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A new look at the neuronal nicotinic acetylcholine receptor pharmacophore

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Abstract

Interest in the field of nicotinic receptors has been recently stimulated both by the discovery of the potential therapeutic effects of new agonists, and by the discovery of an association between nicotinic receptor mutations and human neurological diseases. Expression of human receptors in an exogenous system allows their study in isolation. Receptors reconstituted by pairwise injection of either $\alpha 4$ or $\alpha 3$ with $\beta 2$ or $\beta 4$ subunits displayed important differences between the resulting receptor subtypes. These results were further compared with those obtained with $\alpha 3{:}\alpha 4$ fusion proteins. The modifications of either the ligand-binding site in the N-terminal domain or in the ionic pore domain were found to affect the pharmacological properties of the receptors. Finally, the analysis of non-natural derivatives of epibatidine demonstrates how an agonist can be modified to be selective at one receptor subtype or to become an antagonist. These data are well explained on the basis of a three-state allosteric model.

Keywords: Acetylcholine receptor; Neuron; Property; Ligand binding domain; Pharmacological; Physiological

1. Introduction

Systemic application of a drug often produces a specific effect at one given organ target. For example, drugs that interact with nicotinic acetylcholine receptors have been shown, when injected intravenously, to have a main action at the neuromuscular junction.

Although our knowledge of the neuromuscular junction receptor is rather extensive both in terms of its physiological role and pharmacological sensitivity, much less is known about its peripheral or central neuronal homologues. With a large number of genes coding for neuronal nicotinic acetylcholine receptors, and the demonstration that the corresponding subunits can combine to form receptors displaying distinct physiological and pharmacological profiles, the number of possible receptor combinations has been proposed to be higher than a thousand (Bertrand and Changeux, 1995; McGehee and Role, 1995). Despite the heterogeneity and complexity of receptors, it is interesting to note that nicotine has been shown to be of potential benefit in neurological diseases including Alzheimer's, Parkinson's disease and Tourette's syndrome (Sanberg et al., 1997; Adler et al., 1999; Lemiere et al., 1999; Perry et al., 1999; Potter et al., 1999; White and Levin, 1999). Nicotine use is, however, hampered by its wide spectrum of action, which causes numerous and undesirable side effects. The design of drugs specific to one receptor subtype should, therefore, open new possible therapeutic actions. One of the best examples is the design of new painkilling compounds based on derivatives of the frog toxin epibatidine (Bannon et al., 1998). At present, however, such projects are still limited by the relative lack of knowledge of the exact receptor composition in different areas of the nervous system as well as their properties.

In this work, we examine — using electrophysiological investigation of human and rat receptors reconstituted in *Xenopus* oocytes — the molecular determinants that confer differences in the pharmacological profile between receptors containing either $\alpha 4$ or $\alpha 3$ in pairwise combination with either $\beta 2$ or $\beta 4$. Hypotheses derived from these

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experiments are further investigated by comparison of the effects caused by non-natural derivatives of epibatidine.

2. Material and methods

2.1. Oocyte preparation and cDNA injection

Xenopus oocytes were isolated and prepared as previously described (Bertrand et al., 1991). The oocytes were intranuclearly injected with 1 ng of each cDNA (α and β), bringing the total to 2 ng. Oocytes were kept in a separate well of a 96-well microtiter plate (NUNC) at 18°C. The Barth solution used to store the oocytes consisted of, in mM, 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 10 HEPES, 0.82 $MgSO_4.7H_2O$, 0.33 $Ca(NO_3)_2.4H_2O$, 0.41 $CaCl_2.6H_2O$, pH 7.4 adjusted with NaOH, supplemented with 20 μg/ml of kanamycin, 100 unit/ml penicillin and streptomycin 100 μg/ml.

2.2. Electrophysiological recordings

Oocytes were used for experiments 2–4 days following cDNA injections. They were continuously superfused with OR2-Ca^{2+} composed of, in mM, 82.5 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 5 HEPES and 20 μg/ml bovine serum albumin. Solution exchange was controlled by computer-driven electromagnetic valves (Type III; General Valve, Fairfield, NJ, USA). The gravity driven flow rate was approximately 6 ml/min. To prevent possible activation of endogenous muscarinic current, 0.5 μM atropine was added to all solutions. Electrophysiological recordings were performed with a two-electrode voltage-clamp (GENECLAMP amplifier; Axon Instruments, Foster City, CA, USA). Electrodes made out of borosilicate tubes were pulled, using a BB-CH-PC puller (Mecanex, Switzerland), and filled with 3 M KCl. The holding potential was −100 mV. All experiments were performed at 18°C.

2.3. Data analysis and computation

Data were converted on-line from analogue to digital values with a PC1200 card (National Instruments) connected to a Macintosh G3. Data were usually sampled at 10-ms intervals and stored on a hard disk for further computations, using a personal data acquisition and analysis program. Dose–response relationships were adjusted to the empirical Hill equation: $y = 1/(1 + (EC_{50}/x^{n_H})$, where y equals normalised current response and x agonist concentration.

