British Journal of Pharmacology (1998) 125, 751–760
http://www.stockton-press.co.uk/bjp

Properties of neuronal nicotinic acetylcholine receptor mutants from humans suffering from autosomal dominant nocturnal frontal lobe epilepsy

[1]S. Bertrand, [2]S. Weiland, [3]S.F. Berkovic, [2]O.K. Steinlein & [1,4]D. Bertrand

[1]Department of Physiology, Faculty of Medicine, University of Geneva, CH-1211 Geneva 4, Switzerland; [2]Institute of Human Genetics, University of Bonn, Bonn, Germany; [3]Department of Medicine (Neurology), University of Melbourne, Australia

1 Physiological and pharmacological properties of the human neuronal $\alpha 4\beta 2$ nicotinic AChR and mutants found in patients suffering from autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) were studied.

2 Investigations of nicotinic AChRs reconstituted in *Xenopus* oocytes with the control or mutated $\alpha 4$ subunits revealed that both mutation S248F as well as the Leucine insertion (776ins3) result in major but different changes in the physiological and pharmacological properties of the receptors.

3 Mutation S248F causes a decrease in apparent affinity to ACh of about 7 fold. In addition, this receptor already desensitizes during exposure to agonist concentration 3000 times lower than the control.

4 776ins3 provokes a 10 fold increase of apparent ACh affinity, an increase in the IC_{50} caused by prolonged ACh exposures and a slowing down of the response decay.

5 At saturating ACh concentration cells expressing the S248F mutant display average currents that are about five times smaller than control.

6 When measured at very low concentration, agonist sensitivities of the control and mutated receptors to ACh, nicotine and epibatidine exhibit differences that match those observed for higher agonist concentrations.

7 Mutation 776ins3 increases the apparent efficacy to cytisine.

8 Data presented herein suggest that mutation S248F mainly affects the desensitization properties of the receptor while the leucine insertion (776ins3) increases the probability of transition to the active state. Although these mutations differentially affect the receptor properties they both result in reduced permeability to calcium and enhanced desensitization sensitivity that might account for the ADNFLE phenotype.

Keywords: Nicotinic; acetylcholine; receptor; brain; human; epilepsy

Introduction

Brain function mainly depends upon the activity of neuronal membrane proteins that are activated either by voltage, ligands or intracellular messengers. These proteins form aqueous pores through which ions can flow across the membrane and thereby depolarize or hyperpolarize the cell. A large number of proteins coding for these channels have been identified and mutations in the genes coding for some of these proteins have already been demonstrated to be linked to neurological diseases (Engel *et al*., 1996; Shiang *et al*., 1993; Sine *et al*., 1995; Steinlein *et al*., 1995, 1997). The recent findings of mutations in the gene coding for the $\alpha 4$ subunit, which contributes to the major brain neuronal nicotinic acetylcholine receptor (nicotinic AChR), in autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), suggest that alteration of this ligand-gated channel can induce profound modifications of brain function (Steinlein *et al*., 1995, 1997).

The neuronal nicotinic AChRs are integral membrane proteins made, in the case of heteromeric receptors, by the assembly of five subunits in a probable stoichiometry of two alpha and three beta subunits (Bertrand & Changeux, 1995; Lindstrom, 1996). In contrast, homomeric receptors result from the assembly of five identical subunits (Palma *et al*., 1996). Each subunit, which is about 500 amino acids in length, spans the membrane four times and participates both in the formation of the ligand-binding site and the channel wall (Bertrand & Changeux, 1995; Devillers-Thiery *et al*., 1993). The two mutations found, so far, in patients suffering from ADNFLE are both within the second transmembrane domain and have been shown to modify the receptor physiological properties (Steinlein *et al*., 1997; Weiland *et al*., 1996). In ADNFLE seizures oocur almost exclusively at night particularly in stage II of sleep (Hayman *et al*., 1997; Scheffer *et al*., 1995). The cholinergic system is known to have a major effect on sleep modulation (Stériade & Contreras, 1995) and thus, a cholinergic defect is not surprizing in this particular form of human epilepsy.

In this work we investigated the main physiological and pharmacological properties of the control (CT) and these two mutated nicotinic AChRs. Examination of the activation and desensitization properties revealed striking differences between the two mutants. Analysis of these and other differences on the basis of an allosteric model suggest that mutation S248F strongly alters the desensitization properties whereas the leucine insertion (776ins3) increases the probability of the channel to be in the open state.

[4]Author for correspondence.

Methods

cDNAs and mutagenesis

The neuronal $\alpha 4$ and $\beta 2$ subunits have been cloned as previously described into the pSPoD expression vector (Weiland *et al*., 1996). The mutations found in ADNFLE patients were introduced into the $\alpha 4$ cDNA. For this purpose part of the exon 5 was amplified by PCR from patients DNA using the following primers: ACCTGACCAAGGCCCACCTG and GCTCGGGCCAGAAGCGCGG for S248F; CCTGCCCTCCGAGTGTGGC and GCTCGGGCCAGAAGCGCGG for 776ins3. The amplified DNA fragments were then transferred into the $\alpha 4$ CT subunit using *Dra*III/*Mun*I restriction sites both for the 776ins3 and for the S248F mutation. Plasmids with the S248F mutant allele were identified by a PCR assay with one of the primers creating a new *Hpa*II restriction site only in the wild type allele (Steinlein *et al*., 1995). The subclones carrying the 776ins3 allele were identified by SSCA. The presence of both mutations was further confirmed by sequencing.

Oocyte preparation and cDNA injection

Oocytes were prepared according to the standard procedure (Bertrand *et al*., 1991) and nuclear injected with 10 nl of buffer containing equal concentrations (0.1 μg μl^{-1}) of α and β subunits cDNAs. Cells were kept at 18°C in BARTH medium for 2–3 days prior to electrophysiological investigations. To improve cell survival and minimize possible contamination, each oocyte was placed in a separate well of an 8×12 microtiter plate (NUNC).

Drugs and solutions

BARTH medium was made as follows (mM): NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 10, $MgSO_4 \cdot 7H_2O$ 0.82, $Ca(NO_3)_2 \cdot 4H_2O$ 0.33, $CaCl_2 \cdot 6H_2O$ 0.41 (pH 7.4 adjusted with NaOH), and supplemented with antibiotics (kanamycin, 20 μg ml^{-1}, penicillin, 100 μg ml^{-1}, and streptomycin, 100 μg ml^{-1}). To prevent bacterial and fungal contamination this solution was filtered with a 0.2 μM filter and kept sterile. Control medium contained (mM): NaCl 82.5, KCl 2.5, HEPES 5, $CaCl_2$ 2.5, $MgCl_2$ 1 (pH 7.4 adjusted with NaOH). Acetylcholine, (−)-nicotine and (+)-epibatidine were prepared as stock solutions at 0.1 M and stored at −20°C. Drugs were dissolved at final concentrations in control medium immediately before the experiment. All drugs and chemicals were obtained from either Fluka, Sigma or RBI.

Electrophysiological recording

Recordings were made using the two electrode voltage-clamp technique. Electrodes pulled from borosilicate glass were filled with 3 M KCl. A GENECLAMP (Axon Instruments, Forster City, CA, U.S.A.) was used for the recording and current injection. Data were captured on-line on a personal computer (PC) equipped with an analogue to digital converter (AT-MIO16D from National Instrument). During the experiments oocytes were continuously superfused with control solution and drugs were applied using a fast switching solution system consisting of computer controlled electrovalves (type 3, General Valve).

Data analysis

Data analysis was carried off-line on a Macintosh computer (Apple, type 8500/150) using a home-made program. Curves were adjusted using a least square minimization procedure (SIMPLEX).

