



Binding–gating coupling in a nondesensitizing $\alpha 7$ nicotinic receptor A single channel pharmacological study

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ABSTRACT

The highly conserved α Lys145 has been suggested to play an important role in the early steps of activation of the nicotinic acetylcholine receptor (nAChR) by acetylcholine. Both macroscopic and single channel currents were recorded in the slowly desensitizing mutants L248T- and K145A-L248T- $\alpha 7$ receptors expressed in *Xenopus* oocytes. On ACh-evoked currents, substitution of Lys145 by alanine showed the same effects that in wild type receptors: moderately decreased gating function and a more-than-expected loss of ACh potency, thus validating the experimental model. Single channel analysis quantitatively agreed with macroscopic data and revealed that impaired gating function in the double mutant $\alpha 7$ K145A/L248T is the consequence of a slower opening rate, β . Several nicotinic agonists were also studied, showing important features. Particularly, dimethylphenylpiperazinium (DMPP), acting as an antagonist in $\alpha 7$ K145A, became a full agonist in $\alpha 7$ K145A/L248T. Single channel analysis of DMPP-evoked currents showed effects of Lys145 removal similar to those observed with ACh. Data suggest that $\alpha 7$ Lys145 facilitates the early steps of channel activation. Moreover, the slowly desensitizing mutant $\alpha 7$ L248T could be an interesting tool for the study of channel activation in $\alpha 7$ receptors. Nevertheless, its extensively altered pharmacology precludes the simple extrapolation of pharmacological data obtained in singly mutated $\alpha 7$ receptors.

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1. Introduction

Nicotinic acetylcholine receptors (nAChRs) mediate fast synaptic transmission in muscle and nerve cells. They are members of the Cys-loop family of ligand-gated ion channels, which includes 5-HT₃, γ -GABA_A receptors, and glycine receptors [1,2]. Such receptors are allosteric proteins because the signal generated at the binding-site region must be transmitted to the channel gate located some distance away, and this process involves a more than local conformational change [3]. Recent structural data from the *Torpedo* nAChR [4,5] and from the acetylcholine binding protein (AChBP) of snails [6,7] have been helping to understand the coupling between binding and gating in Cys-loop receptors. However, the precise molecular mechanisms involved in channel activation are still poorly understood [8]. According to the current model of activation, the interaction of the agonist with the binding site would generate a motion of the α subunits that is transmitted to the gate through rearrangements of several extracellular structures. Among them, the highly conserved Lys145, at the β -strand $\beta 7$ of nAChR α subunits, is located close to both the Cys-loop and the binding segment C, the latter containing several aromatic residues involved in binding of nicotinic agonists and

antagonists [6,9]. Such a unique position and its possible role in binding–gating coupling had been pointed out after analysis of crystallographic data obtained in the snail AChBP [10]. This role was confirmed in both muscle- and neuronal-type $\alpha 7$ nAChRs [11,12]. In addition to its role in coupling, it has been reported that Lys145 also determines the pharmacological profile of homomeric $\alpha 7$ nAChRs because DMPP can become a competitive antagonist after mutating Lys145 [12].

However, neuronal type $\alpha 7$ nAChRs currents show fast desensitization which precludes the use of single channel data to study the mechanisms altered by mutations in Lys145 more precisely. The alternative used in the present work is to include such mutations in a slowly- or non-desensitizing $\alpha 7$ nAChR, as it is $\alpha 7$ L248T nAChR. In such receptors, a highly conserved leucine residue of the pore domain has been substituted by a threonine, and the resulting receptors did not show the typical fast desensitization of homomeric $\alpha 7$ nAChR [13], and also presented an unconventional pharmacology [14–17]. This non-desensitizing receptor has also been helpful in establishing the role of several residues both in coupling and calcium modulation of $\alpha 7$ nAChR [18,19].

In the present work, we have further studied the role of Lys145 in binding–gating coupling of neuronal type $\alpha 7$ nAChRs by including the mutation K145A in $\alpha 7$ L248T nAChR, and recording agonist-activated single channel currents. Our data show that impairment of coupling was mostly due to a delay in the channel activation. Moreover, $\alpha 7$ L248T nAChRs has been proven to be a useful tool for studying at

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the single channel level mutations affecting ACh activation of $\alpha 7$ nAChRs. However, the pharmacological profile of single mutant $\alpha 7$ K145A could not be transferred to $\alpha 7$ L248T nAChRs.

2. Materials and methods

2.1. Generation of mutants of the bovine $\alpha 7$ subunit

The bovine $\alpha 7$ cDNA was cloned in a derivative of the pSP64T vector [20] containing part of the pBluescript polylinker. To generate the mutants we annealed single-stranded oligonucleotides with the desired sequences and proper single-strand ends that could be easily ligated to the ends generated through restriction enzymes digestion of the $\alpha 7$ cDNA. The restriction enzyme sites for cloning the annealed oligonucleotides were either present in the original cDNA sequence or introduced as silent mutations [21] by using mutated oligonucleotides and PCR (25 cycles at 94 °C for 10 s, 60 °C for 30 s, 72 °C for 45 s).