2.4. Subunit sequences and comparison

Amino acid sequences for the nicotinic receptor subunits α3, α4, β2, and β4 were obtained from the Swiss-Prot database (Accession numbers P32297, P43681, P17787, P30926, respectively) and compared using Clustal V2 Alignment software.

Table 1
The oligonucleotide primer sequences used for α3:α4 chimeric constructs

α3-F	5′ AGCTTATGGCTCTGGCCGTCTC 3′
α4-R	5′ CGCACTTCCTAGATCATGCCAGCC 3′
χ1-F	5′ GGAGATCTACCCCGACATCACCTATG 3′
χ1-R	5′ CATAGGTGATGTCGGGGTAGATCTCC 3′
χ2-F	5′ GTCTTCTACCTGCCCTCCG 3′
χ2-R	5′ CGGAGGGCAGGTAGAAGAC 3′
χ3-F	5′ TTGGAAGTATGTTGCCATGGTCATC 3′
χ3-R	5′ GATGACCATGGCAACATACTTCCAA 3′

2.5. Polymerase chain reaction construction (PCR) of the chimeric α3/α4 subunit

The chimeric α3/α4 cDNA was generated through two successive PCRs as previously described (Horton et al., 1989). In the first PCR: (24 cycles: 94°C for 1 min; 60°C for 1 min; 68°C for 2 min;10 min final extension at 68°C), 50 ng of template α3 or α4 DNA was amplified with 1 unit Pfu polymerase in 1× supplied buffer, 200 μM dNTPs, 200 nM forward and reverse primers, as summarised in Table 1. In the second PCR (10 cycles: 94°C for 1 min; 60°C for 1 min; 68°C for 10 min; 15 cycles: 94°C for 1 min; 60°C for 1 min; 68°C for 2 min), approximately 50 ng of an α3 5′ and an α4 3′ fragment from the first PCR were annealed and amplified. Final PCR products were incubated 30′ at 72°C in the presence of 2 units Taq polymerase and ligated into a PCR 3.1 TA-cloning expression vector (Invitrogen). Final constructs were sequenced by Sequlab sequencing services (Germany).

2.6. Chemistry

Unless otherwise stated, reagents were obtained in analytical grade from commercial sources (Aldrich, Fluka, Merck, Sigma). Epibatidine derivatives were synthesised and enantiomers were separated as previously described (Bertrand et al., 1999; Patt et al., 1999; Spang et al., 1999).

3. Results

3.1. Molecular determinants at the acetylcholine binding site

The structure–function analysis of the nicotinic acetylcholine receptor benefits from numerous studies which, using a combination of photolabelling and site-directed mutagenesis, have identified the protein segments that participate in agonist binding. For technical reasons, photolabelling studies have been restricted to the *Torpedo* mus-

cle-type receptor, but it has been shown that most of these data readily apply to their closely related neuronal forms. Principal outcomes of these studies can be summarised as follows. Acetylcholine binds at the interface between the N-terminal extracellular domain of two adjacent subunits (Fig. 1A). The α subunit contributes to the principal component while the β subunit constitutes the complementary component. The principal component contains at least three loops (A, B, C) neighbouring the critical residues that have been shown to physically interact with acetylcholine (bold letters, Fig. 1B). In addition, two important loops (D, E) have been identified on the β subunit and their relative position and amino acid sequences at the human β2 and β4 subunits are shown in Fig. 1C.