Agonist affinity Activation curves were adjusted to the empirical Hill equation:

$$y = \frac{1}{1 + \left(\frac{EC_{50}}{x}\right)^{n_H}} \quad (1)$$

where y = the fraction of activated receptors, EC_{50} = the concentration evoking half activation, n_H = the apparent cooperativity, x = agonist concentration.

Slow desensitization Inhibition by low agonist concentrations were adjusted to the empirical Hill equation:

$$y = \frac{1}{1 + \left(\frac{x}{IC_{50}}\right)^{n_H}} \quad (2)$$

where y = the fraction of remaining current, IC_{50} = the concentration causing half inhibition, n_H = the apparent cooperativity, x = agonist concentration.

Agonist sensitivity in the low concentration range Currents plotted as a function of the agonist concentration on a log-log scale were fitted with a straight line adjusted by linear regression.

Desensitization time course Desensitization time constants were fitted to mono-exponential function in the form:

$$y = Ao \cdot e^{-\left(\frac{t}{\tau}\right)} + B \quad (3)$$

where y = ACh-evoked current, Ao = amplitude at time zero, t = time, τ = time constant, B = plateau level.

Allosteric model Curve fitting with the two state allosteric scheme was done using the equation derived from the model proposed by Monod-Wyman-Changeux (Monod *et al*., 1965) which defines that at equilibrium:

$$B \overset{L}{\rightleftharpoons} D$$

$$\bar{D} = \frac{1}{1 + L \cdot \left\{\frac{1 + \frac{x}{K_B}}{1 + \frac{x}{K_D}}\right\}^n} \quad (4)$$

where $\bar{D}$ = fraction of receptors in the desensitized (closed) state, x = agonist concentration, L = isomerization constant between B (basal) and D (desensitized), K_D and K_B = intrinsic affinity constants and n = the number of acetylcholine binding sites. From this relationship it follows that the amount of activatable receptors in the B state corresponds to $1 - \bar{D}$.

All values indicated throughout the text are given with their respective standard deviations (s.d.). Whenever possible significance of the results were assessed using two-sample *t*-test (computed in EXCEL, Microsoft).

Results

Activation and desensitization of $\alpha 4\beta 2$ nicotinic AChR

To assess the physiological properties of CT and mutant receptors their respective activation and desensitization properties were investigated. Oocytes expressing nicotinic AChRs were first challenged with different acetylcholine

(ACh) concentrations applied in gradual order and their dose-response relationships were determined by plotting the peak current as a function of the logarithm of the agonist concentration (squares, Figure 1a, d and g). Measurements done for the CT, 776ins3 and S248F nicotinic AChR yielded an apparent affinity of 2, 0.28 and 19.8 μM and a Hill

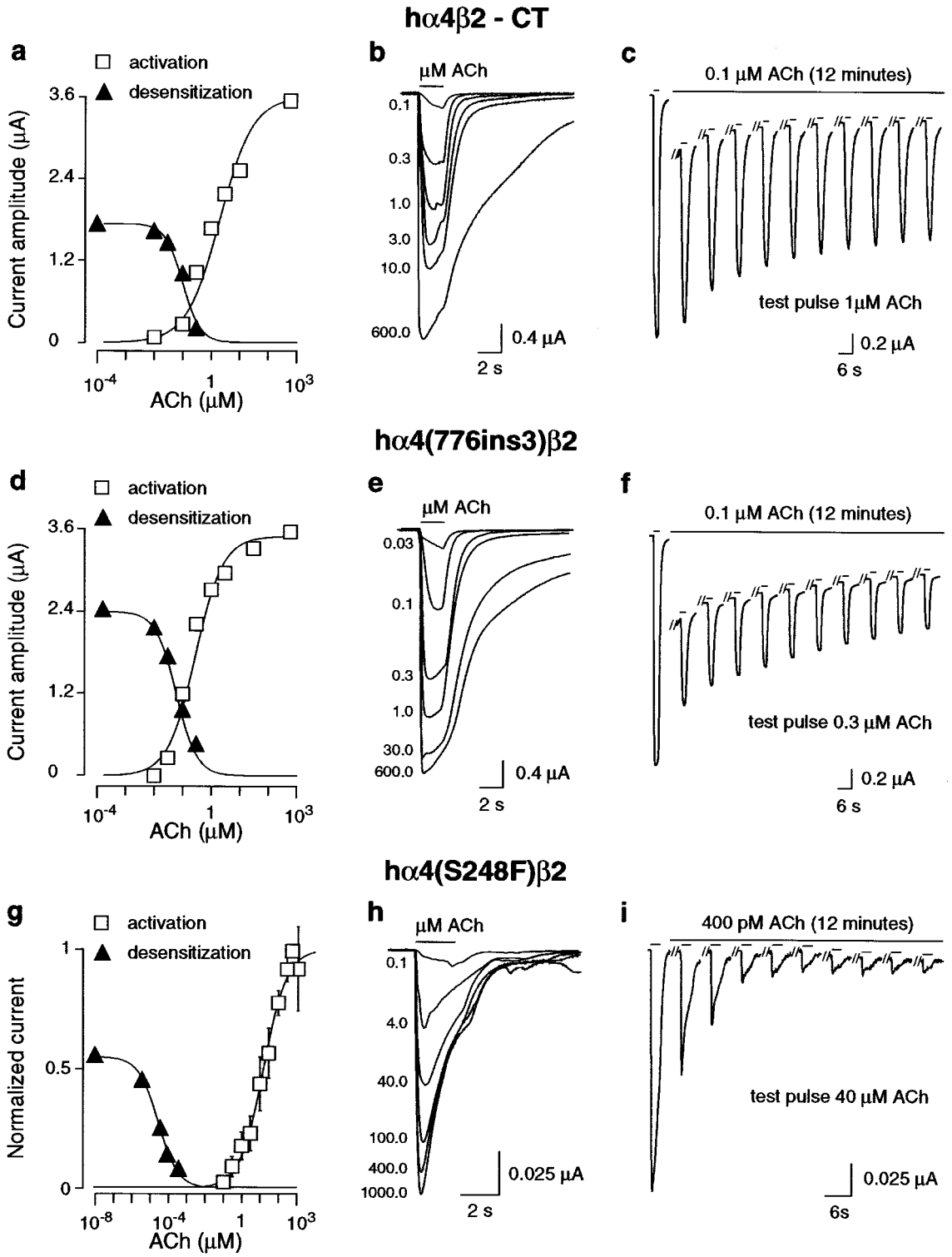

Figure 1 Mutations in the channel domain induce pleiotropic effects on the receptor properties. (a, d, g) Dose-response curves for activation and desensitization measured in cells expressing CT, 776ins3 and S248F containing receptor. Bars represented in g indicate the standard errors obtained for $n=9$. Peak currents evoked by a 2 s ACh pulse were plotted as a function of the agonist concentration. Responses evoked by such ACh pulses are shown in (b, e, and h). To minimize the effects of desensitization ACh pulses were applied at up to 5 min intervals. Apparent EC_{50}'s and Hill coefficients are respectively of: 2 μM and 0.75 for the CT (a), 0.28 μM and 1 for the 776ins3 (d) and 19.8 μM and 0.7 for the S248F (g). Continuous lines correspond to values computed with the Hill equation (1) Desensitization was measured by reporting the current evoked by a short test pulse of ACh (2 s, near the EC_{50}) at the end (12 min) of a steady application of low agonist. IC_{50}'s and Hill coefficients are: 0.1 μM and 1.5 for the CT (a), 0.06 μM and 1.3 for the 776ins3 (d) and 28 pM and 0.8 for the S248F (g). Curves through the data points were computed with the empirical Hill equation (2). (c, f and i) Desensitization protocols are illustrated. Cells were held at -100 mV throughout the experiment. Bars above the currents indicate the time of the drug applications.

