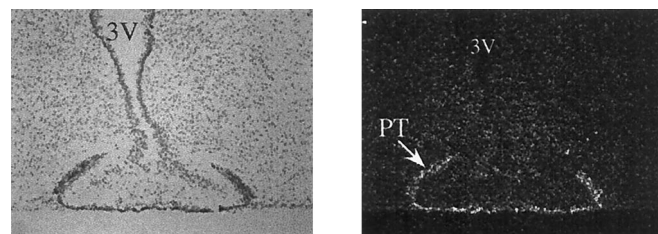


Fig. 3. In situ hybridization of $\alpha 10$ -mRNA in rat pituitary. Bright (A) and dark (B) field pair photomicrographs of adult rat brain section through the third ventricle (3V) hybridized with $\alpha 10$ -cRNA. Hybridization is apparent in the pars tuberalis (PT) of the pituitary.

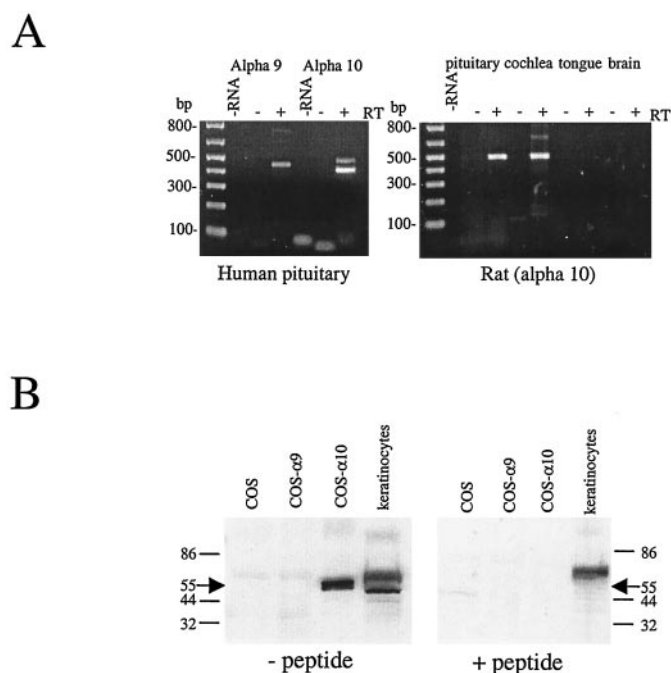


Fig. 2. Analysis of $\alpha 9$ - and $\alpha 10$ -expression by RT-PCR (A) and western blot (B). A, RT-PCR results obtained from human and rat tissue RNAs using $\alpha 9$ - and $\alpha 10$ -specific primers. A sample of $5 \mu l$ of each reaction was loaded on ethidium bromide-stained agarose gels. The two $\alpha 10$ -fragments seen for human pituitary correspond to fully spliced transcript (391 bp) and transcript with a partially spliced intron 2 (453 bp). B, visualization of $\alpha 10$ -subunit expression in human keratinocytes by western blot. A 55-kDa band is specifically recognized. A band of slightly higher molecular mass (about 58 kDa, possibly due to different glycosylation) is recognized for $\alpha 10$ -transfected COS cells. Preincubation of the anti- $\alpha 10$ antibody with 200 nmol of immunizing peptide abolishes the detection of the protein. No staining was detected for COS or $\alpha 9$ -transfected COS cell protein extracts.