2.2. Oocyte expression

Capped mRNA was synthesized *in vitro* using SP6 RNA polymerase, the mMESAGE mMACHINE kit (Ambion, Texas) and the pSP64T derivative mentioned above. Defolliculated *Xenopus laevis* oocytes were injected either with 2.5 ($\alpha 7$ K145A/L248T) or 0.5 ($\alpha 7$ L248T) ng of total cRNA in 50 nl of sterile water. Oocytes were incubated in calcium-free medium containing 1 μ M methyllycaconitine (MLA). All experiments were performed within 3–4 days after cRNA injection.

2.3. 125 I- α -bungarotoxin binding assays

Specific surface expression of 125 I- α -bungarotoxin (α -Bgt) binding sites was tested with 10 nM 125 I- α -Bgt as described [22]. Briefly, oocytes located in 24-well plates were preincubated for 15 min with Barth's buffer containing 5% of fetal calf serum and further incubated in the same medium with 10 nM 125 I- α -Bgt for 2 h at 18 °C in a final volume of 300 μ l. At the end of the incubation, unbound 125 I- α -Bgt was removed, oocytes were passed to 6-well plates, washed five times with 4 ml Barth's buffer and bound radioactivity was counted. Dissociation constants of 125 I- α -Bgt were 3.8 and 2.9 nM for $\alpha 7$ L248T and $\alpha 7$ K145/L248T, respectively (not significant) and, therefore, the errors derived from not using a saturating concentration should be small. Nonspecific binding was determined using uninjected oocytes.

2.4. Electrophysiological recordings

Electrophysiological recordings of whole-cell currents were done as described previously [22]. Some oocytes showed holding currents which were mostly blocked by 1 μ M MLA suggesting spontaneous activity of nAChRs. In order to simplify the analysis, oocytes showing holding currents >1% of the maximal ACh-evoked currents were discarded. Extracellular solution contained (in mM): NaCl 82.5, KCl 2.5, BaCl₂ 2.5, MgCl₂ 1 and HEPES 5 (pH 7.4). The velocity of application of agonists was 18–22 ml min⁻¹. All experiments were done at room temperature (22 °C). Unless otherwise indicated, oocyte membrane potential was held at -40 mV. Data analysis was performed with the software package Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Dose-response curves were fitted using a nonlinear least squares algorithm to the Hill equation, $I/I_{\max} = 1/(1 + (EC_{50}/C)^h)$, where EC_{50} is the agonist concentration that elicits the half-maximal response, h is the Hill coefficient, and C is the agonist concentration.

Single channel records were obtained in the cell-attached mode using an Axopatch 200A amplifier (Axon Instruments, CA). Oocytes were stripped of their vitelline membrane by immersing them in a hypertonic solution to shrink them enough so that the vitelline membrane was visible and then could be removed with thin tip tweezers. They were then transferred to a recording chamber with a

modified extracellular solution where NaCl was substituted by KCl to clamp the membrane potential close to 0 mV [23]. Holding potential was -80 mV. Patch pipettes were pulled from thick-walled borosilicate glass (GC150-15, Clark Electromedical Instruments), with a resistance between 5 and 10 M Ω when filled with standard extracellular solution. ACh or DMPP were added to the pipette solution. Initially 10 μ M gadolinium was added to the pipette to block stretch activated channels [24], but in later experiments gadolinium was not used because stretch activated channels were not observed if the negative pressure was released after making the seal and, furthermore, nicotine activated receptors could be recognized by their kinetics (long openings) and conductance range (with large and uniform currents). Records were low-pass filtered at 1 kHz with an 8-pole Bessel filter (Frequency Devices, Inc.), sampled at 20 kHz and stored on hard disk for later analysis.

2.5. Data analysis

Single channel recordings were analyzed with the Clampfit 9 (Molecular Devices) and the QUB software suite (version 1.4.0.2) [25]. For each patch a voltage pulse protocol was applied to determine the conductance (γ) and reversal potential (V_r) of the channels. Ten second duration pulses were applied from +60 to -120 mV at 20 mV intervals. Single channel current-voltage curves were fitted with Prism 4 (GraphPad Software, Inc.) to the equation $I = \gamma(V - V_r)$. Open probabilities were estimated from all point histograms fitted to a sum of Gaussians with Clampfit routines.

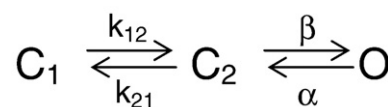
Kinetic analysis was performed using QUB on records with a dead time of 0.1 ms, idealized by the half amplitude threshold method, and restricted to bursts of activity of a single channel. Bursts were identified by a closed interval longer than a given burst terminator time that usually was between 10 and 30 ms, and containing more than 3 opening events. The selected region of the idealized data, obtained as described above, was then fitted to a simple linear three-state model with two closed and one open state, with four kinetic constants, as shown in kinetic Scheme 1, with the MIL method that gives directly an estimate of the kinetic constants their standard errors. Dwell time histograms with the corresponding time constants, and their relative weight, were also obtained with the same program.