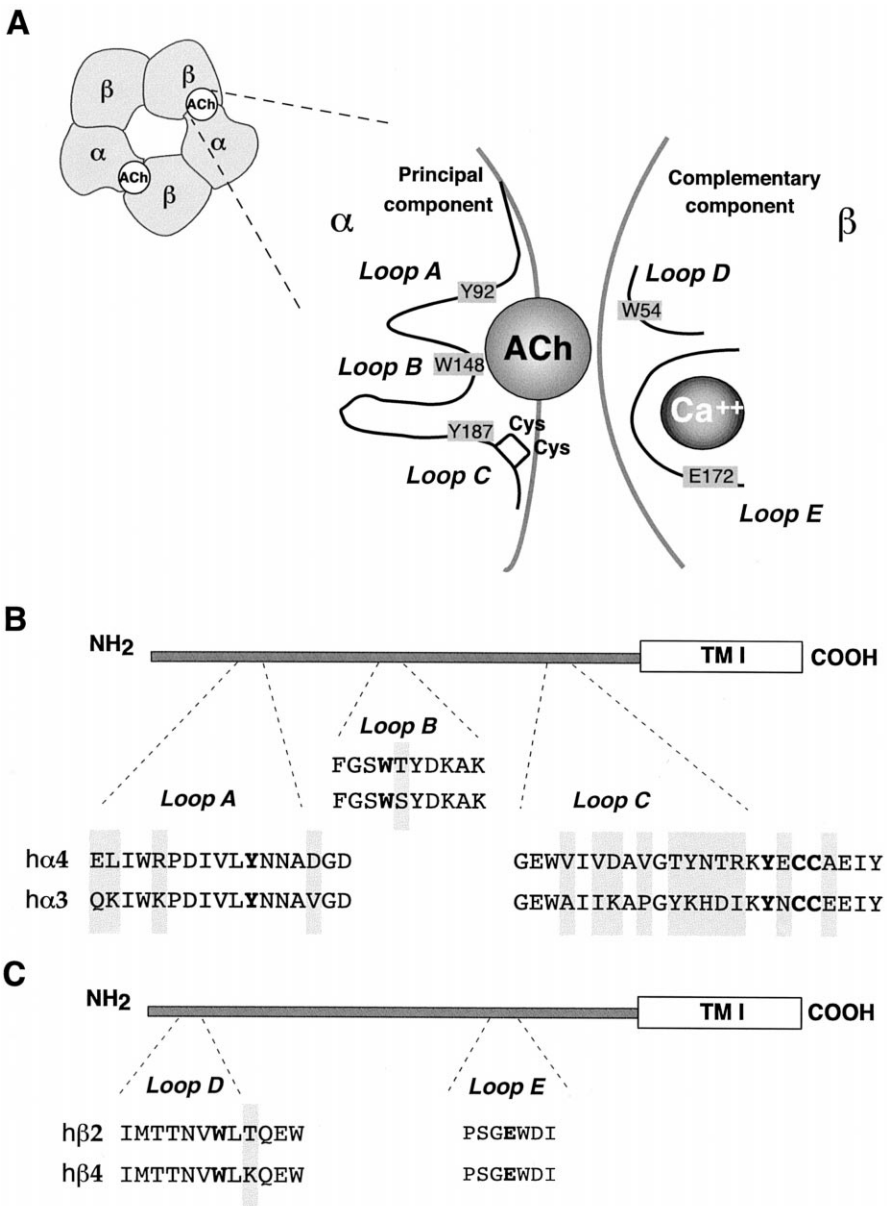

Fig. 1. The principal and complementary components of the acetylcholine binding site. (A) Schematic drawing of the putative organisation of a heteromeric neuronal nicotinic acetylcholine receptor. The five subunits are represented in a 2:3 ratio with the acetylcholine binding site at the interface between an α and the adjacent β subunit. The principal and complementary components are represented in the enlarged section. Loops A, B, and C of the principal component are schematised together with amino acid residues that were shown to participate in acetylcholine binding. The complementary components, contributed by the β subunit, are represented by Loops D and E (corresponding to the calcium binding region). For convenience, amino acid numbering corresponding to those of the chick α7 receptor were kept as reference. (B) The α4 and α3 subunits display important differences in their N-terminal domains. The alignment of the amino acid residues corresponding to loops A, B, and C from the human α4 and α3 are represented. Grey boxes highlight the differences observed between the two subunits while bold characters indicate conserved residues. (C) Protein sequence alignment of the D and E loops from complementary components.

3.2. Both the α and β subunit determine the receptor properties

When characterising receptors reconstituted in expression systems, the physical interaction (binding) or functional interaction (activation leading to ionic currents) of an endogenous agonist (such as acetylcholine) offers a first insight into the possible role of such receptors in vivo. Determination of the dose–response relationship between the agonist concentration and the amount of evoked current therefore constitutes an important step in receptor characterisation.

In agreement with previous findings, we observed that the physiological properties of human nicotinic acetylcholine receptors were defined by both the α and the β subunits (Fig. 2). Receptor reconstituted with α4β2, which is thought to correspond to the major brain combination, displayed an EC_{50} of 8.7 μM ± 8 ($n = 18$). Substitution of the α4 subunit with the α3 subunit caused an increase in the EC_{50} to 38.5 μM ± 17.5 ($n = 11$). Exchange of the β2 for the β4 subunit minimally affected the acetylcholine EC_{50} values. The EC_{50} of α4β4 was 10.7 μM ± 5.1 ($n = 7$) while that of α3β4 was 55 μM ± 37 ($n = 7$).

Comparison of the time course of acetylcholine-evoked currents revealed that α3-containing receptors displayed a greater degree of desensitisation (that is a progressive decrease of the response during steady state exposure to the agonist) than α4-containing receptors. Furthermore, substitution of the β2 with the β4 subunit caused a marked reduction in the desensitisation of either α3- or α4-containing receptors, illustrating that the contribution of the β subunit also influences this property of the receptor.