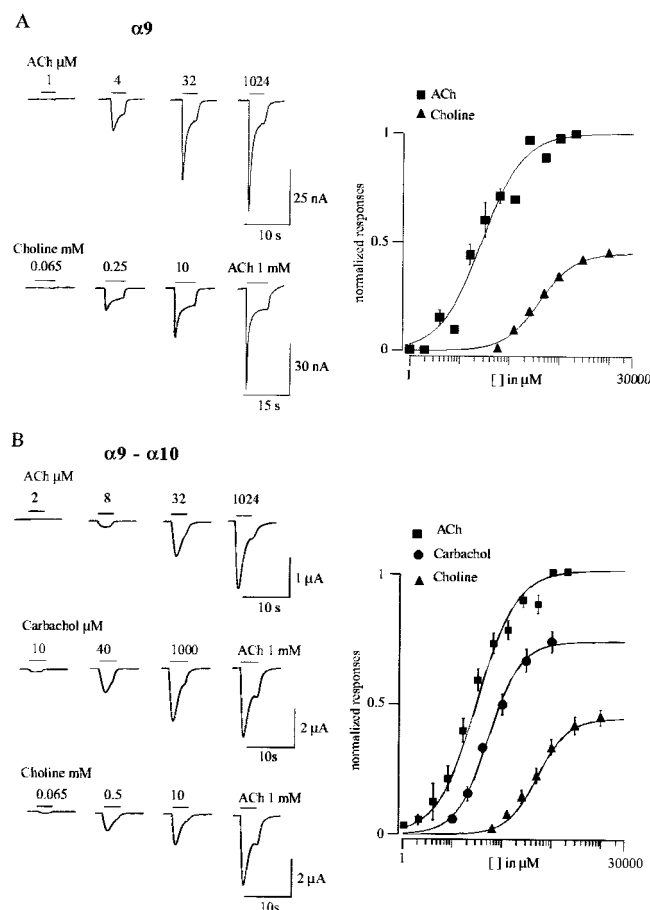


Fig. 4. Properties of $\alpha 9$ - and $\alpha 9$ - $\alpha 10$ -expressing oocytes. A, acetylcholine and choline evoked currents in oocytes expressing the human $\alpha 9$ -receptor. Left illustrates typical currents evoked by three concentrations of these two agonists. Concentration-response curves to acetylcholine and choline are represented in the right. B, oocytes expressing the $\alpha 9$ - and $\alpha 10$ -subunits display robust currents in response to acetylcholine, carbachol or choline. Typical currents are presented in the left and concentration-response relationships in the right. Lines through the data points are best fit obtained with the Hill equation. Corresponding values are given in Table 1.

unit alone (not shown). Coinjection of the available β -subunits ($\beta 2$ and $\beta 4$) remained ineffective, and no current could be detected in any of the configurations tested (not shown). In contrast, robust ACh-evoked currents were recorded in oocytes injected with equivalent amounts of both the $\alpha 9$ - and $\alpha 10$ -cDNAs (Fig. 4B). Comparison of the amplitudes of the ACh-evoked currents in oocytes injected with the $\alpha 9$ - $\alpha 10$ mixture or $\alpha 9$ alone confirmed that presence of the $\alpha 10$ -subunit markedly influences the amplitude of the acetylcholine evoked currents, suggesting that this protein is probably integrated in the $\alpha 9$ -receptor complexes.

To examine further this phenomenon, the physiological and pharmacological profiles of receptors reconstituted in oocytes injected with the $\alpha 9$ - $\alpha 10$ mixture or $\alpha 9$ alone were compared. Although it is known that acetylcholine is the natural agonist of $\alpha 9$ -containing receptors and that these receptors are inhibited by nicotine (Elgoyhen et al., 1994), little information is available regarding other agonists. Choline in the millimolar range is a powerful agonist of the homomeric $\alpha 7$ -receptors (Papke et al., 1996). As shown in Fig. 4A, choline behaves as a partial agonist at $\alpha 9$ -expressing oocytes with an EC_{50} in the high micromolar range (Table 1). Comparison of the concentration-response curves evoked by either acetylcholine or choline reveals no significant differences between oocytes injected with $\alpha 9$ alone or the $\alpha 9$ - $\alpha 10$ mixture (Fig. 4). Concentration-response curves obtained with carbachol illustrate that this substance also acts as a partial agonist that evokes about $76\% \pm 4$ ($n = 6$; Table 1) of the maximal acetylcholine-evoked current in oocytes injected with the $\alpha 9$ - $\alpha 10$ mixture. No differences in response time-courses could be observed between $\alpha 9$ and $\alpha 9$ - $\alpha 10$ expressing oocytes for the agonists tested. Other typical nicotinic receptor agonists such as epibatidine or 1,1-dimethyl-4-phenylpiperazinium only elicited very small responses on $\alpha 9$ - $\alpha 10$ expressing oocytes (Table 1). Moreover, as predicted on the basis of the $\alpha 9$ properties, nicotine acted as an antagonist at the $\alpha 9$ - $\alpha 10$ receptor (not shown).

As complementary characterization, we then examined the effects of competitive and noncompetitive inhibitors. It has been widely documented that the snake toxin α -bgt is a potent competitive inhibitor of homomeric $\alpha 7$ - and $\alpha 9$ -receptors (Couturier et al., 1990; Elgoyhen et al., 1994). Although this toxin blocks in a quasi-irreversible manner homomeric chick $\alpha 7$ nAChRs (Couturier et al., 1990), reversibility has been described on nicotinic receptors from guinea pig OHC

(Lawoko et al., 1995). In agreement with these previous observations, exposures to α -bgt (100 nM, 30 min) caused almost a complete inhibition of the acetylcholine-evoked current (Fig. 5A, upper). Reversibility of this blockade was, however, observed within 15 to 40 min after the toxin had been removed. Homomeric $\alpha 9$ receptors displayed an IC_{50} to α -bgt of about 2.1 nM ($n = 5$), whereas half-blockade was observed only at 14 nM (mean of two to six cells for each data point) in oocytes expressing $\alpha 9$ - $\alpha 10$ (Fig. 5B). The 7-fold difference in sensitivity to α -bgt suggests that $\alpha 10$ must participate in the formation of the receptor binding site and therefore that both $\alpha 9$ and $\alpha 10$ can assemble in the same receptor complex. Challenge with the antagonist *d*-tubocurarine revealed that this compound inhibits the homomeric $\alpha 9$ receptors with an IC_{50} of roughly 2 μ M, whereas half-inhibition of $\alpha 9$ - $\alpha 10$ -expressing oocytes was already observed at 0.73 μ M (Figs. 5, C and D). Both the low Hill coefficient and the increase in the response decay caused by *d*-tubocurarine on $\alpha 9$ - $\alpha 10$ -expressing oocytes suggest that this compound may act as an open channel blocker at this receptor subtype. The higher Hill coefficient of *d*-tubocurarine concentration-response curve at $\alpha 9$ -receptors indicates that this compound may preponderantly act as a competitive inhibitor at the homomeric form of this receptor. In addition, the lower IC_{50} value of these receptors for *d*-tubocurarine indicates an interaction with different amino acid residues.