Data are presented as mean \pm standard error. Statistical significance was calculated by non-paired Student's *t*-test. EC_{50} values were compared with an *F* test. The null hypothesis was rejected when $p < 0.05$.

3. Results

3.1. Effect of the mutation K145A on the functional responses to ACh in the slowly desensitizing L248T receptor

Fig. 1A shows two families of macroscopic inward ACh-evoked currents in the single ($\alpha 7$ L248T) and double ($\alpha 7$ K145A/L248T) mutant receptors, respectively. Inward currents evoked at equiactive ACh concentrations presented the same kinetics both in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors. Maximal currents in $\alpha 7$ K145A/L248T were almost 2-fold larger than in $\alpha 7$ L248T. However, surface expression, as measured by α -bungarotoxin (α -Bgt) binding, was also increased around 4-fold. $\alpha 7$ L248T and $\alpha 7$ K145A/L248T oocytes expressed 0.5 ± 0.1 and 1.8 ± 0.4 fmol of α -Bgt, respectively ($n=4$). Nevertheless, such a difference does not point to a more efficient surface expression of $\alpha 7$ K145A/L248T receptors because of the differences in the amount of



Scheme 1.

cRNA injected (see Methods). After normalizing functional responses to surface expression, $\alpha 7$ K145A/L248T receptors showed a gating function of $41 \pm 12\%$ of $\alpha 7$ L248T receptors ($n=4$). This reduction in gating function is close to that obtained after the inclusion of the same mutation in wild type $\alpha 7$ receptors [12]. Concentration–responses relationship in $\alpha 7$ L248T receptors showed the typical reduction in the EC_{50} value with respect to wild type receptors (around 60-fold lower). However, $\alpha 7$ K145A/L248T receptors showed a shift in ACh potency similar to that observed when starting with $\alpha 7$ wild type receptors, because EC_{50} value increased from 0.65 to 15.7 μ M (Fig. 1B). As occurred in wild type receptors, this change in the EC_{50} value is not quantitatively predicted by only considering the modification in gating function. Thus, a change in the ACh binding properties is likely to have taken place as well.

We have also explored the possible functional interaction between residues located at positions of Lys145 and Leu248 by calculating the coupling coefficient Ω of the two-mutant cycle [12,26,27]. Ω values were 0.42 and 0.57 for the current–binding ratio and for the EC_{50} , respectively, suggesting that both positions are only weakly coupled, and thus, effects of mutating Lys145 are not only qualitatively, but also quantitatively similar in both $\alpha 7$ wild type and $\alpha 7$ L248T receptors.

3.2. Acetylcholine-evoked unitary currents in single and double mutants

So far, substitution of Lys145 by alanine showed the same effects in $\alpha 7$ L248T than in $\alpha 7$ wild type receptors. The slowly desensitizing properties of single mutant $\alpha 7$ L248T allowed the acquisition of single

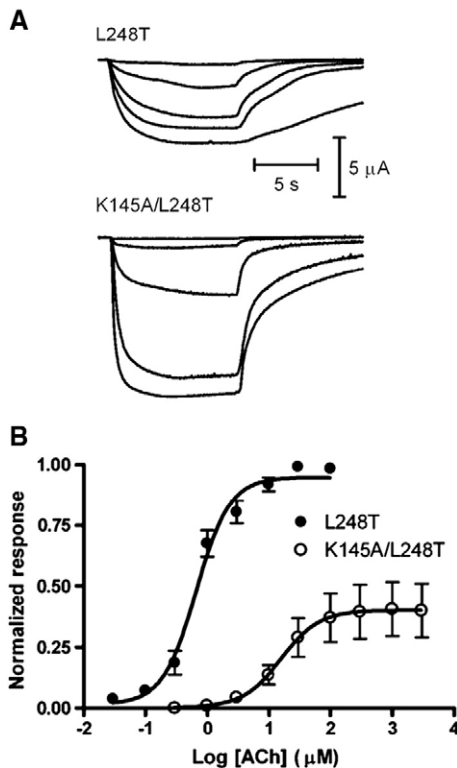


Fig. 1. A comparison between the ACh responses of $\alpha 7$ L248T nAChR and double mutant $\alpha 7$ K145A/L248T. (A) Inward currents obtained upon stimulation with ACh of $\alpha 7$ L248T (top, control) and $\alpha 7$ K145A/L248T (bottom) nAChRs. Traces represent currents obtained at -40 mV. For each family of currents, ACh concentrations were (from top to bottom): 0.1, 0.3, 1, 3 and 10 μ M ($\alpha 7$ L248T), and 1, 3, 10, 30 and 100 μ M ($\alpha 7$ K145A/L248T). (B) ACh concentration–response curves of oocytes expressing $\alpha 7$ L248T (filled circles) and $\alpha 7$ K145A/L248T receptors (open circles). Data are normalized to the estimated maximal response to ACh (both receptors) and corrected for different surface expression ($\alpha 7$ K145A/L248T receptors, error included). Data points represent means \pm SEM obtained in 8–16 oocytes from 3–4 donors. Error bars shown if bigger than symbols. Continuous lines represent fits of data to the Hill equation. $\alpha 7$ L248T: $EC_{50} = 0.65 \pm 0.07$ μ M, $h = 1.51$. $\alpha 7$ K145A/L248T: $EC_{50} = 15.6 \pm 1.3$ μ M, $h = 1.36$.