coefficient (n_H) of 0.75, 1 and 0.7, respectively. Currents recorded in response to different ACh concentrations are superimposed in Figure 1b (control), e (776ins3), and h (S248F). To investigate desensitization of the nicotinic AChR, a protocol of prolonged exposure to a low agonist concentration was designed. Namely, cell responses were tested at regular intervals by a short pulse of ACh (near the EC_{50}, 2 s) first in control solution and then during a prolonged application of agonist. While stabilization of desensitization depended upon the agonist concentration, an equilibrium was obtained at least within 12 min. Typical recordings obtained for the CT and the mutant receptors using this protocol are illustrated in Figure 1c, f and i. Plots of the peak current evoked by the test pulse at the end of the incubation protocol as a function of the desensitizing agonist concentration resulted in a sigmoidal inhibition curve as shown for the CT in Figure 1a (triangles). These data were fitted with the Hill equation (2) and yielded an apparent inhibition value (IC_{50}) and a cooperativity coefficient (n_H).

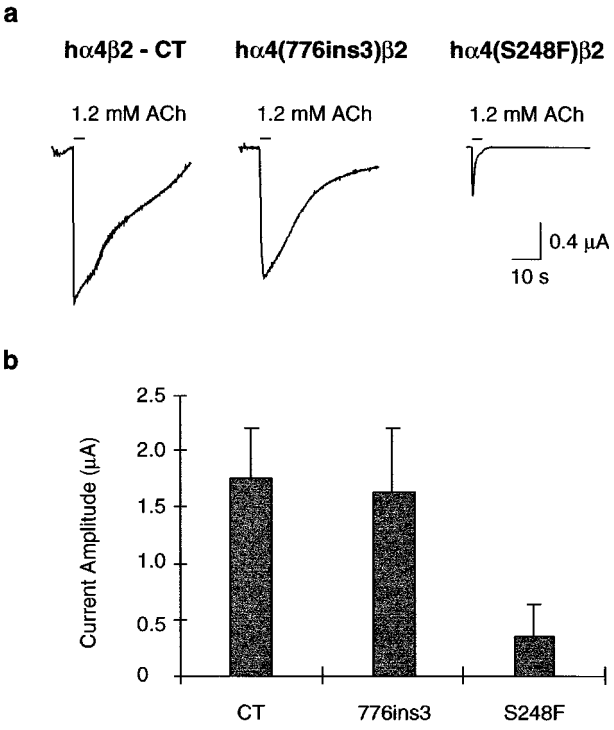

Figure 2 Mutations affect the maximal ACh-evoked current. (a) Typical currents recorded in oocytes expressing (from left to right) the CT h$\alpha4\beta2$ receptor, the hα4(776ins3)$\beta2$ or the hα4(S248F)$\beta2$ receptor combinations are shown. Currents were evoked by a saturating (1.2 mM) ACh pulse (3 s). Bars indicate the time of application. (b) Mean current amplitudes recorded in several oocytes in the same conditions as in (a).

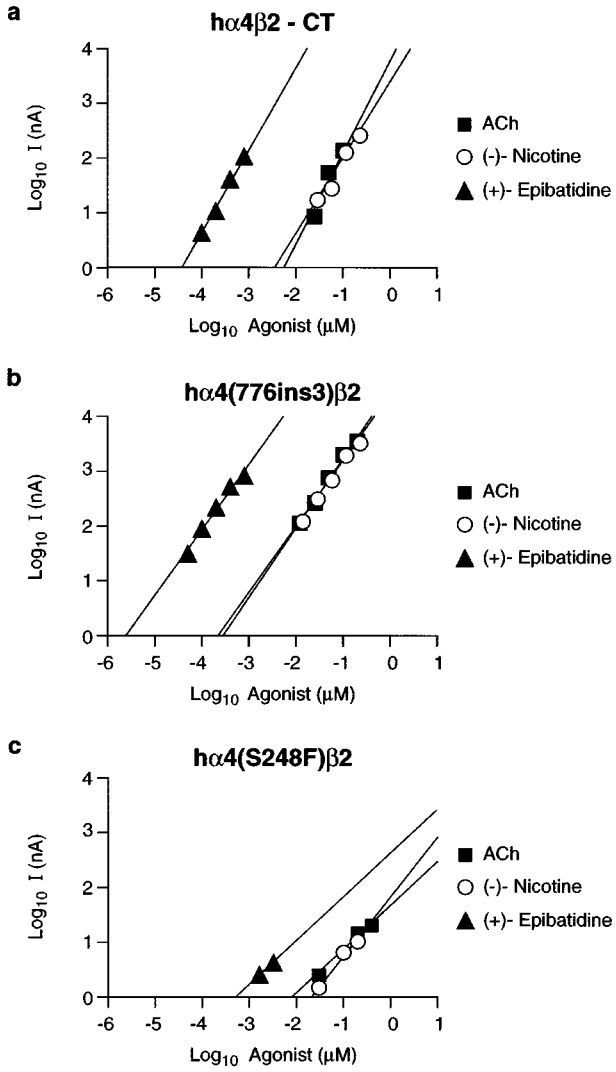

Figure 3 Currents evoked by low concentrations of three different agonists. (a) Currents recorded in a single oocyte expressing the CT receptors in response to long exposures (several seconds) to ACh, nicotine and epibatidine are plotted on a log-log scale as a function of the agonist concentration. (b and c) Plots of the currents recorded in the same conditions as in (a) for the 776ins3 or S248F containing receptors. Straight lines were computed by linear regressions. Values for the slopes and offsets are presented in Table 2.

Table 1 Pharmacological properties of h$\alpha4\beta2$ AChR and its mutants

cDNA type	*EC_{50} ACh* (μM)	n_H	*IC_{50} ACh* (μM)	n_H	*EC_{50} cytisine* (μM)	n_H	*IMax ACh* (nA)
h$\alpha4\beta2$-CT	3.4 ± 2.7 ($n=10$)	0.70 ± 0.05	0.11 ± 0.02 ($n=4$)	1.4 ± 0.15	2.12 ± 1.7 ($n=9$)	0.79 ± 0.14	1762 ± 439 ($n=15$)
hα4(776ins3)$\beta2$	0.34 ± 0.2 ($n=9$) $P=0.002$	0.92 ± 0.20	0.038 ± 0.016 ($n=5$) $P=0.011$	1.2 ± 0.11	0.042 ± 0.02 ($n=5$) $P=0.006$	0.67 ± 0.08	1624 ± 569 ($n=22$) $P=0.4$
hα4(S248F)$\beta2$	19.8 ± 7.0 ($n=9$) $P=7.4\times10^{-5}$	0.70 ± 0.09	0.000028 ($n=2$) $P=0.005$	0.8	1.4 ($n=2$) $P=0.26$	0.9	357 ± 276 ($n=12$) $P=4\times10^{-10}$

All measurements were done at a holding potential of –100 mV. Results are presented as mean $\pm$ s.d., values in () indicate the number of cells tested in each condition. *P* values indicate the significance of the differences versus control values computed with the *t*-test (see Methods).

For the CT receptor, desensitization is observed only when an agonist is applied that evokes a detectable current. Mean values obtained in several cells, for both activation and desensitization are summarized in Table 1.

