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Role of negatively charged amino acids in β4 F-loop in activation and desensitization of α3β4 rat neuronal nicotinic receptors

Jiří Lindovský [a,1], Martina Kaniaková [a,1], Lucie Svobodová [a], František Vyskočil [a,b], Jan Krůšek [a,*]

[a] Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[b] Department of Animal Physiology and Developmental Biology, Charles University, Viničná 7, 120 00 Prague 2, Czech Republic

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Abstract

The role of negatively charged amino acids in the F-loop of the β4 subunit in channel activation and desensitization was studied using the patch-clamp technique. The selected amino acids were changed to their neutral analogs via point mutations. Whole-cell currents were recorded in COS cells transiently transfected with the α3β4 nicotinic acetylcholine receptor. The application of acetylcholine (ACh), nicotine (Nic), cytisine (Cyt), carbamylcholine (CCh) and epibatidine (Epi) to cells clamped at −40 mV produced inward currents which displayed biphasic desensitization. The EC_{50} of Epi and Nic were increased by a factor of 3–6 due to mutations D191N or D192N. Only Epi remained an agonist in the double-mutated receptors with EC_{50} increased 17-fold. The interaction of the receptors with the competitive antagonist (+)tubocurarine (TC) was weakened almost 3-fold in the double-mutated receptors. The mutations increased the proportion of the slower desensitization component and increased the response plateau, resulting in decreased receptor desensitization. The double mutation substantially accelerated the return from long-term desensitization induced by Epi.

Keywords: Nicotinic acetylcholine receptor; α3β4; Epibatidine; F-loop

1. Introduction

Nicotinic types of acetylcholine receptors (nAChR) are ligand-gated ion channels comprised of five subunits forming the central pore. nAChRs are activated from agonist binding sites localized to the boundary between the α and neighbouring subunits (for a review, see [1]). The binding of two agonist molecules to the receptor is necessary to induce or stabilize conformational changes of the channel complex which lead to the opening of the cation-selective channel [2,3]. In the prolonged presence of the agonist, the receptor enters an inactive desensitized state [4,5]. Significant structural parts of the neuronal receptor controlling fast and slow desensitization were summarized by Giniatullin et al. [6].

Binding sites are composed of two parts, principal and complementary, the first of which is always formed by an α subunit. The complementary part is formed by a β subunit in heteromeric neuronal nAChRs, whereas this role is played by a δ, γ or ε subunit in muscle types. Thus, from the point of view of function and position in the receptor, neuronal β4 subunits are more related to muscle δ, γ and ε subunits than to the muscle β1 subunit. In contrast to muscle nAChRs with two different binding sites, it is reasonable to suppose that both binding sites in α3β4 receptors are identical or almost identical, since not only their principal but also their complementary parts are formed by identical subunits. Several conserved negatively charged amino acids in the F-loop of the δ, γ and ε subunits are functionally important and hypothetically could interact with the positively charged moiety of cholinergic agonists. Nevertheless, it was concluded that at least in the case of the ε and δ subunits, their influence on channel gating or allostery is more important than their disputable participation in agonist binding [7–10]. Fig. 1 shows an amino-acid sequence comparison of the

* Corresponding author. Tel.: +420 29644 2551; fax: +420 29644 2488.
E-mail address: krusek@biomed.cas.cz (J. Krůšek).
[1] These authors contributed equally.

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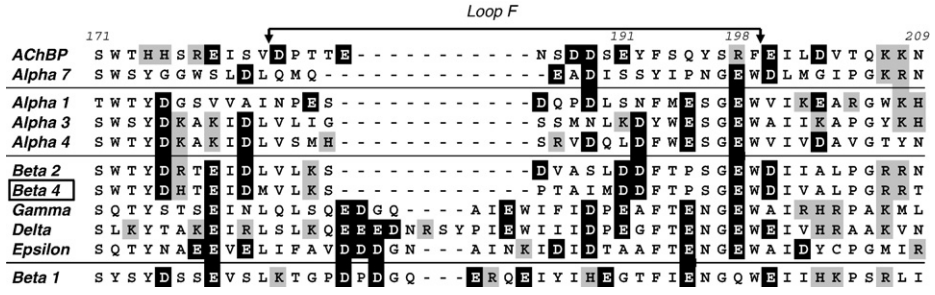