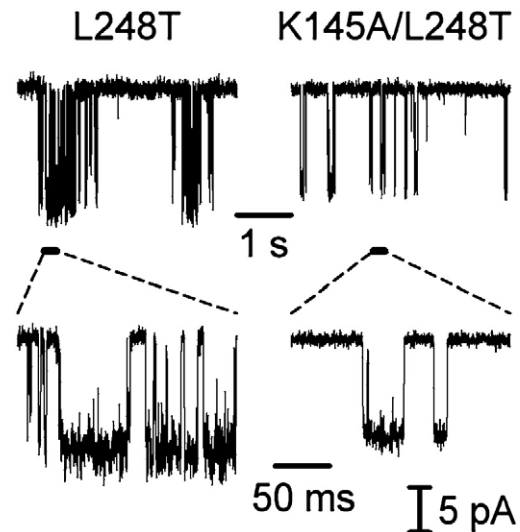


Fig. 2. Acetylcholine-evoked unitary currents. Top panels: Representative traces of single channel currents evoked by 100 μ M ACh in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors. Bottom panels: Expanded scale representations of the horizontal bars in top panels. Holding potential was -100 mV.

channel recordings in the cell-attached configuration of the patch-clamp technique. In the absence of agonist $\alpha 7$ L248T receptors showed very brief spontaneous openings. However, when the oocyte membrane is exposed to a high ACh concentration (100 μ M) large and frequent unitary inward currents of about 12 pA are observed with both receptors (Fig. 2). Differences in single channel conductances were small but significant (105 ± 3 and 114 ± 2 pS in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors, respectively). However, these results discard that reduced gating function in $\alpha 7$ K145A/L248T was due to a decrease in the unitary conductance. Very brief events with a smaller conductance could also be seen in both receptors, but they contribute very little to the overall probability and were not included in the dwell time analysis. $\alpha 7$ K145A/L248T receptors show both longer closings and somewhat shorter openings than $\alpha 7$ L248T receptors. Single channels showed open probabilities of 0.17 and 0.06 in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors, respectively. Those probabilities were estimated from fitting all-points histograms with Gaussian functions and comparing the areas. The estimated ratio of open channel probabilities (35% of $\alpha 7$ L248T receptors) is close to that obtained previously from macroscopic currents.

Dwell time analysis was also carried out in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors. Fig. 3 shows the analysis of two representative membrane patches. These data could be analyzed in terms of a given kinetic model. For the sake of simplicity, a simple linear three-state kinetic model (two closed states and one open state; Scheme 1) was applied to data, and open time distributions were fitted with mean open times of 28 and 19 ms, for $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors, respectively (Table 1). Such a difference was not statistically significant. Accordingly with Scheme 1, closed times showed a two-exponential distribution with mean closed times of 1.0 (weight: 0.78), and 31 ms in $\alpha 7$ L248T receptors, with a clear dominance of the shortest closures. When the same analysis is performed in $\alpha 7$ K145A/L248T receptors, mean closed times were longer (1.6 (weight: 0.45), and 62 ms), and longer closures were much more frequent (Table 1).

Values for the several first order kinetic rates could be estimated as well (Table 2). Such analysis revealed that the reduction in open probability in $\alpha 7$ K145A/L248T receptors is mostly attributed to an impairment of channel opening due to statistically significant decrease in the opening rate constant, β , being all other rate constants not statistically different from $\alpha 7$ L248T receptors. Interestingly, the gating constant, β/α , in $\alpha 7$ K145A/L248T receptors was 30% of $\alpha 7$ L248T