As described previously the activation curve of the 776ins3 mutant displays a shift of about 10 fold toward higher sensitivity that is accompanied by a small shift of the desensitization IC_{50}. In contrast, oocytes expressing the S248F mutant show a marked difference in both activation and desensitization. While this mutant displays a lower apparent affinity to ACh of roughly 7 fold, its desensitization is shifted toward lower ACh concentrations by a factor of 3000 or more. In addition, the absence of overlap between the activation and desensitization curves is notable (Figure 1g). In agreement with previous observations ACh-evoked currents recorded in this mutant (Figure 1h) show a high degree of desensitization (Weiland *et al*., 1996). It is important to note that given their small response amplitude and extremely high desensitization oocytes expressing the S248F mutant are much more difficult to study than those with CT or 776ins3 mutant. Thus, although complete activation and desensitization profiles were determined on only two cells single points obtained from several other measurements have confirmed the EC_{50} and IC_{50} reported in Table 1.

Table 2 Relative sensitivity and cooperativity to three agonists

cDNA type	*Epibatidine*		*Acetylcholine*		*Nicotine*		ΔLog_{10} *intercept Epi-ACh*	ΔLog_{10} *intercept Epi-Nico*
	n_H	*Log_{10} intercept*	n_H	*Log_{10} intercept*	n_H	*Log_{10} intercept*		
hα4β2-CT	1.21±0.17 (n=6)	−5.13±0.32	1.2±0.27 (n=6)	−3.16±0.47	1.24±0.27 (n=6)	−3.10±0.62	−1.97	−2.03
hα4(776ins3)β2	1.12±0.13 (n=6)	−5.63±0.4	1.15±0.14 (n=6)	−3.55±0.36	1.12±0.22 (n=6)	−3.62±0.64	−2.08	−2.01
hα4(S248F)β2	1.25±0.78 (n=3)	−3.70±0.8	1.05±0.38 (n=6)	−2.1±0.8	0.71±0.3 (n=5)	−3.12±1.48	−1.60	−0.58

Slopes and intercepts of lines passing through data points (Figure 3) are presented as mean±s.d., values in () indicate the number of cells tested in each condition. Correlation coefficients were of 0.988±0.08 for all 50 linear regression computed.

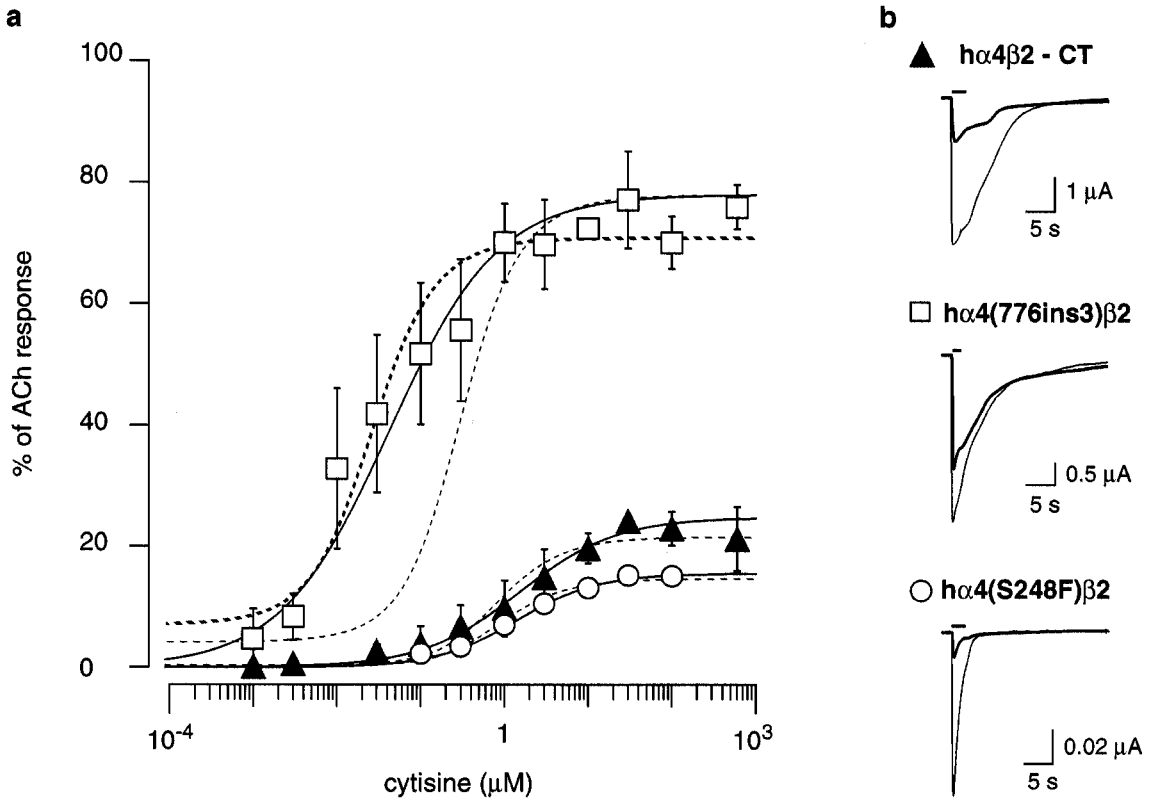

Figure 4 Leucine insertion 776ins3 changes the pharmacological profile of the nicotinic AChR. (a) Currents evoked by several concentrations of cytisine are plotted as a function of the logarithm of the agonist concentration for CT, 776ins3 and S248F. Values were normalized with respect to the currents evoked by saturating ACh concentration (600 μM). Lines through the data points are best fitted with the Hill equation (1). Respective EC_{50}'s and Hill coefficients are: 0.042 μM 0.67 for the 776ins3, 2.12 μM, 0.79 for the CT and 1.4 μM, 0.9 for the S248F. Thin dashed lines indicate theoretical data computed with a two state allosteric model using the following coefficients: K_A=0.0555 μM, K_B=0.5 μM, n=2 and L_1=23 for the 776ins3, L_1=295 for the CT and L_1=472 for the S248F. Thick dashed line corresponds to the best fit obtained with the allosteric equation without constrain on the K values. Constants were: L_1=13, K_A=0.03 μM, K_B=0.0006 μM. Cells were held at −100 mV throughout the experiment. (b) Typical currents evoked by a saturating concentration of ACh (600 μM) and cytisine (600 μM) for 1 or 2 s indicated by horizontal bars in the three constructs are illustrated.

Maximal ACh-evoked current in α4β2 control and mutant receptors

In view of the low amplitude of the currents recorded in the S248F expressing oocytes, a large number of cells were tested to determine the possible variations in saturating ACh-evoked currents. Typical currents evoked by a short pulse of saturating ACh concentration (1.2 mM, 3 s) recorded in the same batch of oocytes for the CT, 776ins3 and S248F constructs are shown in Figure 2a. Mean current values obtained for the same experimental protocol in 15, 22 and 12 cells, for the CT, the 776ins3, and S248F receptors respectively, are illustrated in