Fig. 1. Section of amino-acid sequence alignment of rat (*Rattus sp.*) nicotinic receptor subunits and *Lymnaea stagnalis* acetylcholine-binding protein (AChBP). Horizontal lines separate four functionally distinct groups of subunits. From top to bottom: subunits participating in the formation of both the principal and complementary face of the agonist binding site (AChBP, α7), subunits forming exclusively the principal face (α1, α3, α4), subunits forming exclusively the complementary face (β2, β4, γ, δ, ε), and β1, which is thought to not contribute to binding site formation. An abundance of negative-charged residues (highlighted with black background) can be seen in the F-loop region in all cases, yet no overall pattern of their distribution can be deduced. Sporadic positively charged residues are highlighted with grey background. Sequence numbering corresponds to the β4 subunit.

F-loop and its vicinity in β4 with several related subunits and acetylcholine-binding protein (AChBP). Note that AChBP, which is used for constructing models of nicotinic receptors [11,12] shows only a low homology to the receptor subunits in the region of loop F [13]. In general, F-loops are rich in negatively charged amino acids, though their exact position within the loop differs. Of the most preserved, the glycine-glutamate-tryptophan motif (GEW) at positions 197–199 is present in all subunits except for muscle β1 and AChBP. Two, three or four consecutive negatively charged residues (starting after position 185 in Fig. 1) can be found in muscle γ, ε and δ subunits, respectively. Another motif of two consecutive aspartates at positions 191–192 is unique to the closely related β4 and β2 subunits (Ligand-gated ion channel database: http://www.ebi.ac.uk/compneur-srv/LGICdb/HTML/catphylogen.php). The E198, D200 pair is also present in human, chick and bovine α7 subunits and in δ mouse, the aspartate is replaced by a glutamate. In chick α7, the receptor amino acids 161–172 (belonging to loop V, partially homologous to loop F) are thought to participate in Ca^{2+} and agonist binding. Mutation of the negative glutamate 172 strongly influences the Hill coefficient and EC_{50} in a calcium-dependent manner [14].

Our goal was to identify those negatively charged amino acids in the loop F of the β4 subunit which could be important both for interaction with a positively charged moiety of agonists and competitive antagonists and for receptor activation and desensitization.

2. Materials and methods

Experiments were performed on COS cells, transiently transfected using a Lipofectamine 2000 procedure (Gibco BRL) with plasmids coding the appropriate subunit combination. Cells were cultivated in a minimal essential medium which was supplemented with 10% fetal calf serum (both from Sigma Chemical, St. Louis, MO). The cDNA coding the α3 and β4 subunits of rat neuronal nAChR were obtained from Dr. S. Heinemann. The cDNAs were subcloned into the pcDNA3.1 expression vector (Invitrogen) at the multiple cloning site. Point mutations were introduced using Pfu Turbo DNA polymerase (Stratagene) and primers coding mutated nucleotides (VBC Biotech, Austria). Successfully transfected cells were detected by cotransfection with the CD4 coding plasmid (kindly provided by Dr. G. Westbrook) and Dynabeads M-450 CD4 (Dynal Biotech, Norway) aggregation control. More than 48 h after the transfection, whole-cell patch-clamp measurements were performed using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA). Fire-polished glass micropipettes with an outer diameter of about 3 μm were filled with a solution of the following composition (in mM): CsF 110, CsCl 30, $MgCl_2$ 7, EGTA 2, HEPES–CsOH 10, and Na_2ATP 5; pH was 7.4. The resulting resistances of the microelectrodes were from 2.5–6 MΩ. The cell bath solution contained (in mM): NaCl 160, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 2, HEPES–NaOH 10, and glucose 10; pH was 7.3. Cells were kept at a holding potential of −40 mV. Solutions of drugs (all from Sigma Chemical, St. Louis, MO) were applied using a rapid perfusion system consisting of an array of ten parallel quartz–glass tubes, each 400 μm in diameter. Both the positioning of the tubes and switching on and off of the flow of different solutions were synchronized under microcomputer control [15,16]. A complete change of the solution around the cell could be carried out in 30–60 ms. For signal recording and evaluation of the data, an Axon Instruments Digidata 1320A digitizer and pClamp 9 software package (Axon Instruments, Foster City, CA) were used. Data were low-pass filtered at 1 kHz and digitized at 2 kHz. Concentration–response curves were fitted to the Hill equation:

$$I(C_a) = \frac{C_a^{nH}}{C_a^{nH} + EC_{50}^{nH}} \quad (1)$$

where C_a is the agonist concentration, $I(C_a)$ is the relative value of membrane current, EC_{50} is the agonist concentration inducing 50% of the maximal response, and nH is the Hill coefficient.