Whether the difference between current amplitude obtained with $\alpha 9$ alone or $\alpha 9$ - $\alpha 10$ -subunits was due to either a low level of $\alpha 9$ -surface expression or the fact that functional receptors require the assembly of the two subunits was analyzed by measuring α -bgt binding on oocyte surface. Interestingly, a significant amount of α -bgt binding was observed in oocytes injected with the $\alpha 9$ -subunit alone (Table 2). Significant amount of α -bgt binding was also observed in $\alpha 9$ - $\alpha 10$ oocytes but for technical difficulties, no attempt was made to correlate the current amplitude and amount of binding.

One of the common biophysical properties of all the neuronal nicotinic acetylcholine receptors is their strong inward rectification (Couturier et al., 1990; Mathie et al., 1990; Elgoyhen et al., 1994). Highly nonlinear current-voltage (*I-V*) relationships were also reported for the $\alpha 9$ -receptor (Katz et al., 2000), indicating that these receptors may be more complex than more classical nAChRs. A typical rectification was observed when voltage ramps protocols were performed from positive to negative, whereas an outward rectification was

TABLE 1
Comparison of responses of $\alpha 9$ and $\alpha 9$ - $\alpha 10$ expressing oocytes to different nicotinic ligands. Values are indicated with their respective S.E.M. The number of cells tested in each condition is indicated in parenthesis.

Agonists	$\alpha 9$ - $\alpha 10$			$\alpha 9$		
	ACh Response	EC_{50}	n_H	ACh Response	EC_{50}	n_H
	%	μ M		%	μ M	
ACh		27 ± 6 (8)	1.05 ± 0.04		30 ± 6 (10)	1.1 ± 0.08
Carbachol (1 mM*)	76 ± 4 (6)	57 ± 2 (4)	1.35 ± 0.1			
Choline (10 mM*)	57 ± 4 (8)	519 ± 41 (3)	1.4 ± 0.1	45 ± 2 (4)	400 ± 23 (4)	1.2 ± 0.1
Suberyldicholine (1 mM*)	39 ± 4 (3)					
DMPP (100 μ M*)	7 ± 4 (4)					
ATP (1 mM*)	3 ± 2 (4)					
(\pm)-Epibatidine (10 μ M*)	0.14 ± 0.07 (4)					
Cytisine (100 μ M*)	0 (4)					
(-)-Nicotine (100 μ M*)	0 (4)			0 (4)		

* Concentration used to determine % of ACh response.
 n_H , Hill coefficient.

observed when the ramp was effectuated in the opposite direction (Fig. 6A). The comparable inward rectification observed with different steady holding current (Fig. 6B) suggests that the difference between positive to negative ramp is attributable to a slowly appearing channel blockade. Because of the time persistence of this blockade, negative to positive ramps failed to relieve the block, and only the outward rectification is observed. Thus, coexpression of the $\alpha 10$ -subunit causes no detectable modification of the receptor I-V curve. Because a negative reversal potential was observed both in BAPTA-AM treated cells and in absence of extracellular calcium (data not shown), it must be attributed to a permeability ratio of sodium versus potassium slightly lower than unity (best fit was obtained with a pNa/pK of 0.65).

Chimeric $\alpha 9$ - $\alpha 10$ -Subunits Form Fully Functional Acetylcholine Receptors. Considering the relatively high homology between $\alpha 9$ - and $\alpha 10$ -amino acid sequences, it was surprising to find that homomeric $\alpha 10$ -receptors could not form functional ligand-gated channels. To get a better understanding of the structural features behind this difference we constructed and analyzed the properties of a chimeric $\alpha 9$: $\alpha 10$ -subunit. The chimeric $\alpha 9$: $\alpha 10$ -cDNA was obtained by fusing the amino-terminal region of $\alpha 9$ up to the first predicted membrane-spanning domain with from this point the remaining 3' sequence coding for the $\alpha 10$ -subunit (Fig. 7A). The resulting construct was injected into *X. laevis* oocytes and the electrophysiological responses to acetylcholine analyzed. As shown in Fig. 7B, robust currents were evoked in