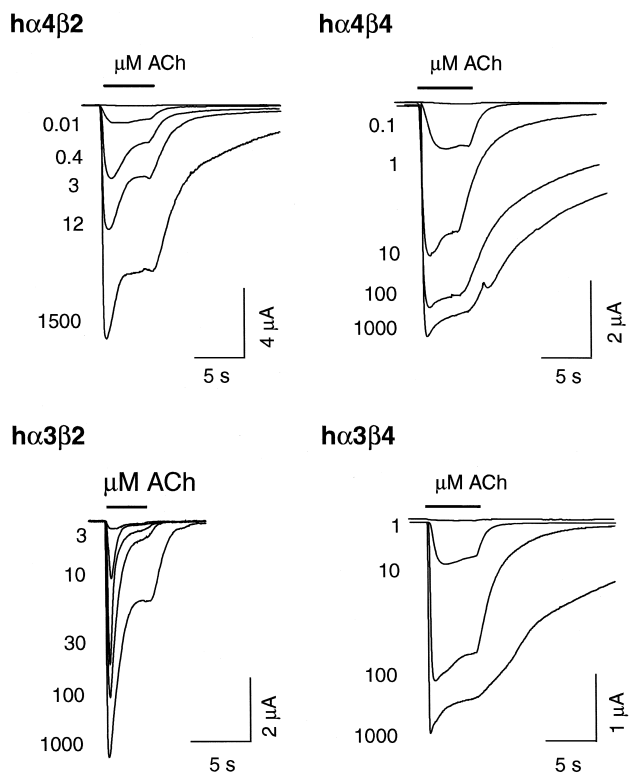

Fig. 2. Both α and β subunits contribute to receptor properties. Currents evoked by several concentrations of acetylcholine in oocytes expressing a pairwise combination of α and β subunits are superimposed. Acetylcholine concentrations are indicated on the left of each current trace while pulse duration and timing are symbolised by the horizontal bars. Agonist test pulses were applied once every 2 min.

3.3. Chimeras: a tool to explore the receptor properties

As shown in Fig. 2, the α3 containing receptors displayed a more important desensitisation and a displaced EC_{50} compared to those containing α4. To take advantage of these differences, a series of chimeras was constructed to further evaluate the properties of these receptors. A schematic representation of the first construct is shown in Fig. 3A. As seen in this figure, a clear dissociation between the receptor sensitivity and desensitisation profile was obtained with this chimera. Introduction to the α4 subunit of the N-terminal domain from α3 (1–202) caused a displacement of the EC_{50} toward a higher α3 value (8.7 μM to 58 ± 7 μM; $n = 4$, right panel Fig. 3A). Exchange of this domain, which encompasses the ligand binding site, had, however, comparably smaller effects on receptor desensitisation (middle trace from left panel Fig. 3A). These data suggest that determinants of acetylcholine sensitivity and desensitisation may be related to distinct amino acid segments. Construction of chimeras with a progressive increase in the α3 corresponding portion further confirmed this observation. Desensitisation profiles progressively resembling those of the α3β2 receptor were obtained as the chimeras contained greater portions of α3 (Fig. 3B).

3.4. The nicotinic acetylcholine receptor: a prototype allosteric protein

Although complex, the physiological and pharmacological properties of the nicotinic acetylcholine receptor, including its activation and desensitisation as well as blockade by competitive antagonists, were readily described using a three-state allosteric model (Fig. 4A). The three states were minimally required to account for the receptor properties: a closed resting state (B, basal), the active open state (A) and the desensitised closed state (D). An interesting and characteristic feature of the allosteric model is that ligand can bind to any of these states. On the basis of this model, agonists are defined as compounds that preferentially stabilise the active A state whereas competitive antagonists are substances that bind to the closed B (basal) or D (desensitised) state (Changeux and Edelstein, 1998).

Another feature of the allosteric model is that transition between states can occur even in the absence of ligand and that the fraction of the receptor in any given state is defined by the ratio of transition probabilities. According to this concept, the characteristics of the receptor are best