The decay phases of the current responses were fitted to two exponentials with an added constant plateau component using the program Clampfit 9 (Axon Instruments, Foster City, CA) and the relationship:

$$A(t) = A_1 \cdot e^{-\frac{t}{\tau_1}} + A_2 \cdot e^{-\frac{t}{\tau_2}} + C \quad (2)$$

where A_1, τ_1 and A_2, τ_2 are the amplitudes and time constants of the two components of desensitization, respectively. C is the amplitude of the non-desensitizing component of the response, the final plateau. For further analysis, the amplitudes were normalized to the peak value for each response: $a_1 = 100\% \cdot (A_1/A_{max})$; $a_2 = 100\% \cdot A_2/A_{max}$; $c = 100\% \cdot C/A_{max}$.

Inhibition curves were fitted to the simple inhibition curve:

$$I(C_i) = 1 - \frac{C_i}{C_i + IC_{50}} \quad (3)$$

where C_i is the antagonist concentration, $I(C_i)$ is the relative value of the membrane current and IC_{50} is the antagonist concentration that inhibits 50% of the response. IC_{50} was used to calculate the inhibition constant K_i, which is independent of the EC_{50} and concentration of the agonist, according to the Cheng–Prusoff formula modified for a receptor with cooperative agonist binding [17–19]

$$K_i = \frac{IC_{50}}{\left(2 + \left(\frac{C_a}{EC_{50}}\right)^{nH}\right)^{\frac{1}{nH}} - 1} \quad (4)$$

Statistical analysis was performed after transforming the concentration data to −log values indicated as pEC_{50}, pIC_{50} and pK_i as the distribution of errors

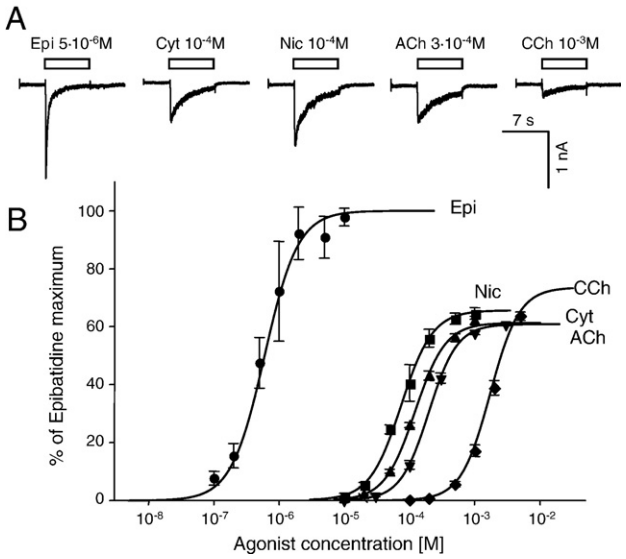

Fig. 2. Activation of wild-type α3β4 receptors by various agonists. A) Examples of current responses induced by 7 s application of Epi (5 μM), Cyt (100 μM), Nic (100 μM), ACh (300 μM) and CCh (1000 μM) in cell clamped at −40 mV. B) Concentration–response curves for the peak currents induced by various agonists of the α3β4 nicotinic receptor (Epi, Cyt, Nic, ACh, CCh). Experimental data points were fitted to the Hill equation. Note that Epi acts with the highest potency and efficacy. COS cells were clamped at −40 mV.

corresponds better to the Gaussian distribution after this transformation. All values of EC_{50}, IC_{50} and K_i are therefore given as means and their −log value (indicated as pEC_{50}, pIC_{50} and pK_i)±S.E.M. in parenthesis. The statistical significance of the differences between K_i values was determined by *T*-test (SigmaPlot 6, SPSS Inc. Chicago, IL) performed on sets of pK_i values with a significance level of 0.05.