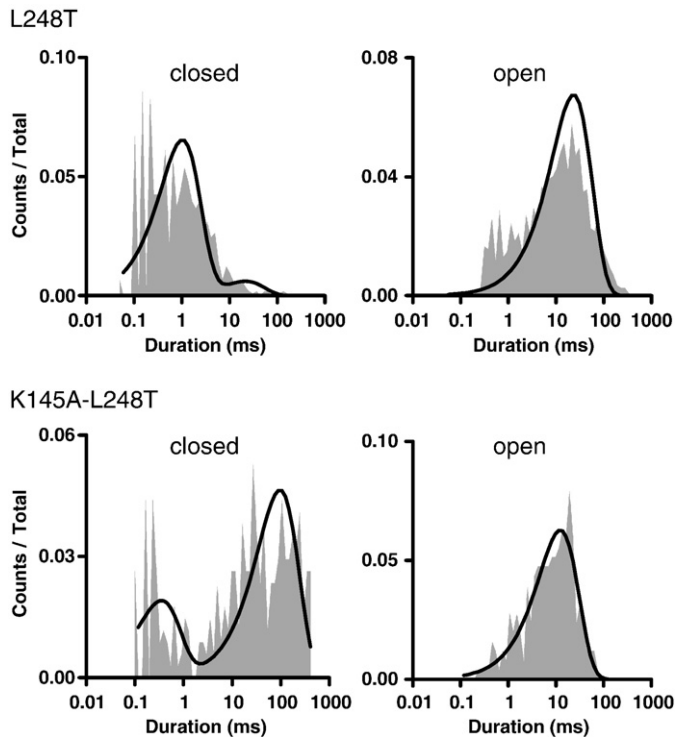


Fig. 3. Dwell-time histograms of acetylcholine-evoked single channel events. Closed (left) and open (right) time histograms for $\alpha 7$ /L248T (top) and $\alpha 7$ K145A/L248T (bottom) ACh-evoked unitary currents. Logarithmic binning and linear scale for relative frequency of events were used. Superimposed continuous lines are fits for Scheme 1. Holding potential was -100 mV.

receptors, indicating that the changes in these gating rates quantitatively account for most of the reduction in overall currents. Taken together, data suggest that the reduction of whole-cell currents in $\alpha 7$ K145A/L248T receptors is mainly caused by impairment in the channel activation rather than a destabilization of the open configuration.

3.3. Pharmacological profile

Several mutations of Lys145 in wild type receptors induced changes in the pharmacological profile of the resulting receptors [12]. Therefore, we checked the functional responses of $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors to several nicotinic drugs. All additional agonists tested evoked ionic currents that were kinetically indistinguishable from ACh-evoked currents both in $\alpha 7$ L248T and in $\alpha 7$ K145A/L248T receptors (not shown). Fig. 4 shows concentration–response relationship for nicotine, epibatidine, cytosine and DMPP. When compared to $\alpha 7$ L248T curves, those obtained with $\alpha 7$ K145A/L248T receptors were consistently shifted to the right, showing statistically significant higher EC_{50} values. When the same mutation was inserted in wild type $\alpha 7$ receptors, there were also changes in the agonist potency, but they were small except for ACh. However, the shifts observed in the present work were not always of the same

Table 2
Kinetic parameters estimated from a fit to Scheme 1

Agonist	Receptor	K_{12}	K_{21}	β	α	β/α
ACh	$\alpha 7$ L248T	55 ± 17	274 ± 109	863 ± 92	43 ± 7	24 ± 5
	$\alpha 7$ K145A/L248T	54 ± 14	730 ± 272	$474 \pm 96^*$	86 ± 21	$7.3 \pm 1.8^*$
DMPP	$\alpha 7$ L248T	119 ± 42	1081 ± 642	1168 ± 177	813 ± 294	1.8 ± 0.5
	$\alpha 7$ K145A/L248T	68 ± 3	172 ± 75	$692 \pm 87^*$	1129 ± 251	$0.6 \pm 0.1^*$

Data analyzed and agonist concentrations are as in Table 1. Rate constants are in s^{-1} .

* Statistically different from $\alpha 7$ L248T ($p < 0.05$).

magnitude but strongly depended on the agonist used. The loss of potency in $\alpha 7$ K145A/L248T receptors ranged from 1.8-fold with epibatidine up to 125-fold with cytosine. Nicotine and DMPP showed in-between values, similar to those obtained with ACh (Fig. 1). When maximal currents were considered, all tested agonists showed the same extent of functional response when compared to ACh in the same receptor (Fig. 5). Only cytosine in $\alpha 7$ L248T receptors showed a small but statistically significant reduction in maximal currents. This is in great contrast to results obtained with $\alpha 7$ wild type and $\alpha 7$ K145A receptors [12]. In $\alpha 7$ wild type receptors, all five agonists evoked similar responses and, when Lys145 was substituted by Ala, responses to epibatidine and cytosine were only moderately reduced, ACh and nicotine were significantly less efficacious, and DMPP became a competitive antagonist. The same behavior of DMPP was observed with carbamylcholine, which was ineffective in the single mutant $\alpha 7$ K145A, but evoked full responses in the double mutant $\alpha 7$ K145A/L248T (not shown).