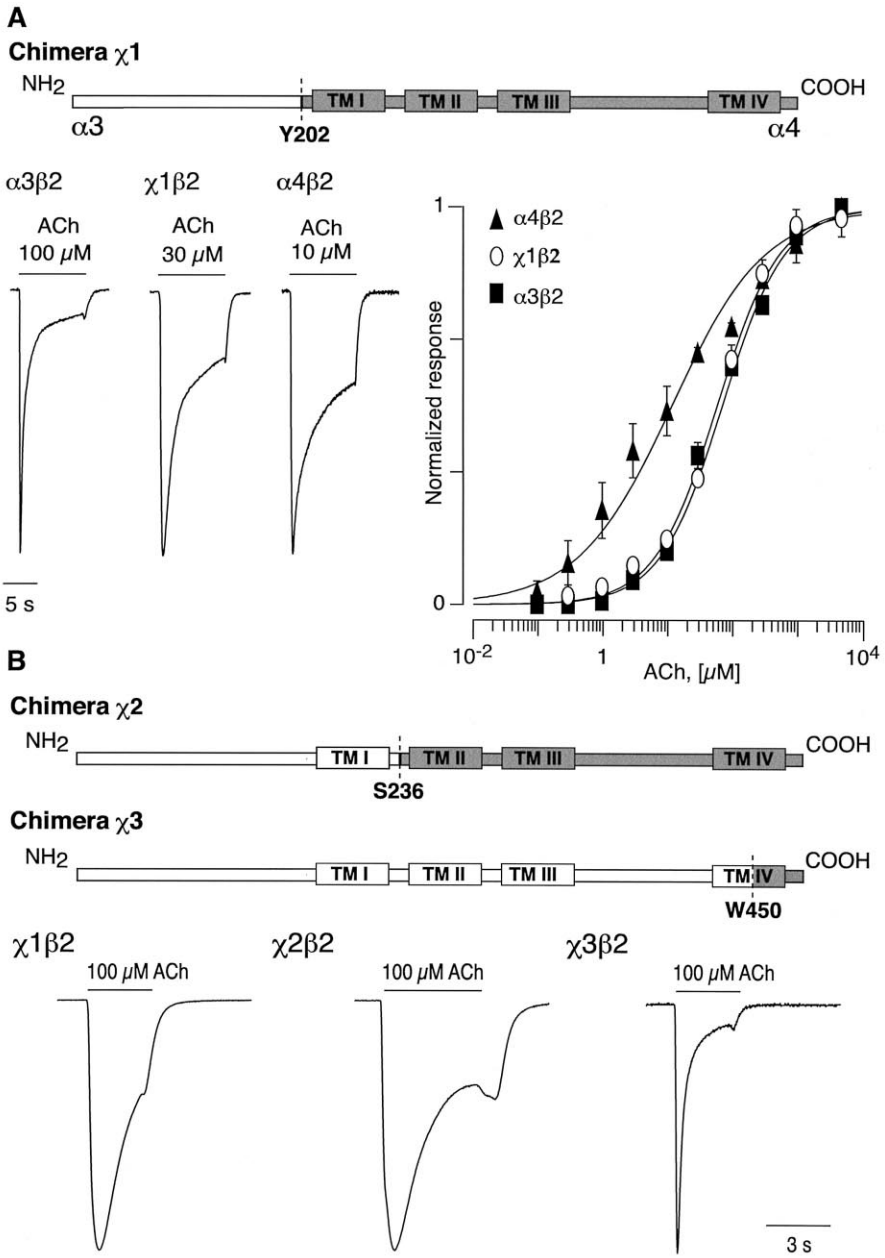

Fig. 3. Chimeras to explore receptor properties. (A) Schematic representation of the α3:α4 construct and typical acetylcholine-evoked currents recorded in oocytes expressing the control α3β2, χ1β2, and α4β2 receptors are represented (left panel). For the best comparison of the response time course, recordings were normalised to unity. Acetylcholine dose–response relationships recorded from control and chimeric receptors are illustrated (right panel). Lines through the data points correspond to the best fit obtained with the empirical Hill equation. The respective EC_{50}s and Hill coefficients were 12 μM, $n_H = 0.6$ for α4β2; 72 μM, $n_H = 0.9$, for α3β2; and 60 μM, $n_H = 0.9$ for χ1β2. (B) The typical acetylcholine-evoked currents recorded in oocytes expressing the χ1β2, χ2β2, and χ3β2 receptors are illustrated. To allow for the comparison of the current time course, responses were normalised to unity. Fusion positions are indicated on the linear representation of the receptor proteins.

defined by an isomerisation coefficient L_0–L_2 that depends upon the overall properties of the protein. Thus, when considering the activation profile of a receptor, it is necessary to examine the properties of both the ligand binding site as well as other possible factors that could modify the transition energy barriers.

From these allosteric principles, it can be hypothesised that the transition from the closed to the open state is critically influenced by the properties of the pore-forming domain. In nicotinic acetylcholine receptors it has been shown that the wall of the ionic pore is formed by the juxtaposition of each of the second transmembrane domains (2) of the subunits that compose a single receptor (Hucho et al., 1986; Giraudat et al., 1989; Miyazawa et al., 1999). Sequence alignment of the α3, α4, β2, and β4 transmembrane domain 2 segments revealed important dif-