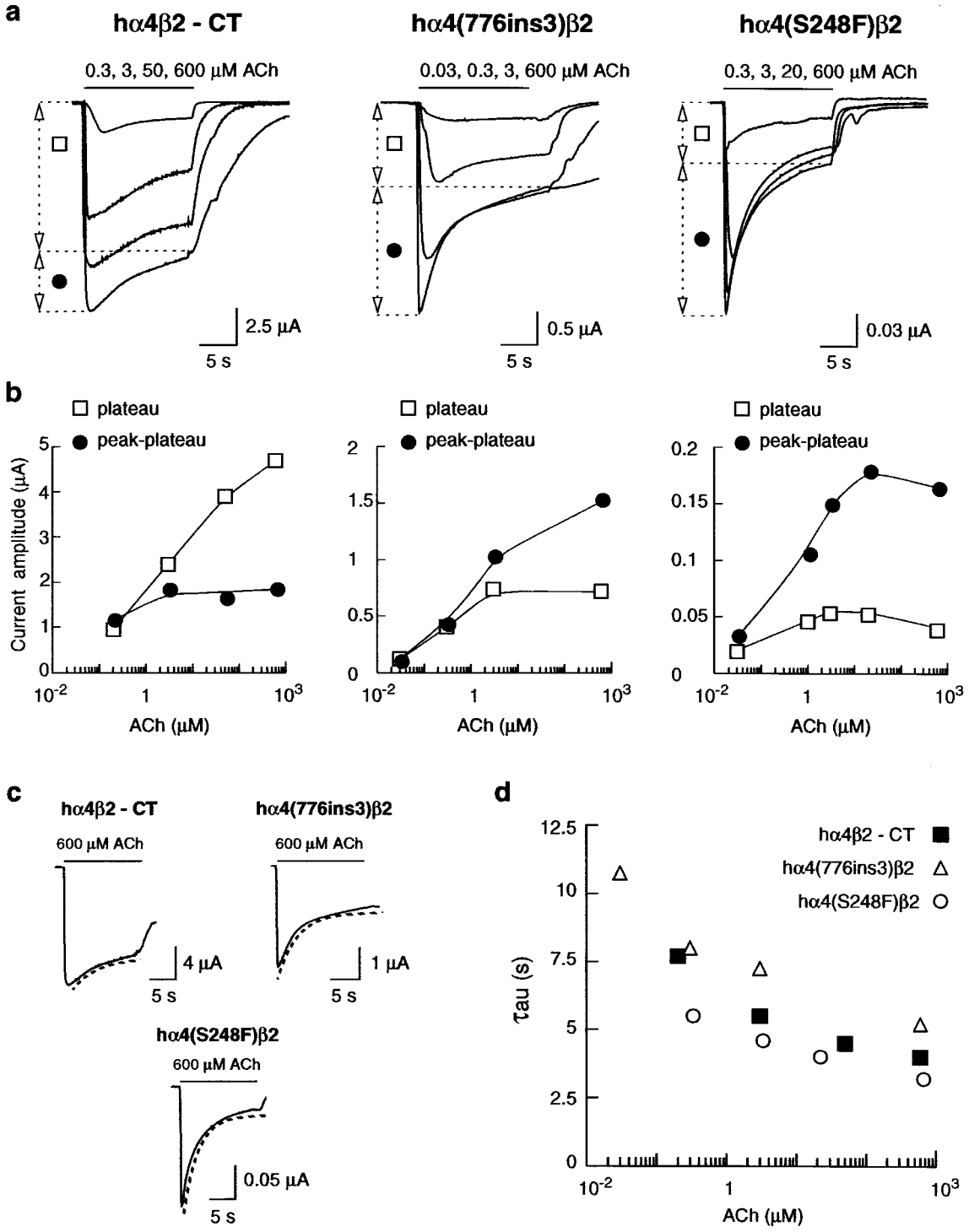

Figure 5 Mutations in the channel domain alter desensitization to ACh. (a) Typical currents recorded in response to four ACh pulses (15 s) in the CT, 776ins3 and S248F containing nicotinic AChRs. Bars indicate the application time. Dashed lines and squares, indicate the level taken to measure the amplitude of the plateau response for the maximal ACh concentration whereas dashed lines and circles indicate the corresponding peaks minus plateau values. Cells were held in voltage clamp at −100 mV. (b) Plots of the peak-plateau and plateau values measured as indicated in (a) as a function of the logarithm of ACh concentration. Lines through data points were drawn to illustrate the results. (c) Typical currents recorded as in (a) are shown with the curve fitting of the decay time. Dashed lines below the current traces correspond to a mono-exponential function (equation 3). (d) Time constant of the decay from peak to plateau are plotted as a function of the logarithm of the ACh concentration.

Figure 2b and Table 1. Similar results were observed in all the oocyte batches tested with these constructs.

Agonist pharmacological profile

Relative sensitivity of a given receptor to several agonists is easily quantified by examining the amplitude of currents evoked by very low concentrations. Under these conditions, plot of the logarithm of the current measured at steady state as a function of the logarithm of the agonist concentration yields a straight line. The slope of this line corresponds to the Hill coefficient. Data presented in Figure 3a illustrates the relationships obtained for ACh (squares), (−)-nicotine (circles) and (+)-epibatidine (triangles) in an oocyte expressing CT nicotinic AChR. The horizontal shift observed between ACh or nicotine and (+)-epibatidine reflects the difference in apparent affinity of the $\alpha4\beta2$ nicotinic AChR to these three compounds. While (+)-epibatidine is about 100 fold more potent than ACh and/or nicotine the apparent Hill coefficient remains constant. Data obtained for the 776ins3 and S248F mutants are shown in Figure 3b and c. Mean values obtained with this protocol in several cells are recapitulated in Table 2.

Leucine insertion increases the efficacy of a partial agonist

It is known that cytisine is a weak agonist of the human $\alpha4\beta2$ nicotinic AChR that can evoke only a fraction of the ACh responses (Buisson *et al*., 1996). Since it was shown that conditions which affect the probability of the transition to the active (open) state also affect the pharmacological profile of a given receptor (Krause *et al*., 1998) we investigated if the 776ins3 mutation induced any detectable changes of the receptor sensitivity to cytisine. Currents evoked by short pulses of cytisine were measured in several cells and normalized to the maximal ACh response (Figure 4a). Typical traces recorded in one of the cells tested for ACh and cytisine are shown in Figure 4b. In CT receptors cytisine evoked only about 24.77% ± 15 ($n=9$) of ACh currents while 776ins3 receptors exhibited a ratio of 78.2% ± 9.2 ($n=5$) and S248F receptors 15.5% ($n=2$). Oocytes expressing the S248F mutant displayed constantly smaller current amplitude when challenged with cytisine which is confirmed by the mean dose-response values obtained for two cells over a broad range of cytisine concentrations. Furthermore, a shift of the apparent affinity to cytisine from an EC_{50} of 2.12 μM in CT receptors to 0.042 μM in the 776ins3 receptor was observed. These data illustrate that the leucine insertion in the channel domain modifies the cytisine pharmacological profile of the receptor. Although a modification of the isomerization L_1 value can explain an important part of the effects caused by the leucine insertion theoretical values plotted in Figure 4 illustrate that adjustment of the intrinsic affinities K_A and K_B are also necessary to achieve the best fit.

Time course of desensitization

To quantify the modifications of the response desensitization, analysis of the current decay observed during a 15 s exposure to various concentrations of ACh were examined. Typical recordings obtained for four different ACh concentrations in the CT, 776ins3 and S248F receptors are shown in Figure 5a. Measures of the plateau (squares) and delta currents (circles) between peak and plateau, give a first estimation of the fraction of desensitization occurring during these brief exposures to agonist. Plots of these values as a function of the ACh concentrations for the three constructs illustrate the difference in behaviour between these receptors (Figure 5b). The CT, which is a low desensitizing receptor, displays a higher ratio of plateau versus delta (peak-plateau) current while contrary to this the S248F mutant shows a small plateau but large delta values. The 776ins3 being an intermediate between these two extremes. The difference in desensitization time course was further analysed by measuring the response decay time constant approximated as a single exponential function (see Equation 3). Dashed curves plotted in Figure 5c illustrate the good agreement between the experimental data and these theoretical values. Plotting of the time constants (τ) as a function of the ACh concentrations reveal that, as expected from the previous observations, the S248F containing receptor (circles) presents a decay time course faster than any other receptor at saturating ACh concentration (Figure 5d, and Table 3). In contrast, the 776ins3 mutant displays a slower desensitization rate.