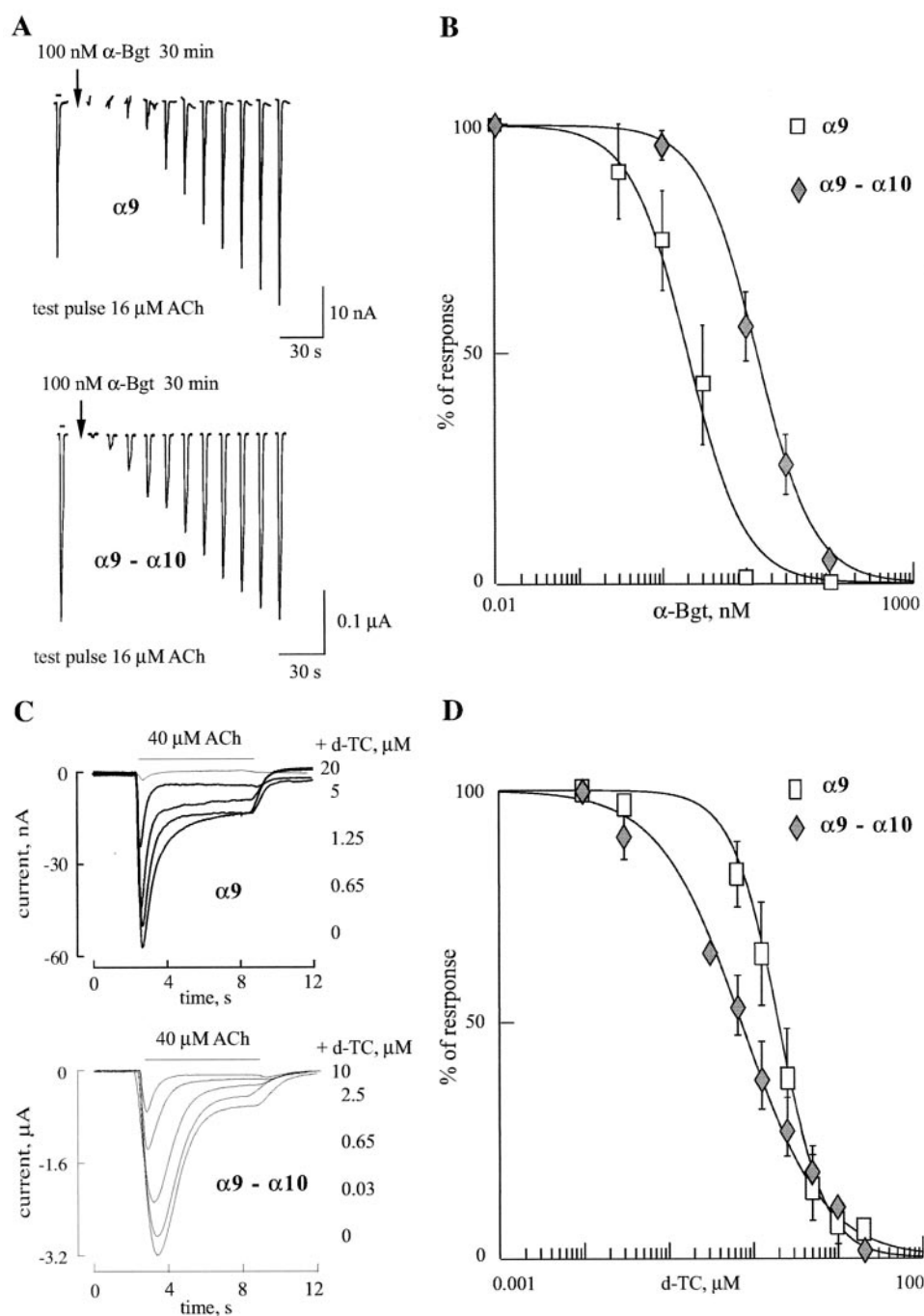


Fig. 5. Sensitivity of $\alpha 9$ and $\alpha 9$ - $\alpha 10$ to nicotinic receptor antagonists, α -Bgt and *d*-tubocurarine. A, the $\alpha 9$ and $\alpha 9$ - $\alpha 10$ acetylcholine-evoked currents are reversibly blocked by α -Bgt. Time course of the recoveries from blockade for two typical recordings are illustrated. B, concentration response inhibition relationship for $\alpha 9$ (squares) and $\alpha 9$ - $\alpha 10$ (diamonds). Lines through the data points are the best fit obtained with a Hill equation with respective IC_{50} values of 2.1 and 14 nM and nH of 1.3 and 1.3 for $\alpha 9$ and $\alpha 9$ - $\alpha 10$. C, effects of *d*-tubocurarine on the amplitude and time course of acetylcholine evoked currents. *d*-Tubocurarine was both pre- (20 s) and coapplied with a fixed concentration of acetylcholine (40 μ M, 5 s). D, dose-response inhibition profile, measured at the peak current, for the $\alpha 9$ (squares) and $\alpha 9$ - $\alpha 10$ (diamonds). Lines correspond to the best fits obtained with the Hill equation with respective IC_{50} values of 2 and 0.73 μ M and nH of 1.6 and 0.9 for $\alpha 9$ and $\alpha 9$ - $\alpha 10$ ($n = 4$).

Values are indicated in femtomoles/oocyte with their respective standard error, with the number of cells tested in parenthesis.