3. Results

3.1. Activation

The locations for point mutations in the glutamate and aspartate in the F-loop of the β4 subunit were chosen to be analogous to the negatively charged amino acids in the F-loops of other subunits forming the complementary part of the agonist binding sites. Cells with wild-type α3β4 receptors and receptors with the β4 subunit mutated in the E198Q, D200N, D191N, D192N, and D191N+D192N positions were stimulated by the application of a solution containing the indicated concentration of Epi, Nic, Cyt and ACh. Mutated receptors formed functional chemically activated channels, but their sensitivity to particular agonists was different. The responses of all constructs were compared with those of wild-type receptors. Generally, the rapid application of Epi to successfully transfected cells (10–90% from total in the dish), evoked desensitizing inward ionic currents as high as 5 nÅ in whole-cell patch-clamp mode at −40 mV. The whole-cell responses of all variants of α3β4 receptors are strongly rectified (not shown) and no measurable responses could be induced at positive membrane potentials [20,21].

3.1.1. Wild-type

Wild-type (WT) concentration–response curves were fitted to the Hill Eq. (1) and a comparison of its sensitivity to various agonists is given in Fig. 2 and Table 1. Epi is the agonist with the lowest EC_{50}. The EC_{50} values for Nic, ACh and Cyt were similar, but the estimated EC_{50} value for CCh was one order of magnitude higher. The maximal responses to Nic, ACh, Cyt and CCh were smaller and only reached 60–70% of the maximal responses to Epi.

Table 1
Activation of wild-type α3β4 receptor

Agonist	pEC_{50}	EC_{50} (μM)	*n*H	I_{max}/I_{max}Epi (%)	*n*
Epi	6.2±0.1	0.6	1.7±0.25	100	4
Nic	4.13±0.07	75	1.8±0.1	65±4	6
ACh	3.70±0.03	198	2±0.1	61±5	7
Cyt	3.92±0.01	120	1.9±0.1	61±9	7
CCh	2.76±0.07	1700	1.9±0.04	73±19	3

$pEC_{50}=-\log(EC_{50})$, *n*H is the Hill coefficient, I_{max}/I_{max}Epi is the maximal response relative to Epi and *n* is the number of cells.

3.1.2. Mutations

As the difference in the maximal current amplitude of the responses to a specific agonist between wild-type and mutated receptors could not be estimated directly, only the relative changes in amplitude between agonists were estimated. It was only possible to construct reliable concentration–response curves for all mutated receptors with Epi. Therefore, we compared the saturating response to Epi in every receptor variant with the maximal responses to the various agonists to estimate relative efficacy changes. Mutations E198Q and D200N have virtually no effect on receptor activation and EC_{50} values (not shown).

The change in functionality of D191N- and D192N-mutated receptors was different for the various agonists. Only for two agonists (Epi and Nic) could concentration–response curves be constructed for single mutations (Fig. 3) and the results are shown in Table 2. The EC_{50} for the Epi activation of D191N and

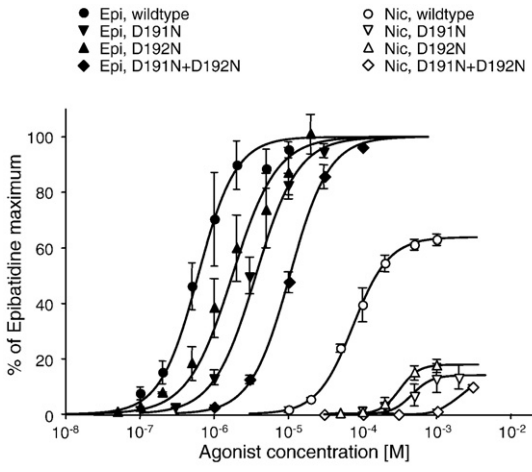

Fig. 3. Effect of mutations within the F-loop on concentration–response curve for Epi and Nic. The relative amplitudes of the responses of wild-type and mutated receptors are normalized to the corresponding maximal response to Epi (set to 100%). Information comparing the maximal response amplitudes between different types of receptors (efficacy of channel opening) is not available. Experimental data points were fitted to the Hill equation with the exception of the Nic responses of the double-mutated receptor.