Modeling of the desensitization

A typical model that can be proposed to take into account the desensitization of nicotinic AChRs, comprises at least three states which are: the basal resting (B, closed) , the active (A, open) and the desensitized (D, closed) states (Figure 6a). Assuming that the nicotinic AChR behaves as an allosteric protein and that in absence of agonist the fraction of receptors in the A state is negligible but that the B and D states are at equilibrium, it follows that the amplitude of the response to an ACh test pulse can be described by 1 − D (Equation 4). Data presented in Figure 6b, c and d illustrate that experimental values are adequately described by this equation for the CT,

Table 3 Decay time constants of ACh-evoked currents

cDNA type			τau (s)			
ACh (μM)	0.03	0.3	3	20	50	600
h$\alpha4\beta2$-CT	n.d.	7.7 ± 0.8 ($n=6$)	5.4 ± 1.2 ($n=6$)	n.d.	4.4 ± 0.7 ($n=6$)	4.0 ± 0.6 ($n=6$)
h$\alpha4$(776ins3)$\beta2$	10.8 ± 1.5 ($n=4$)	8.0 ± 0.8 ($n=4$)	7.2 ± 1.0 ($n=4$)	n.d.	n.d.	5.2 ± 0.9 ($n=4$) $P=0.08$
h$\alpha4$(S248F)$\beta2$	n.d.	5.5 ($n=2$)	4.7 ± 0.8 ($n=7$)	4.0 ± 0.4 ($n=3$)	n.d.	3.05 ± 0.56 ($n=6$) $P=0.02$

Results obtained from curve fitting of data shown in Figure 4 are presented as mean ± s.d., values in () indicate the number of cells tested in each condition. n.d. = not determined. *P* values indicate the significance of differences versus control values computed with the *t*-test (see Methods).

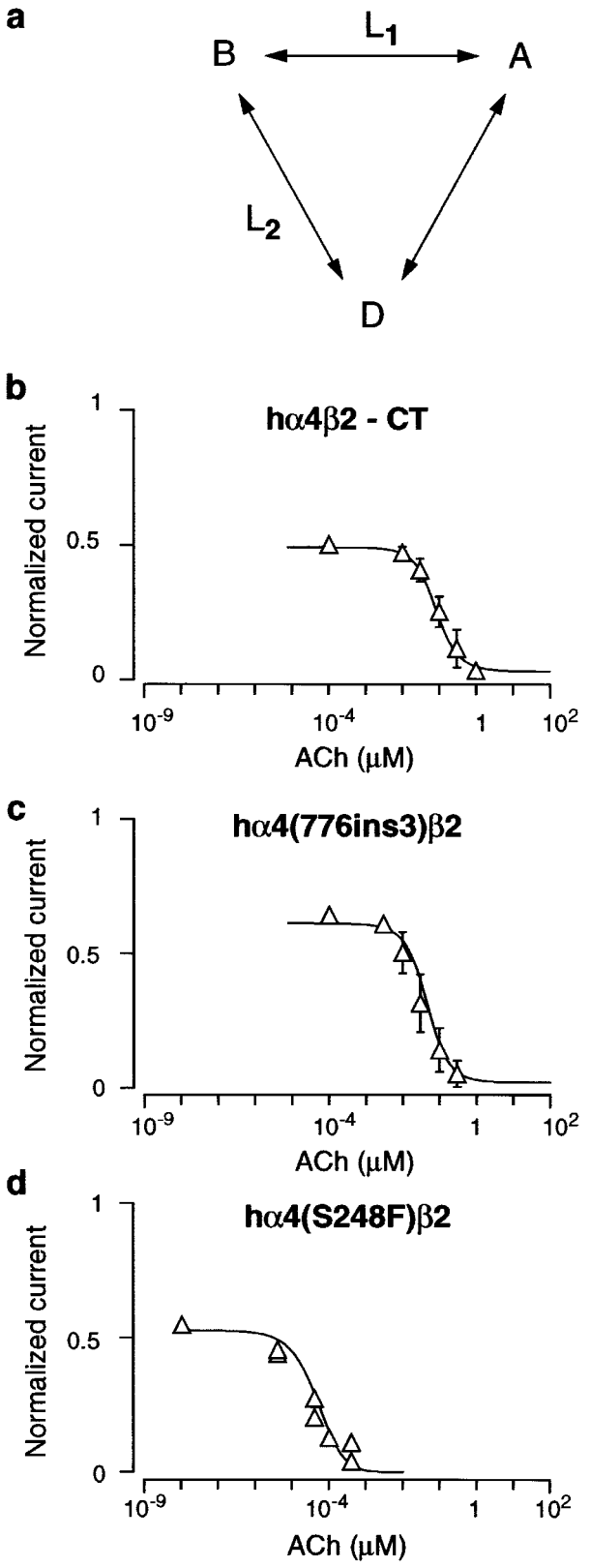

Figure 6 Allosteric modeling of the desensitization. (a) Three state allosteric model where B corresponds to the basal (closed) state, A to the active (open) state and D to the desensitized (closed) state. (b, c and d) Modeling of the desensitization for the CT, 776ins3 and S248F mutants. Continuous lines were computed using a two state model corresponding to the B and D state (equation 4). Symbols correspond to mean values of data obtained as in Figure 1. Bars indicate standard deviations and respective number of cells were b ($n=4$), c ($n=5$) and d ($n=2$). Values for the allosteric curve fitting were $K_B=0.28$ μM, $K_D=0.01$ μM, $L_2=50$ for the CT, $K_B=0.28$ μM, $K_D=0.01$ μM, $L_2=20$ for the 776ins3 whereas $K_B=1$ μM, $K_D=0.00011$ μM, $L_2=0.1$ for the S248F mutation.

776ins3 and S248F containing α4 subunit. Although curve fitting of the CT and 776ins3 data can be done by adjustment of the isomerization coefficient (L_2), results obtained with the S248F mutant require important modifications of both the L_2 as well as K_B and K_D coefficients.

Discussion

The aim of this work was to better characterize the similarities and differences in the physiological and pharmacological properties of nicotinic AChRs reconstituted from human CT and spontaneous mutant subunits found in ADNFLE patients. For this purpose, neuronal nicotinic AChRs were expressed in *Xenopus* oocytes and their properties examined using the dual voltage-clamp technique.

Activation and desensitization

Dose–response curves determined in oocytes expressing the $\alpha4\beta2$ CT nicotinic AChR displayed an apparent affinity that matches rather well the apparent affinity found in transfected HEK-293 cells (Buisson *et al*., 1996). However, the steepness of the curve or apparent cooperativity, was lower than in transfected HEK-293 cells. Since drug application in an oocyte system, using the whole cell recording, is significantly slower than in patch-clamp experiments, receptor desensitization may partly mask the responses and thus reduce the apparent cooperativity. In agreement with this hypothesis, the apparent cooperativity determined in the low agonist concentration range was significantly higher than the value deduced for higher concentrations. This conclusion was already proposed in earlier experiments done with oocytes (Covernton *et al*., 1994).

Although activation curves may be partly impaired by the desensitization, it is of value to note that 776ins3 results in an increase of the apparent affinity whereas mutation S248F causes a small decrease of the EC_{50}. These data suggest a differential effect of the two mutations on the receptor properties.

Investigation of the receptor desensitization properties, using a protocol similar to that described previously (Corringer *et al*., 1998), revealed striking differences between the two mutants. Whilst the 776ins3 mutation affects the IC_{50} to ACh rather little, desensitization of the S248F mutant occurs at concentrations roughly 3000 times lower than those needed to induce comparable effects in the CT nicotinic AChR. By analogy to other studies, the marked differences in the IC_{50} and EC_{50} can be attributed to an increase in the probability of transition from the basal B to the desensitized D state.