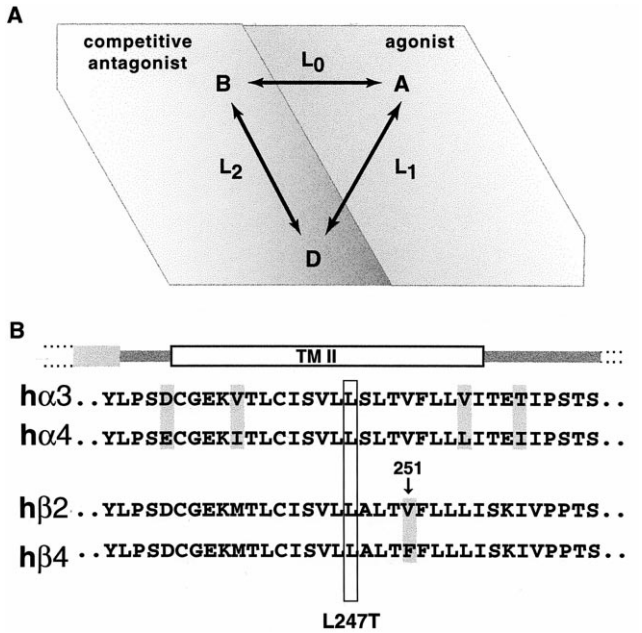

Fig. 4. The neuronal nicotinic acetylcholine receptor: a prototype allosteric protein. (A) The minimal three-state allosteric scheme with the respective isomerisation constants is illustrated. Closed states are represented by the basal (B) and desensitised (D) states whereas the open state corresponds to the active (A) state. Competitive antagonists are compounds that stabilise either the B or D states, whereas agonists are compounds that stabilise the A state. (B) The sequences of the second transmembrane segment of the α3, α4, β2, and β4 subunits are aligned for comparison. Grey boxes highlight the amino acid differences observed between the sequences. The empty rectangle corresponds to the α7 leucine 247.

ferences in the amino acid residues (Fig. 4B). Moreover, the equivalent to α7 V251, which has been shown to play a key role in the receptor energy barrier (Devillers-Thiéry et al., 1992; Galzi et al., 1996), was changed to a phenylalanine in the β4 subunit (arrow).

3.5. The second transmembrane domain: a key determinant

While any mutation can be introduced by site-directed mutagenesis, its relevance to physiological conditions may be argued. Mutations that cause major modifications of receptor function could turn out to be lethal and therefore would never be observed *in vivo*. The analyses of mutants that spontaneously occur in a genetically transmissible form of epilepsy have led to important findings regarding the pharmacological properties of the receptor. One of the mutations is the insertion of three base pairs in the α4 coding gene, which causes the addition of an extra leucine in the transmembrane domain 2 at the outer end of the channel (Fig. 5A) (Steinlein et al., 1997). As shown in Fig. 5B, this insertion caused a decrease in the receptor EC_{50} for cytisine from 2.12 μM ± 1.7; $n_H = 0.8$ (control CT, measured in sibling oocytes) to 0.042 μM ± 0.2; $n_H = 0.7$ (776ins3). This effect was accompanied by an increase in the efficiency, from 0.25 (control CT, filled triangles) to 0.78 (776ins3, open squares), of the partial agonist cytisine. Modifications of the pharmacological profile observed with this mutated subunit can be explained on the basis of the allosteric model, assuming that this mutation predominantly reduces the isomerisation coefficient L_0 but not the coefficient L_1 or L_2.

3.6. Use of non-natural ligands to characterise the binding site

A complement to site-directed mutagenesis experiments to approach the properties of the ligand binding site is to explore the effects caused by non-natural ligands. The discovery of the unusual properties of the frog toxin epibatidine and its way of chemical synthesis opened new and important ways to design new artificial and specific agonists (Daly, 1998; Holladay et al., 1998; Spang et al., 1999). Since some of these compounds were designed for positron emission tomography studies with rodents (Bertrand et al., 1999), all experiments presented herein were carried out with rat receptors.

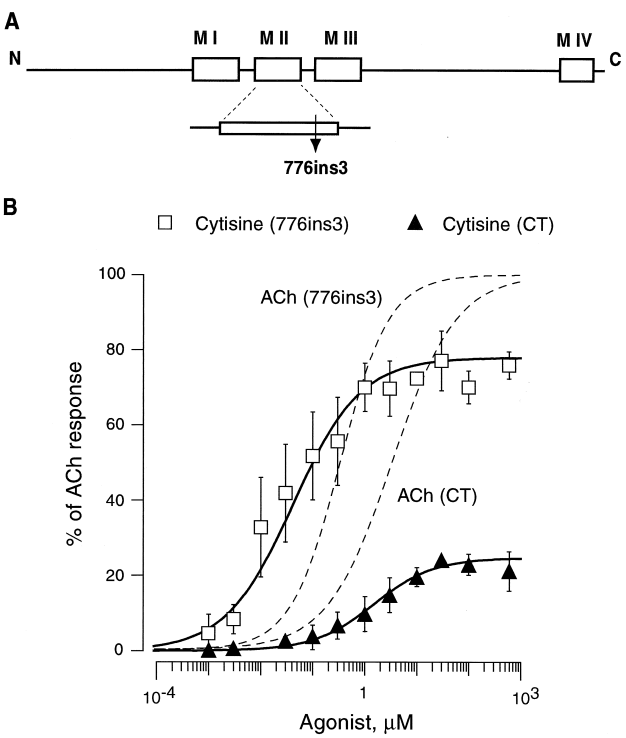

Fig. 5. Spontaneous mutation 776ins3 modifies the receptor pharmacological profile. (A) Schematic representation of the receptor sequence with the four transmembrane domains. Position of the 776ins3 insertion (leucine) relative to the second transmembrane segment is represented in the inset. (B) Leucine insertion increases cytisine efficiency. The cytisine dose–response curves determined in oocytes expressing the control (α4β2 CT, filled triangles) or mutated receptors (α4(776ins3)β2, open squares) were measured. Plot of the peak evoked currents as a function of the agonist concentrations yielded the dose–response relationship. Continuous lines correspond to the best fits obtained with the empirical Hill equation for mean cytisine-evoked currents while dashed lines illustrate the corresponding acetylcholine dose–response curves.