Table 2
Activation of mutated α3β4 receptors

Agonist – receptor	pEC_{50}	EC_{50} (μM)	nH	EC_{50}mut/ EC_{50}WT	n
Epi – D191N	5.44±0.07	3.65	1.52±0.04	6.1	6
Epi – D192N	5.75±0.18	1.76	1.4±0.2	2.9	4
Epi – D191N+ D192N	4.98±0.05	10.5	1.64±0.1	17.5	4
Nic – D191N	3.35±0.09	440	–	5.9	3
Nic – D192N	3.5±0.03	315	–	4.2	5

$pEC_{50}=-\log(EC_{50})$, nH is the Hill coefficient, EC_{50}mut/EC_{50}WT is a relative increase of EC_{50} and n is the number of cells.

D192N was a 6- and 3-fold increased, respectively. In the double-mutated receptor D191N+D192N, Epi EC_{50} was 17.5 times as high as in the WT, which almost perfectly corresponds to the additive action of the two individual mutations.

Nic remained an agonist with the D191N and D192N mutations but its relative efficacy was decreased (14±9% and 17±5% of the Epi maximal response in D191N and D192N, respectively) and the concentration–response curves could have been deformed. With D191N+D192N, Nic was only able to induce responses at a concentration of 3 mM. It was therefore impossible to construct a concentration–response curve and no EC_{50} value was estimated. The amplitude of this response amounts to 9.9±0.7% of the maximal Epi response.

The activation ability of ACh and Cyt was inhibited by the D191N and D192N mutations to such a degree that their maximal responses only reached 10–20% of the Epi response value at high agonist concentration (higher than 3 mM) and no concentration–response curve could be constructed. CCh was completely ineffective at activating these two mutated receptors (not shown). ACh, Cyt and CCh were completely ineffective at activating D191N+D192N receptors. To clarify to what extent these ligands could bind to double-mutated receptors, they were used as competitive antagonists to Epi as described in Section 3.3.

3.2. Desensitization

In control wild-type responses to 200 μM ACh, 75 μM Nic and 1 μM Epi, the time course of current decay was fitted to two exponential components plus a constant plateau. The time constants τ_1, τ_2, as well as the normalized amplitudes a_1, a_2 of two exponentials and the normalized plateau c varied between cells and also between the responses in one cell. The time constants of the wild-type responses to ACh, Nic and Epi were similar but the relative proportions of the individual components differed markedly between ACh and Nic on one side and Epi on the other side (Table 3). In Epi responses, it was predominantly the shorter component that formed the shape of the responses and the constant plateau was hardly detectable. In contrast, the longer time constant components made a major contribution to the responses of ACh and Nic and the shorter components were slightly smaller. Also, the constant plateau was more expressed in ACh and Nic responses.

The desensitization parameters of different mutated receptors were estimated using the same approach. However, in order to ensure reliable and comparable responses, the Epi concentration for each receptor variant had to be chosen close to its appropriate EC_{50} value. Therefore concentrations of 10 μM, 5 μM and 30 μM were used for the D191N, D192N and D191N+D192N mutations, respectively. The time constants and component amplitudes as well as examples of individual responses of wild-type and mutated receptors to Epi are summarized in Fig. 4.

Table 3
Parameters of wild-type receptors desensitization

Agonist	τ_1 (s)	τ_2 (ms)	a_1 (%)	a_2 (%)	c (%)
ACh	1.1±0.1	190±20	64±2	28±3	6.3±0.5
Nic	1±0.03	200±20	56±3	40±3	4.4±0.6
Epi	1.6±0.3	200±20	11±1	82±3	2±0.5

τ_1 and τ_2 are time constants of desensitization, a_1, a_2 and c are the corresponding relative amplitudes of the individual components and the plateau.

Both, the time course of decay and degree of desensitization were profoundly altered, particularly in D191N and the D191N+D192N double mutation. The amplitudes of plateau c and of slower component a_1 were increased and its time constant τ_1 was prolonged. The amplitude of faster component a_2 was decreased. The overall result of such changes was that