S248F presents lower ACh-evoked currents at saturating concentration

The lower current amplitudes evoked by saturating ACh concentrations observed in the S248F versus the CT receptor can either be attributed to a lower density of proteins on the cell surface or to a lower current carried by this receptor. In agreement with this second hypothesis it was recently shown that single channel recordings of the S248F mutant display a significantly lower conductance than the CT (Kuryatov *et al*., 1997). Taken together these data suggest that mutation S248F does not impair protein synthesis or expression at the cell surface but rather the properties of the receptors ionic pore.

Agonists pharmacological profile

The $\alpha 4\beta 2$ nicotinic AChRs have been shown to display a roughly equal sensitivity to ACh and nicotine but a different sensitivity to the frog toxin epibatidine. To assess the relative sensitivity of the CT and mutated receptors to these three agonists, with a minimum effect of desensitization, responses to pulses of very low agonist concentrations were measured. A direct comparison of the sensitivity is given by measuring the current amplitudes in the same oocyte. From these measurements (see Figure 3) it is clear that ACh and nicotine cannot be distinguished in any of the three receptor types and display equivalent apparent affinity and cooperativity. As expected, however, responses of the same amplitude were evoked by epibatidine concentrations about a hundred times lower than ACh. While the 776ins3 receptor displays a higher apparent affinity for ACh, a comparable horizontal translation between ACh and epibatidine responses is observed for the CT and 776ins3 mutant suggesting that this mutation affects the receptor allosteric properties without modifying the ligand binding site itself. Although results obtained for the S248F mutant are less clear, no significant differences are observed between ACh and nicotine and this receptor also shows a higher apparent affinity for epibatidine. Results obtained for epibatidine are, however, partly impaired by the extreme desensitization of the S248F receptor even at the lowest concentrations.

Leucine insertion modifies the cytisine efficacy

Examination of the response evoked by the partial agonist cytisine in the CT and 776ins3 mutant unveiled a striking difference in the current amplitudes with the mutant being more responsive than the CT. Although at first surprising, this difference can easily be explained on the basis of allosteric modeling. Assuming that the leucine insertion in the upper portion of the channel domain reduces the isomerization coefficient (L_1) between the basal (B, closed) and active (A, open) state, it follows that compounds that partially stabilize the A state may become more effective (Galzi *et al*., 1996). A similar effect can be observed under the influence of a positive effector (Krause *et al*., 1998). In contrast, currents evoked by cytisine in the S248F containing receptor are slightly reduced. These data reinforce the hypothesis that modifications observed for the S248F mutant cannot be attributed to a reduction of the L_1 coefficient. As described above, further interpretation of the allosteric properties of the nicotinic AChR suggest that the S248F mutation stabilizes the desensitized (D, closed) state.

Time course of desensitization

Evaluation of the desensitization during different ACh concentrations confirmed that the S248F containing receptor displays the fastest kinetic and exhibits the largest difference between the peak and plateau which confirms that its equilibrium must be displaced in favour of the desensitized state. By comparison, the 776ins3 mutant displays a desensitization rate slower than the CT (see Table 3).

Physiological implications of the nicotinic AChR mutants

The contribution of neuronal nicotinic AChRs in the brain function still remains of debate but it is now well established that they play at least two roles with: (a) the modulation of other neurotransmitters release (Gray *et al*., 1996; Léna *et al*., 1993; McGehee & Role, 1996a,b and (b) fast synaptic transmission (Heft *et al*., 1997). Therefore, the possibility that the two mutations have distinct physiological effects needs to be considered.

Examination of the mechanisms underlying the modulatory role of nicotinic AChRs implies that calcium entering through these receptors during their activation is sufficient to increase the intracellular calcium concentration and thereby the probability of fusion of synaptic vesicles with the presynaptic membrane (Gray *et al*., 1996; Léna *et al*., 1993). This function has been clearly documented for the mesolimbic system both at the synaptosomes as well at the cellular level (Pidoplichko *et al*., 1997; Wonnacott *et al*., 1990, 1996) The modulatory role of nicotine on the dopamine release has been proposed by several authors as the key determinant in the addictive process caused by this natural alkaloid (Clarke, 1993; Nestler, 1992; Piciotto *et al*., 1998; Stolerman & Shoiab, 1991; Wonnacott *et al*., 1996) Although at first dissimilar, the effects of mutations 776ins3 and S248F may, however, have an ultimate similar effect. The mutant S248F is mainly characterized by an increase in the desensitization accompanied with a slight decrease in apparent affinity that both result in a reduction of calcium influx during nicotinic AChR activation. A further diminution of the calcium influx can also be foreseen in the view of the lower calcium permeability reported for this mutant receptor (Kuryatov *et al*., 1997). Similarily, the 776ins3 mutant is also characterized by a reduction of calcium influx (Steinlein *et al*., 1997).

When considering fast transmission across neurons, the key issue is the amount of current flowing through the receptor which must be sufficient to depolarize the postsynaptic membrane. Desensitization of the receptor may again be the main determinant for this receptor property.

In order to explain the high frequency spike discharges characteristic of seizures on the basis of impaired nicotinic AChR function we suggest the following. As the neurotransmitter (ACh) accumulates in the synaptic cleft, excessive desensitization of receptors containing mutant subunits occurs. The decrease of function of the nicotinic AChR may influence other inhibitory regulating pathways such as those mediated by $GABA_A$ receptor (Léna *et al*., 1993) and could therefore lead to abnormal high frequency spike discharges. High frequency discharges of thalamo-cortical volleys, particular to the frontal lobes, are observed during the sleep and could explain the linkage between the sleep and the epileptic syndrome.

We have shown that mutations S248F and 776ins3 profoundly modify, although in a distinct manner, the human $\alpha 4\beta 2$ nicotinic AChR. Common features of both mutants are their restricted calcium permeability and enhanced desensitization sensitivity. On the basis of these observations it can therefore be proposed that both mutants result in a loss of function of the receptor properties that may be the cause of epileptic discharges observed during the sleep in this particular epilepsy syndrome.

We thank Drs Buisson and Picard for their helpful discussions. This work was supported by the Swiss National Foundation No 31-37191.93, the 'Office Fédéral de l'Education et des Sciences' and the Swiss Priority Program (PNR38) 4038-044050 to D.B. Work done in O.S. laboratory was supported by a grant by the Deutsche Forschungsgemeinschaft (SFB 400/B5).

References

BERTRAND, D. & CHANGEUX, J.P. (1995). Nicotinic receptor: An allosteric protein specialized for intercellular communication. *Seminar in Neuroscience,* **7,** 75–90.

BERTRAND, D., COOPER, E., VALERA, S., RUNGGER, D. & BALLIVET, M. (1991). Electrophysiology of neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes following nuclear injection of genes or cDNA. In *Methods in Neuroscience*. ed. Conn, M. pp. 174–193. New York: Academic Press.

BUISSON, B., GOPALAKRISHNAN, M., ARNERIC, S.P., SULLIVAN, J.P. & BERTRAND, D. (1996). Human alpha 4 beta 2 neuronal nicotinic acetylcholine receptor in HEK 293 cells: A patch-clamp study. *J. Neurosci.,* **16,** 7880–7891.

CLARKE, P.B. (1993). Nicotine dependence–mechanisms and therapeutic strategies. *Biochem. Soc. Symp.,* **59,** 83–95.

CORRINGER, P.J., BERTRAND, S., BOHLER, S., EDELSTEIN, S.J., CHANGEUX, J.P. & BERTRAND, D. (1998). Critical elements determining diversity in agonist binding and desensitization of neuronal nAChR. *J. Neurosci.,* **18,** 648–657.