Competitive nicotinic antagonists and other non-nicotinic drugs have been reported to activate $\alpha 7$ /L248T receptors. Thus, we checked whether the double mutant $\alpha 7$ K145A/L248T retained these unusual

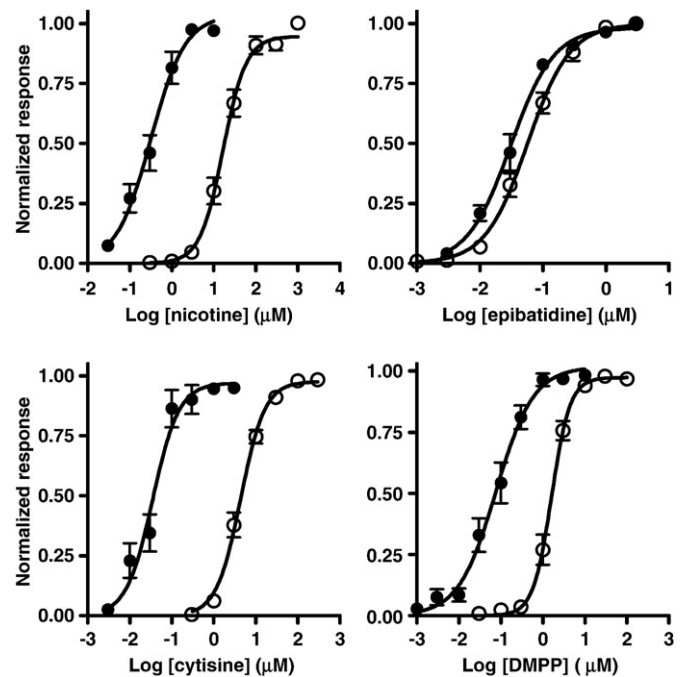


Fig. 4. Concentration–response relationships. Agonist concentration–response curves of oocytes expressing $\alpha 7$ L248T (filled circles) and $\alpha 7$ K145A/L248T (open circles) nAChRs for nicotine, epibatidine, cytosine and DMPP. For each receptor and agonist, data are normalized to their maximal response. Data points represent means \pm SEM obtained in 4–7 oocytes from 3 donors. Error bars shown if bigger than symbols. Continuous lines represent fits of data to the Hill equation. Nicotine = 0.32 ± 0.07 μM , $h = 1.06$; Nicotine – $\alpha 7$ K145A/L248T: $EC_{50} = 16.7 \pm 1.6$ μM , $h = 1.58$. Epibatidine – $\alpha 7$ L248T: $EC_{50} = 0.031 \pm 0.004$ μM , $h = 1.27$; Epibatidine – $\alpha 7$ K145A/L248T: $EC_{50} = 0.057 \pm 0.006$ μM , $h = 1.29$. Cytosine – $\alpha 7$ L248T: $EC_{50} = 0.035 \pm 0.007$ μM , $h = 1.39$; Cytosine – $\alpha 7$ K145A/L248T: $EC_{50} = 4.38 \pm 0.29$ μM , $h = 1.51$. DMPP – $\alpha 7$ L248T: $EC_{50} = 0.077 \pm 0.016$ μM , $h = 0.97$; DMPP – $\alpha 7$ K145A/L248T: $EC_{50} = 1.61 \pm 0.11$ μM , $h = 1.98$.

Table 1
Time constants extracted from the dwell-time distributions

Agonist	Receptor	τ_{c1} (weight)	τ_{c2}	τ_o	N
ACh	$\alpha 7$ L248T	0.97 ± 0.13 (0.78 ± 0.08)	31.5 ± 5.9	28.0 ± 4.6	6
	$\alpha 7$ K145A/L248T	1.62 ± 0.52 (0.45 ± 0.08)	62.4 ± 12.8	18.5 ± 4.9	8
DMPP	$\alpha 7$ L248T	0.75 ± 0.23 (0.55 ± 0.14)	19.9 ± 2.4	1.94 ± 0.49	5
	$\alpha 7$ K145A/L248T	1.51 ± 0.25 (0.76 ± 0.05)	15.5 ± 2.7	1.05 ± 0.24	6

Agonist concentrations used were 100 and 10 μM for ACh and DMPP, respectively. Time constants are in ms. N stands for the number of patches analyzed.

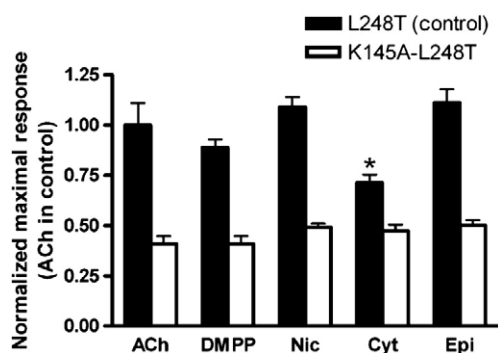


Fig. 5. Maximal currents. Mean maximal currents \pm SEM upon stimulation with nicotinic agonists in $\alpha 7$ L248T (filled bars) and $\alpha 7$ K145A/L248T receptors (open bars). Inward currents were obtained at -40 mV with saturating concentrations of each agonist, and normalized to the maximal response achieved by ACh in the same oocyte. Data of $\alpha 7$ K145A/L248T receptors were corrected for different surface expression of receptors.

pharmacological features. Dihydro- β -erythroidin, d-tubocurarine, strychnine and serotonin, all of them were capable to elicit agonist responses in this double mutant (not shown).