As shown in Fig. 6, which summarises results obtained with an entire group of epibatidine derivatives, even small changes of the chemical structure caused important modifications of the receptor responses. It is important to note that while neuronal nicotinic acetylcholine receptors displayed almost no differences in their sensitivities to the two stereo-enantiomers of epibatidine, a marked difference was observed with methylepibatidine. A 10-fold smaller EC_{50} was observed for the (−) enantiomer compared to the (+) enantiomer at the three receptors tested (Fig. 6B,C,D). Interestingly, however, the efficiency, that is the fraction of current evoked by saturating concentrations of the agonist vs. maximal responses evoked by acetylcholine, of methyl-(−)-epibatidine remained unaltered at α4β2 or α3β4. A distinct picture was obtained with the *exo*-2-(2-pyridyl)-7-azabicyclo[2.2.1]heptane (2PABH). Two modifications of the original epibatidine were made to obtain this compound: (i) first removal of the chloride residue and (ii) displacement of the nitrogen position from meta to para on the pyridine ring (Fig. 6A). These changes caused a marked difference in the properties of the compounds with a reduction of roughly a 100-fold in affinity

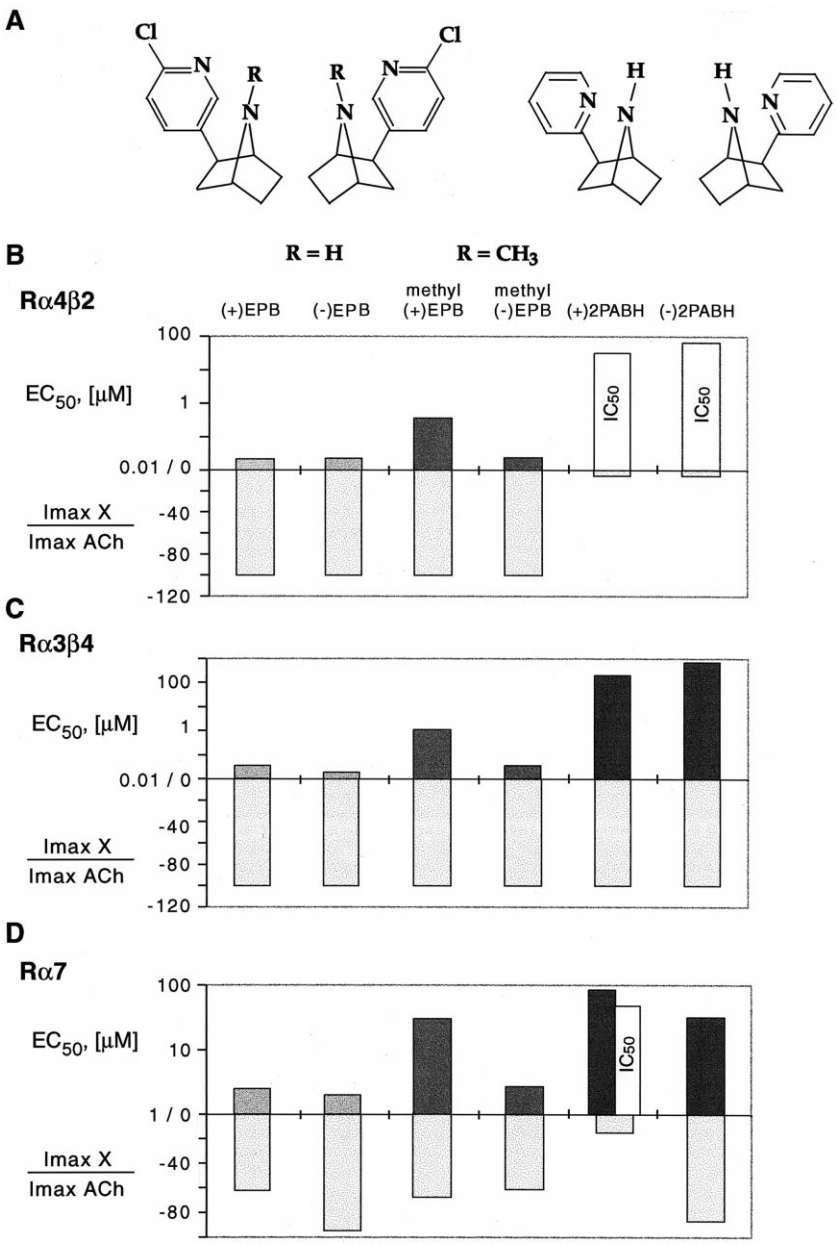

Fig. 6. Properties of non-natural derivatives of epibatidine (EPB). (A) The chemical structures of EPB and two non-natural derivatives. (B, C, D) Effects of EPB, methyl-EPB, and 2PABH on rat α4β2, α3β4, and α7 receptors are compared. The (+) and (−) enantiomers of each of the chemicals have been tested in isolation. The EC_{50} values are indicated for each of the compounds that elicited a current when applied alone. The IC_{50} values, represented by empty boxes, indicate that the compound was found to inhibit the acetylcholine (at EC_{50} concentrations) responses. Note that EC_{50} and IC_{50} values are indicated on a logarithmic scale. Lower grey boxes indicate the respective efficiencies of the different compounds at each of the receptor tested.