COVERNTON, P.J., KOJIMA, H., SIVILOTTI, L.G., GIBB, A.J. & COLQUHOUN, D. (1994). Comparison of neuronal nicotinic receptors in rat sympathetic neurones with subunit pairs expressed in Xenopus oocytes. *J. Physiol.,* **481,** 27–34.

DEVILLERS-THIÉRY, A., GALZI, J.L., CHANGEUX, J.P., BERTRAND, S. & BERTRAND, D. (1993). Functional architecture of the nicotinic acetylcholine receptor: A prototype of ligand-gated ion channel. *J. Memb. Biol.,* **136,** 97–112.

ENGEL, A.G., OHNO, K., BOUZAT, C., SINE, S.M. & GRIGGS, R.C. (1996). End plate acetylcholine receptor deficiency due to nonsense mutations in the epsilon subunit. *Ann. Neurol.,* **40,** 810–817.

GALZI, J.L., EDELSTEIN, S.J. & CHANGEUX, J.P. (1996). The multiple phenotypes of allosteric receptor mutants. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 1853–1858.

GRAY, R., RAJAN, A.S., RADCLIFFE, K.A., YAKEHIRO, M. & DANI, J.A. (1996). Hippocampal synaptic transmission enhanced by low concentrations of nicotine. *Nature,* **383,** 713–716.

HAYMAN, M., SCHEFFER, I.E., CHINVARUN, Y., BERLANGIERI, S.U. & BERKOVIC, S.F. (1997). Autosomal dominant nocturnal frontal lobe epilepsy: demonstration of focal frontal onset and intrafamilial variation. *Neurology,* **49,** 969–975.

HEFT, S., BERTRAND, D. & MULLER, D. (1997). Synaptic transmission mediated by nicotinic receptors in hippocampal slice cultures. *Soc. for Neurosci.,* **23,** 265.7.

KRAUSE, M.R., BUISSON, B., BERTRAND, S., CORRINGER, P.J., GALZI, J.L., CHANGEUX, J.P. & BERTRAND, D. (1998). Ivermectin: A positive allosteric effector of the α7 neuronal nicotinic acetylcholine receptors. *Mol. Pharmacol.,* **53,** 283–294.

KURYATOV, A., GERZANICH, V., NELSON, M., OLALE, F. & LINDSTROM, J. (1997). Mutation causing autosomal dominant nocturnal frontal lobe epilepsy alters Ca^{2+} permeability, conductance, and gating of the human $\alpha 4\beta 2$ nicotinic acetylcholine receptors. *J. Neurosci.,* **17,** 9035–9047.

LÉNA, C., CHANGEUX, J.P. & MULLE, C. (1993). Preterminal nicotinic receptors on GABAergic axons : a study on thin slices and freshly isolated neurons in the rat interpeduncular nucleus. *J. Neurosci.,* **13,** 2680–2688.

LINDSTROM, J. (1996). Neuronal nicotinic acetylcholine receptors. In *Ion Channels*. ed. Narahashi, T. pp. 377–450. New York: Plenum Press.

MCGEHEE, D.S. & ROLE, L.W. (1996a). Neurobiology–Memories of nicotine. *Nature,* **383,** 670–671.

MCGEHEE, D.S. & ROLE, L.W. (1996b). Presynaptic ionotropic receptors. *Curr. Opin. Neurobiol.,* **6,** 342–349.

MONOD, J., WYMAN, J. & CHANGEUX, J.P. (1965). On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.,* **12,** 88–118.

NESTLER, E.J. (1992). Molecular mechanisms of drug addiction. *J. Neurosci.,* **12,** 2439–2450.

PALMA, E., BERTRAND, S., BINZONI, T. & BERTRAND, D. (1996). Neuronal nicotinic α7 receptor expressed in *Xenopus* oocytes presents five putative binding sites for methyllycaconitine. *J. Physiol.,* **491,** 151–161.

PICIOTTO, M.R., ZOLI, M., RIMONDINI, R., LÉNA, C., MARUBIO, L.M., MERLO PICH, E., FUXE, K. & CHANGEUX, J.-P. (1998). Acetylcholine receptors containining the α2 subunit are involved in the reinforcing properties of nicotine. *Nature,* **391,** 173–177.

PIDOPLICHKO, V.I., DEBIASI, M., WILLIAMS, J.T. & DANI, J. (1997). Nicotine activates and desensitizes midbrain dopamine neurons. *Nature,* **390,** 401–404.

SCHEFFER, I.E., BHATIA, K.P., LOPES-CENDES, I., FISH, D.R., MARDSEN, C.D., ANDERMANN, E., ANDERMANN, F., DESBIENS, R., KEENE, D., CENDES, F., MANSON, J.I., CONSTANTINOU, J., MCINTOSH, A. & BERKOVIC, S.F. (1995). Autosomal dominant nocturnal frontal lobe epilepsy: a distinctive clinical disorder. *Brain,* **118,** 61–73.

SHIANG, R., RYAN, S., ZHU, Y.-Z., HANHN, A.F., O'CONNELL, P. & WASMUTH, J.J. (1993). Mutations in the α1 subunit if the inhibitory glycine receptor cause the dominant neurologic disorder, hyperekplexia. *Nature Genetics,* **5,** 351–357.

SINE, S.M., OHNO, K., BOUZAT, C., AUERBACH, A., MILONE, M., PRUITT, J.N. AND ENGEL, A.G. (1995). Mutation of the acetylcholine receptor alpha subunit causes a slow-channel myasthenic syndrome by enhancing agonist binding affinity. *Neuron,* **15,** 229–239.

STEINLEIN, O.K., MAGNUSSON, A., STOOD, J., BERTRAND, S., WEILAND, S., BERKOVIC, S.F., NAKKEN, K.O., PROPPING, P. & BERTRAND, D. (1997). An insertion mutation of the CHRNA4 gene in a family with autosomal dominant nocturnal frontal lobe epilepsy. *Human Molecular Genetics,* **6,** 943–947.

STEINLEIN, O.K., MULLEY, J.C., PROPPING, P., WALLACE, R.H., PHILLIPS, H.A., SUTHERLAND, G.R., SCHEFFER, I.E. & BERKOVIC, S.F. (1995). A missense mutation in the neuronal nicotinic acetylcholine receptor alpha 4 subunit is associated with autosomal dominant nocturnal frontal lobe epilepsy. *Nature Genetics,* **11,** 201–203.

STÉRIADE, M. & CONTRERAS, D. (1995). Relations between cortical and thalamic cellular events during transition from sleep patterns to paroxysmal activity. *J. Neurosci.,* **15,** 623–642.

STOLERMAN, I.P. & SHOIAB, M. (1991). The neurobiology of tobacco addiction. *Trends Pharmacol. Sci.,* **12,** 467–473.

WEILAND, S., WITZEMANN, V., VILLARROEL, A., PROPPING, P. & STEINLEIN, O. (1996). An amino acid exchange in the second transmembrane segment of a neuronal nicotinic receptor causes partial epilepsy by altering its desensitization kinetics. *Febs Lett.,* **398,** 91–96.

WONNACOTT, S., DRASDO, A., SANDERSON, E. & ROWELL, P. (1990). Presynaptic nicotinic receptors and the modulation of transmitter release. *Ciba Found Symp.,* **152,** 87–101.

WONNACOTT, S., SOLIAKOV, L., WILKIE, G., REDFERN, P. & MARSHALL, D. (1996). Presynaptic nicotinic acetylcholine receptors in the brain. *Drug Develop. Res.,* **38,** 149–159.

(Received February 10, 1998
Revised July 31, 1998
Accepted August 8, 1998)