	Non-Injected	$\alpha 9$ -Injected	$\alpha 9 + \alpha 10$ -Injected
^{125}I - α -Bgt binding (fmol/oocyte)	0.09 ± 0.04 (24)	1.08 ± 0.9 (16)	0.49 ± 0.32 (16)

A high calcium permeability of acetylcholine receptors ex-

Discussion

Despite its sequence homology with $\alpha 9$, the $\alpha 10$ -subunit failed to reconstitute a functional receptor alone or in combination with other nAChR subunits. However, the coexpression of human $\alpha 9$ - and $\alpha 10$ -subunits resulted in a dramatic increase (about 100-fold) of the amplitude of the acetylcholine-evoked currents compared with that obtained with $\alpha 9$ alone. The binding experiments carried out with the ^{125}I - α -bgt on oocytes injected either with $\alpha 9$ alone or the mixture $\alpha 9$ - $\alpha 10$ yielded surprising results. First, oocytes injected with $\alpha 9$ alone displayed a significant amount of α -bgt binding, whereas very small or no detectable currents could be measured in sibling oocytes. This suggests that $\alpha 9$ -subunits are properly synthesized by the oocyte machinery and inserted in the plasma membrane where they form high-affinity α -Bgt binding sites. For some unknown reasons, however, these

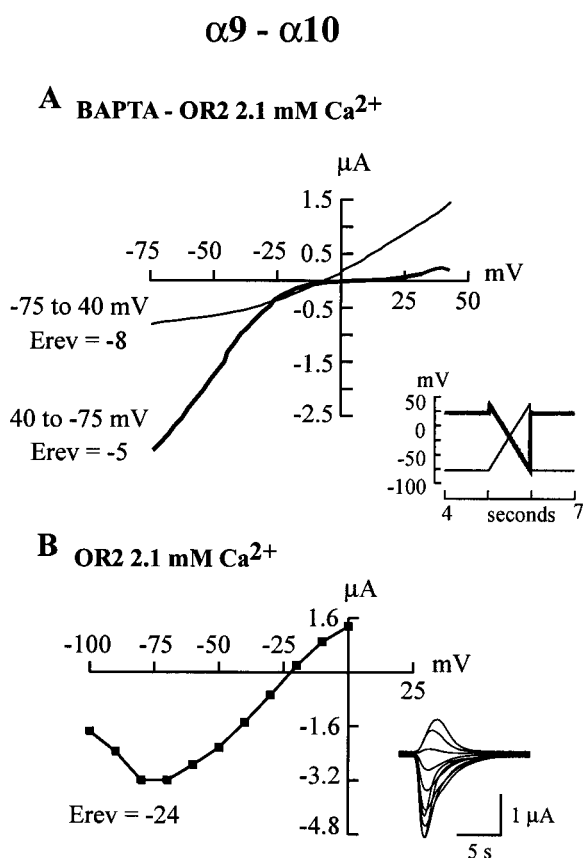


Fig. 6. Reversal potential of the ACh-evoked currents at $\alpha 9-\alpha 10$ receptors. A, current-voltage (I-V) was measured using a sawtooth voltage protocol (ranging from 40 to -75 mV, 1800 ms, thick trace) at the peak of the ACh-evoked current. Subtraction of the passive cell properties was obtained by repeating the same measure in absence of ACh. The thin line illustrates the I-V curve recorded in the same condition for a ramp starting at -75 mV and ending at $+40$ mV. To minimize chloride contamination, oocytes were incubated for at least 6 h in BAPTA-AM (100 μ M). B, I-V relationship measured at steady holding potentials. To support the eye, data points have been connected by straight line. Inset, superposition of the current traces measured in B (ACh 1 mM, 3s).

proteins lack functionality. Second, coinjection of $\alpha 9$ and $\alpha 10$ yielded functional nAChRs and robust currents could be recorded without displaying a significant difference in α -bgt labeling than oocytes injected with $\alpha 9$ alone. These data illustrate that failure of $\alpha 9$ -subunit to produce functional receptors must be attributed to the assembly and formation of an activatable receptor but not to the transport and insertion of $\alpha 9$ in the membrane. To challenge this hypothesis further we have compared the pharmacological profile of $\alpha 9$ -expressing oocytes versus sibling cells injected with the $\alpha 9$ - $\alpha 10$ -mixture. Experiments carried out with antagonists such as α -bgt or *d*-tubocurarine revealed that addition of $\alpha 10$ significantly modified the $\alpha 9$ pharmacological profile. Because it is known that the ligand binding site resides at the interface between the α - and the adjacent subunit (Corringer et al., 1998; Sine et al., 1998), this result indicates that the

$\alpha 10$ -subunit must contribute to the formation of the agonist binding site.