3.4. DMPP-evoked unitary currents in single and double mutants

Since DMPP was able to activate nicotinic currents in $\alpha 7$ K145A/L248T receptors to the same extent than ACh, DMPP-evoked single channel currents were recorded in the cell-attached configuration (Fig. 6). Unitary conductances were similar to those observed with ACh. For DMPP-evoked currents, they were estimated in 102 ± 9 and 114 ± 13 pS, in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors, respectively (not significant difference). Open probabilities were estimated to be 0.16 and 0.07, very close to those of ACh and in good agreement with macroscopic data. However, both $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors also showed several significant differences regarding the activating agonist. First of all, DMPP-evoked openings are much shorter and, besides, a subconductance state makes a clear contribution to the total current (Fig. 6).

Dwell time distributions were also analyzed (Fig. 7). According to Scheme 1, DMPP-activated channels in $\alpha 7$ L248T receptors showed mean open times of 1.9 ms (Table 1), more than one order of

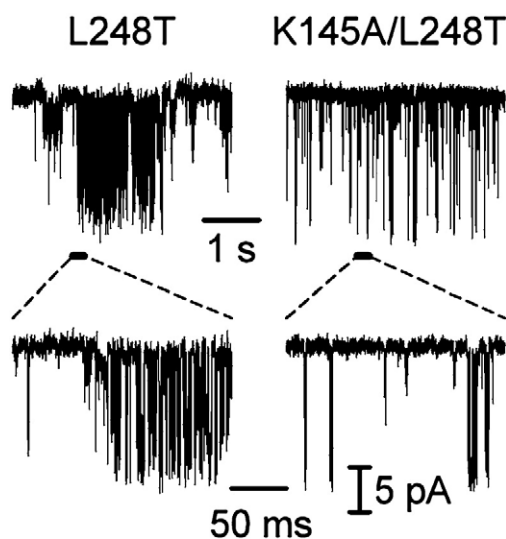


Fig. 6. DMPP-evoked unitary currents. Top panels: Representative traces of single channel currents evoked by $10 \mu\text{M}$ DMPP in $\alpha 7$ L248T and $\alpha 7$ K145A/L248T receptors. Bottom panels: Expanded scale representations of the horizontal bars in top panels. Holding potential was -100 mV.

magnitude shorter than with ACh. Closed times were also briefer (0.8 (weight: 0.55), and 20 ms), but the difference with ACh was not large. However, in $\alpha 7$ K145A/L248T receptors, DMPP evoked slightly shorter openings (mean open time of 1.1 ms, not significant difference), but the brief closures were longer and more abundant than in $\alpha 7$ L248T receptors (mean closed times of 1.5 (weight: 0.76), and 16 ms). Thus, the effects of mutating Lys145 to Ala in $\alpha 7$ L248T are similar for both agonists. This could also be observed in the estimates for the first-order rate constants shown in Table 2. Similarly to ACh, opening rate constant, β , was significantly slower in $\alpha 7$ K145A/L248T receptors. However, gating constant, β/α , obtained with DMPP in $\alpha 7$ K145A/L248T receptors was 33% of $\alpha 7$ L248T receptors, quantitatively accounting for the reduction in open channel probability.

4. Discussion

When ACh was used as the activating molecule, the effects of mutation K145A in $\alpha 7$ L248T receptors were similar to those observed in wild type $\alpha 7$ nAChRs [12], i.e.: a decrease in gating function (estimated by the current-to-binding ratio) accompanied by a shift to the right in the dose-response curve which cannot be quantitatively accounted for by the reduced gating function [28]. Single channel events were recorded in the presence of nicotinic agonists showing a unitary conductance around 110 pS, which roughly doubles those reported for chicken $\alpha 7$ -L247T [17] and human $\alpha 7$ -L248T receptors [29]. The higher conductance observed here is likely to be due to a different gating mode found in bovine $\alpha 7$ L248T receptors, rather than to the simultaneous activation of two lower conductance channels, because of two reasons: 1st) microscopic kinetics are slower [29], and 2nd) not half-conductance events were recorded.