and a switch from agonist to antagonist at the α4β2 receptor (Fig. 6B). An even more important change was found at the α7 receptor, with a switch between agonist and antagonist between the (−) and (+) enantiomers (Fig. 6D). Note that (+)-2PABH was still able to evoke a very small fraction of current at both the α4β2 and α7 receptors, suggesting that it probably acts as a partial agonist.

4. Discussion

Ligand-gated channels constitute a large family of proteins that, for a long time, were thought to participate exclusively in the transmission of the electrical influx at chemical synapses. Several lines of evidence have, however, shown that neuronal nicotinic acetylcholine receptors are not solely expressed in the postsynaptic membrane and that some receptors are also present in the presynaptic boutons, perisynaptic membranes or on the axon (Léna et al., 1993; Coggan et al., 1997; Shoop et al., 1999). An additional complexity in understanding of the role of nicotinic receptors is the presence of an entire family of genes coding for α and β subunits and their possible combinatorial expression (Bertrand and Changeux, 1995; McGehee and Role, 1995). While the assessment of protein expression and localisation is an approach to obtain a better understanding of the role of these receptors in the function of the nervous system, another important step is the study of receptors in isolation.

The pairwise expression of the human α4 or α3 subunits with either β2 or β4 subunits confirmed previous findings that the most sensitive receptors are the α4β2 receptors while receptors displaying the fastest desensitisation are those containing α3 and β2 (Couturier et al., 1990; Chavez-Noriega et al., 1997). Results obtained with the α3:α4 chimera further indicated that acetylcholine sensitivity and desensitisation can be separated. Furthermore, the characterisation of these fusion proteins revealed that, as for the chick receptors, functional domains can be interchanged between subunits while conserving receptor function (Gross et al., 1991; Eiselé et al., 1993). When analysed in terms of the allosteric model, this suggests that transition from the B to A state mainly depends on the N-terminal domain of the α3 subunit whereas this does not hold for transition to the D state.

Displacing the fusion point after the first transmembrane segment (1) did not significantly modify desensitisation. A desensitisation profile close to that of the α3β2 receptor was, however, obtained when the fusion point was displaced further towards the fourth transmembrane segment. These results are compatible with previous studies suggesting an important role of the second transmembrane segment (2) in receptor desensitisation and corroborate the allosteric nature of the nicotinic acetylcholine receptor (Bertrand and Changeux, 1995; Edelstein et al., 1996; Changeux and Edelstein, 1998).

While the sensitivity of the receptor to acetylcholine depends upon the N-terminal ligand-binding site, we found that a mutation spontaneously occurring in the second transmembrane domain of the α4 subunit (776ins3) altered the sensitivity to cytisine. These data further illustrate the allosteric nature of the acetylcholine receptor and can be interpreted as a reduction of the isomerisation coefficient L_0, which defines the transition from the basal to the active state, and are in good agreement with results obtained in other studies (Bertrand, 1999).

Non-natural ligands constitute an important complement to study the properties of the ligand-binding site. With the discovery of the frog toxin epibatidine, an important opening was created for the synthesis of non-natural ligands (Daly, 1998). The synthesis and separation of active enantiomers allowed the identification of the major features that confer agonist selectivity to epibatidine derivatives such as methylepibatidine (Bertrand et al., 1999; Spang et al., 1999). Moreover, the observation that modification of the pyridine nitrogen position from meta to para changed epibatidine from a full to a partial agonist or to an antagonist indicates that this new compound, 2PABH, preferentially binds to a non-active state. Comparison of these data with results recently obtained for the *Torpedo* receptor with photoaffinity labelling (Grutter et al., 1999) suggests that the epibatidine quaternary ammonium penetrates more deeply between the α and β subunits to interact with the aromatic residues of the α subunit.

In conclusion, analysis of the properties of natural and modified subunits of the nicotinic acetylcholine receptor demonstrates the crucial role of the N-terminal domain as well as the ionic pore domain. In addition, the use of non-natural epibatidine derivatives illustrates how a small chemical modification can lead to profound changes, with an agonist becoming antagonist. Although difficult to reconcile at first, all these data can be discussed on the basis of a three-state allosteric model. Furthermore, they exemplify how electrophysiological studies can contribute to the development of new drugs of potential therapeutic interest.

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