The $\alpha 9$ - and $\alpha 10$ -subunits are clearly structurally related and display important differences with the other known nAChR α -subunits. The discovery that both subunits needed to be associated to form a functional receptor contrasts with the supposed homomeric assembly of $\alpha 9$. The only other example of functional heteromeric nAChR resulting from assembly of α -subunits is the $\alpha 7$ - $\alpha 8$ found in chick retina (Gotti et al., 1994), although in this case both subunits are able to form a functional homomeric receptor. We thus sought to understand the structural features behind the impossibility for $\alpha 10$ to form a functional homomeric receptor and the poor functional expression of $\alpha 9$ alone by studying an $\alpha 9$: $\alpha 10$ -chimera in which the extracellular domain of the $\alpha 9$ -subunit was maintained, whereas all the rest of the pro-

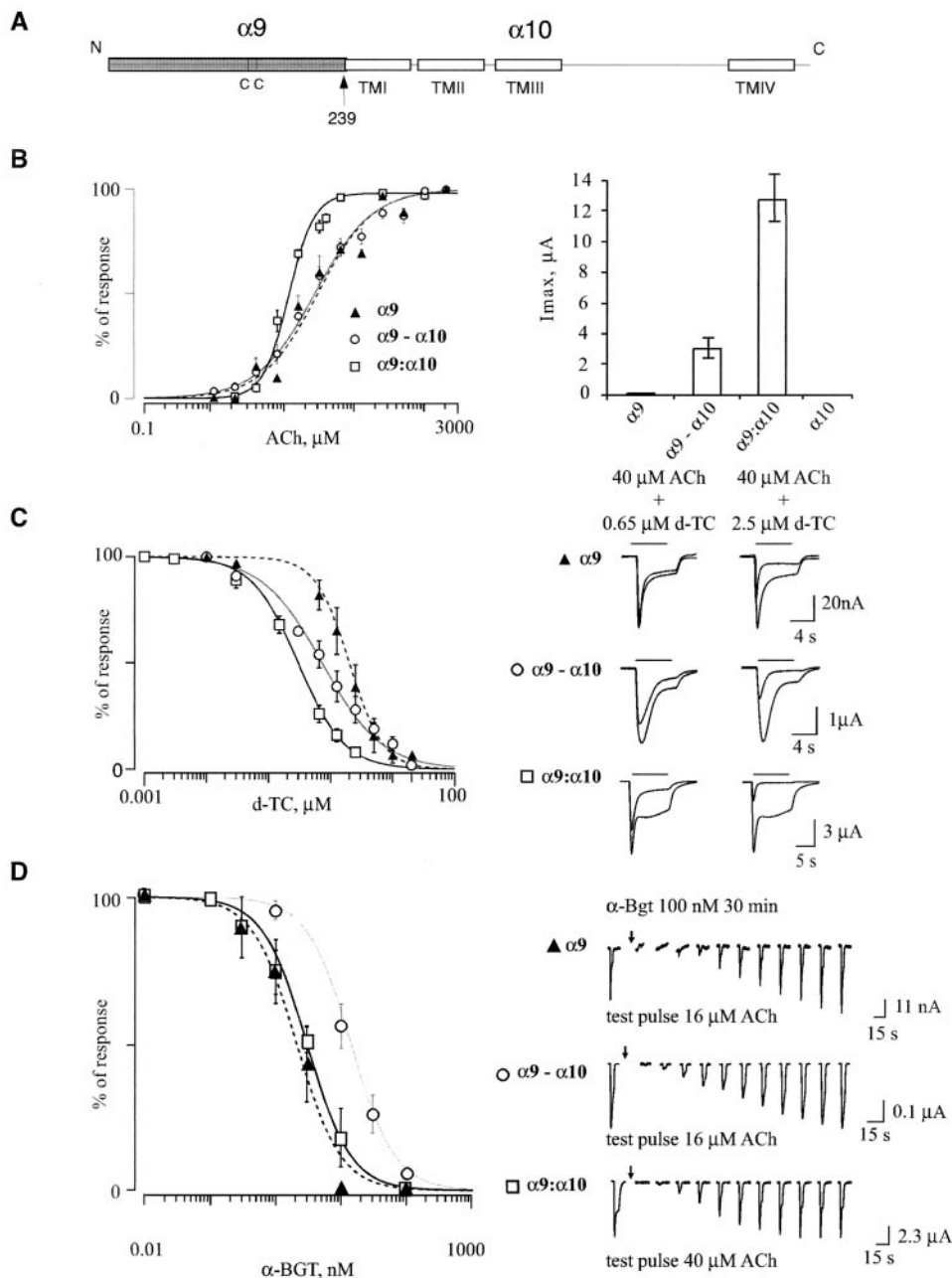


Fig. 7. The $\alpha 9$: $\alpha 10$ -chimera reveals properties of the ligand binding site and the ionic pore. **A**, schematic representation of the $\alpha 9$: $\alpha 10$ -chimera (dark area = $\alpha 9$ -segment). The arrow indicates the point of fusion between the two proteins. **B**, acetylcholine sensitivity of the $\alpha 9$: $\alpha 10$ -chimera is compared with those of oocytes expressing either $\alpha 9$ - or $\alpha 9$ - $\alpha 10$ -receptors (left). Lines are the best fit obtained with the empirical Hill equation with an EC_{50} of 10 μ M and nH of 1.5. Average currents evoked by saturating acetylcholine concentrations are represented for the different cDNA expression (right). Numbers of cells tested in each condition were, respectively, of 18 for $\alpha 9$, 29 for $\alpha 9$ - $\alpha 10$, 12 for $\alpha 9$: $\alpha 10$, and 24 for $\alpha 10$. **C**, differential sensitivity of $\alpha 9$: $\alpha 10$ to *d*-tubocurarine. Lines through the data points are the best fits obtained with the empirical Hill equation an IC_{50} of 0.3 μ M \pm 0.04 and nH of 1.16 (n = 7). Typical acetylcholine evoked currents recorded in control and presence of *d*-tubocurarine are shown in the right. **D**, concentration-response inhibition profile of α -Bgt reveals a higher sensitivity of the $\alpha 9$: $\alpha 10$ -chimera. Best fit of the data points (continuous line) was obtained with the Hill equation with an IC_{50} of 3 nM and nH of 1.3. Typical recovery from blockade are represented by the successive acetylcholine evoked currents presented in the right.

tein was substituted by the $\alpha 10$ -sequence. The very large acetylcholine-evoked currents recorded in oocytes injected with this $\alpha 9$: $\alpha 10$ -chimera alone indicate that exchange of the $\alpha 10$ N-terminal domain was enough to restore its functionality. This finding can be interpreted either by the lack of homomerization of the unmodified $\alpha 10$ -subunit or its incapacity to form a functional acetylcholine-binding site. In addition, these data illustrate that the ionic pore and gating properties are maintained in the $\alpha 10$ -subunit.