ACh was applied at saturating concentrations and $\alpha 7$ K145A/L248T receptors showed slightly shorter openings and considerably longer closings, when compared $\alpha 7$ L248T receptors. When studied under a

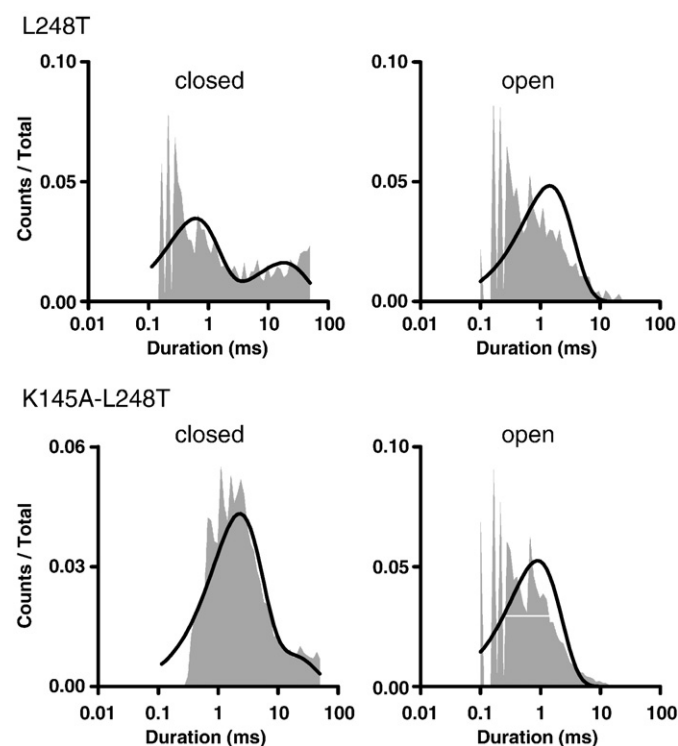


Fig. 7. Dwell-time histograms of DMPP-evoked single channel events. Closed (left) and open (right) time histograms for $\alpha 7$ /L248T (top) and $\alpha 7$ K145A/L248T receptors (bottom) DMPP-evoked unitary currents. Logarithmic binning and linear scale for relative frequency of events were used. Superimposed continuous lines are fits for Scheme 1. Holding potential was -100 mV.

simple kinetic scheme (two closed and one open states), those data resulted in changes of the opening rate constant (β). These results are in qualitative agreement with those obtained in muscle nAChRs showing that substitution of Ala for α K145 intensely affected receptor activation mostly by a large reduction in opening rate constant [11]. Thus, data confirm that Lys145 is involved in the early steps of muscle nAChR activation, as could have been advanced by the strategic position of Lys145. However, the effects of mutating Lys145 in neuronal-type α 7L248T receptors are more modest, which is not surprising because both quantitative and qualitative discrepancies between muscle- and neuronal-type nAChR gating have been reported [12,21,27,30,31].

Several residues and/or regions of the extracellular domain of neuronal-type subunits have been identified as determinants of pharmacological selectivity in nAChRs [32–37]. The pharmacological profile of α 7 AChRs is also determined by the residue present at position 145. Thus, single mutant α 7K145A showed a great influence in the effectiveness of some nicotinic agonists (ACh, nicotine, carbamylcholine and DMPP), but not others (cytisine, epibatidine), being the most remarkable changes those of carbamylcholine and DMPP, which became competitive antagonists in mutant α 7K145A [12]. We have checked the effectiveness and potency of those nicotinic agonists in both α 7L248T and α 7K145A-L248T receptors, and found that they behaved as full agonists in both receptors. This was not absolutely unexpected as α 7L248T receptors have been shown to present a rather peculiar pharmacology, capable of being activated by a series of typical nicotinic antagonists such as tubocurarine, dihydro- β -erithroidine, hexamethonium, serotonin, strychnine or bicuculine [14–16,29]. All the latter were also able to activate the doubly mutated α 7K145A/L248T receptors. Moreover, changes in the potency of the conventional agonists tested were much larger than those observed in the single mutant α 7K145A. All these data indicate that the pharmacological phenotype of single mutant α 7K145A cannot be transferred to α 7L248T receptors, probably because the gating mechanisms of α 7L248T receptors are deeply altered. Whatever the case, we have also explored the effects of the K145A mutation on DMPP-activated single channel currents. Although open channel block cannot be ruled out (DMPP is used at saturating concentrations), single channel currents activated by DMPP greatly differed from those by ACh. Openings are much shorter, gating constants, β/α , are much smaller and several subconductance states might significantly contribute to the overall current. Nevertheless, it should be pointed out that single channel analysis was performed only on the higher conductance openings. This bias and the one originated by the method of selecting the bursts of activity, which excludes very long closures (more frequent with ACh), could partially explain that the quantitative agreement with macroscopic currents data is not complete. However, the effect of substituting Lys145 by Ala is roughly similar to that obtained by ACh: i.e.: closings are longer and openings are slightly shorter in α 7K145A/L248T receptors, both resulting in a reduced open probability.

At the single channel level the present work has addressed the mechanism by which Lys145 is involved in the gating of neuronal-type homomeric α 7 nAChRs [12] by using the non-desensitizing α 7L248T receptor as control. In spite of some quantitative differences, results are similar to those obtained in muscle-type nAChRs, showing that Lys145 is involved in the early steps of channel activation, and suggesting again that the coupling between agonist binding and gating could depend on the type of nAChR studied. Nevertheless, the otherwise deeply altered gating of α 7L248T receptors precludes the direct extrapolation of pharmacological data obtained in singly mutated α 7 receptors.

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