The exchange of functional domain implies, as demonstrated with the serotonergic receptor (Eiselé et al., 1993), that ligand-binding properties belong to the protein constituting the N-terminal domain, whereas the ionic pore characteristics are defined by the fusing protein segment. In agreement with this prediction, the difference between $\alpha 9$ and $\alpha 10$ for the competitive antagonist α -bgt was conserved in the chimera as similar to that of $\alpha 9$, whereas the blockade by *d*-TC was closer to that of $\alpha 10$ -containing receptors suggesting again the heteromeric nature of $\alpha 9$ - $\alpha 10$ -receptors.

This $\alpha 9$ - $\alpha 10$ -receptor is a peculiar nicotinic receptor, both in terms of structure and functional property. One of the main issue is the functional significance of this novel nAChR. Efferent modulation of the cochlea OHCs is mediated by acetylcholine through a nicotinic receptor that exhibits a pharmacological profile resembling that described for $\alpha 9$ - and $\alpha 9$ - $\alpha 10$ -receptors (Guth and Norris, 1996). Activation of this nAChR causes a transient influx of calcium that in turn activates hyperpolarizing calcium-dependent potassium channels (Blanchet et al., 1996). The pharmacology of $\alpha 9$ -subunit reconstituted in oocytes (Elgoyhen et al., 1994) corresponds to that of native receptors expressed by vertebrate hair cells. Moreover, $\alpha 9$ -null mice were shown to exhibit an absence of suppression of cochlear responses during efferent

fiber activation (Vetter et al., 1999), thus demonstrating the role of $\alpha 9$ -containing nAChR in the modulation of the cochlear response. The poor functional response of homomeric $\alpha 9$ -receptor in oocyte suggests that additional subunit(s) might be required to obtain the calcium influx required to produce the activation of the calcium-dependent potassium conductance observed in vitro. Correctly processed $\alpha 10$ -mRNA was found in the cochlea and we showed that the presence of $\alpha 10$ -subunit together with $\alpha 9$ not only dramatically increased ACh-evoked currents but also preserved the receptor pharmacology similar to that observed in OHCs. In addition, affinity of α -bgt reported for isolated guinea pig OHCs ($K_d = 62$ nM; Lawoko et al., 1995) further illustrates a closer match to $\alpha 9$ - $\alpha 10$ than $\alpha 9$ alone. This evidence supports the hypothesis that the $\alpha 10$ -subunit must be contained in functional receptor complexes expressed by these sensory cells.

The coexpression of both subunits in the same region of the pituitary gland, the pars tuberalis, suggests another role for $\alpha 9$ - $\alpha 10$ -receptor. This region is in rat and in other mammals a major neuroendocrine target for melatonin, which regulates photoperiodical changes in prolactin secretion. Activation of pars tuberalis-specific cells is thought to trigger the release of a yet uncharacterized peptide, "tuberalin," which would in turn provoke the liberation of prolactin hormone from the pars distalis region (Morgan, 2000). Nicotinic receptors have been shown to modulate hormonal secretion in pituitary and adrenal gland (Gu et al., 1996; Matta et al., 1998). $\alpha 9$ - $\alpha 10$ -receptors could thus be involved in the control of a specific pars tuberalis endocrine system.

Recently, studies have suggested a role for $\alpha 9$ -containing nAChRs in regulating keratinocyte adhesion (Grando, 1997). In particular, Nguyen et al. (2000) have shown that anti- $\alpha 9$

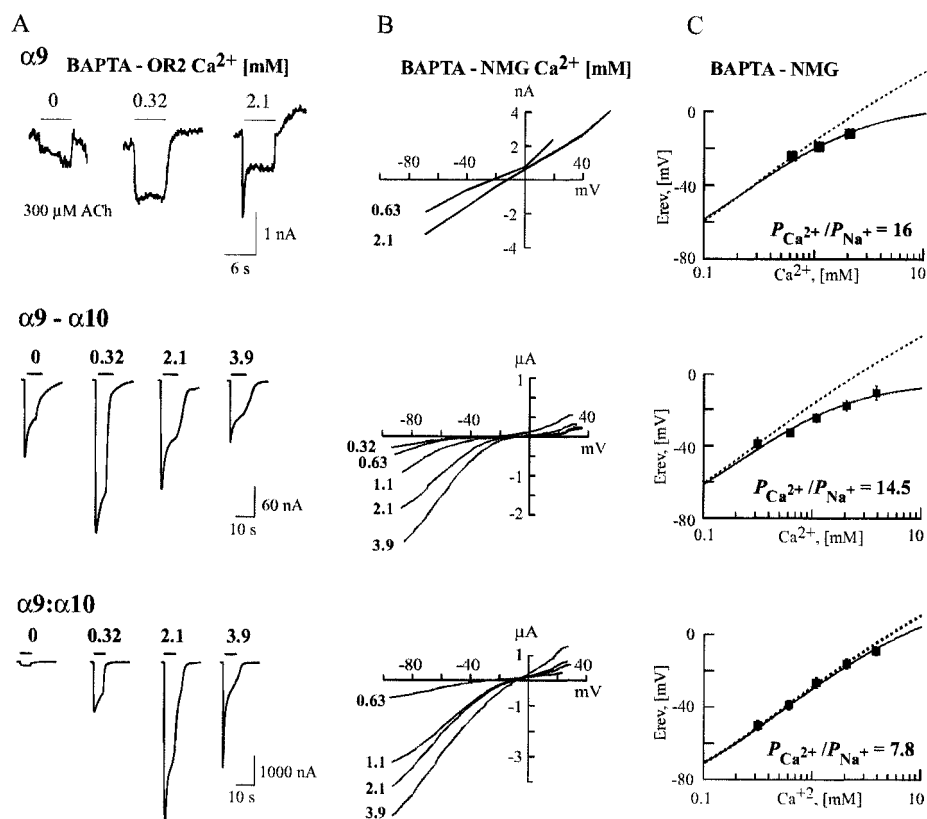


Fig. 8. Effects of extracellular calcium and calcium permeability of $\alpha 9$, $\alpha 9$ - $\alpha 10$, and $\alpha 9$: $\alpha 10$ receptors. A, Typical ACh-evoked currents recorded in $\alpha 9$ (top traces), $\alpha 9$ - $\alpha 10$ (middle traces), and $\alpha 9$: $\alpha 10$ (lower traces) expressing oocytes. Cells were held at -70 mV and challenged with 300μ M ACh (indicated by the bars) in different external calcium concentrations, values indicated above the traces. B, current-voltage relationships recorded in different external calcium concentration in *N*-methyl-D-glucamine medium and BAPTA-AM. C, plot of the reversal potential, measured in C, as a function of the extracellular calcium concentration. Dashed lines indicate the best fit obtained with eq. 1, derived from the Goldman-Hodgkin-Katz equation with a pNa/pK of 0.65. Continuous lines were obtained using the same equation multiplied by an empirical Hill equation to take into account the calcium blockade. Relative calcium permeability for the three receptor types are indicated.

antibodies were present in the serum of patients affected by the autoimmune disease *Pemphigus vulgaris* and that the acantholysis resulting from this disease could be linked to a block of $\alpha 9$ -containing receptors. Interestingly, this antibody effect could be reversed by the addition of carbachol, a cholinergic agonist that we have demonstrated to be active on $\alpha 9$ - $\alpha 10$ -receptors. We have shown that $\alpha 10$ -subunit is also expressed in these cells and therefore that $\alpha 9$ - $\alpha 10$ -receptors are probably the functional nAChRs involved in the modulation of keratinocyte adhesion. The cholinergic pathway involved in this process is still unknown but the distinctive pharmacological profile of $\alpha 9$ - $\alpha 10$ -receptor suggests that specific agonists could have a therapeutic effect on such skin diseases.

The conclusion that $\alpha 10$ -subunit is probably associated in vivo with $\alpha 9$ to form a novel subtype of nicotinic receptor involved in different physiological systems further illustrates the complexity of this receptor family. In a very recent publication, Elgoyhen et al. (2001) have reported the cloning and characterization of the rat $\alpha 10$ -subunit. Similarly to the human $\alpha 10$, the rat $\alpha 10$ can assemble with $\alpha 9$ to form functional receptors and is expressed in cochlear hair cells. Whether $\alpha 10$ might be involved in the composition of other atypical nAChRs remains to be determined.

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