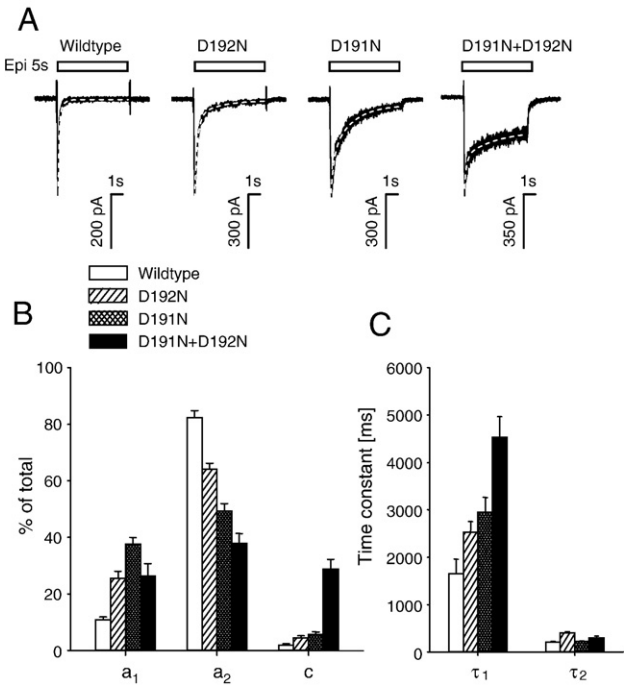

Fig. 4. Effects of selected mutations on desensitization kinetics of α3β4 receptor responses. Epi was used as the agonist in all cases; its concentration was set near the EC_{50} value of the individual receptor variants: 1 μM, 10 μM, 5 μM and 30 μM in the wild-type, D191N, D192N and D191N+D192N, respectively. A) The time course of responses of wild-type and mutated α3β4 receptors. B) The relative contribution of the slow (a_1) and fast (a_2) desensitization components and the final plateau (c) component to the total peak current amplitude. C) The slow (τ_1) and fast (τ_2) desensitization time constants. Note marked prolongation of the slower component τ_1 in all mutated receptors, substantial increase in the plateau level in the double-mutated receptor and decreased contribution of the faster component a_2.

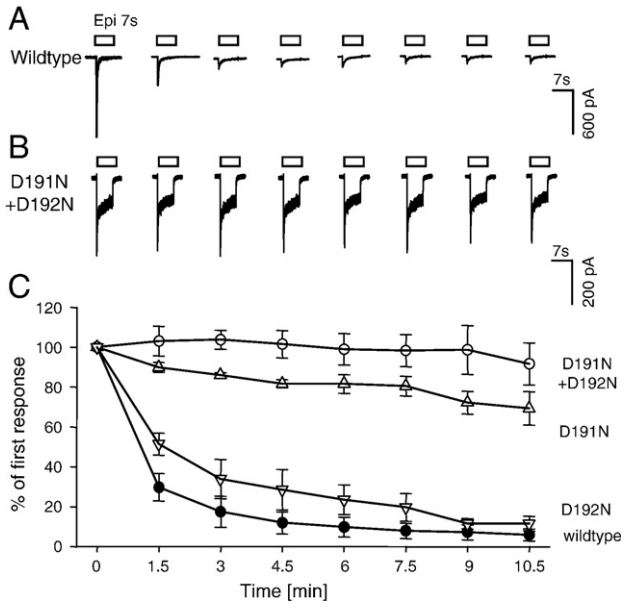

Fig. 5. Effect of selected mutations on Epi-induced long-term desensitization and receptor recovery. Receptors were stimulated by 7 s application of Epi at 90 s intervals. Epi was used at the concentration of its EC_{50} for individual receptor variants. A) In the wild-type, repetitive application of Epi strongly diminished responses after several successive applications. B) The double mutation D191N +D192N hardly exhibited this progressive decrease of responses at all. C) Summary of effect of repetitive stimulation on selected mutations. Mutations D191N and D191N+D192N caused almost complete recovery from desensitization during each 90 s gap.

the responses desensitize more slowly and less completely than the Epi responses of the α3β4 WT, which desensitized almost completely in a few seconds. The plateau was 2% in the WT and 5% in the D191N and D192N single mutations, whereas double-mutated receptor responses desensitized markedly slowly and had their plateau at about 30% of the maximum. The application of higher concentrations of Epi to the WT or D192N mutation led to long-term desensitization and to a pronounced decrease in subsequent responses to any cholinergic agonist. This long-term desensitization was removed after at least 10 min of washing with control solution. Such a slow recovery prevented any experimental mapping of the time course of recovery from desensitization. Instead, the decrease in the response peak value during repetitive 7-second applications of Epi at 90 s intervals was measured (Fig. 5). In the WT, after 3 successive applications the response dropped to less than 20% of the control and decreased even more with further applications. Such a progressive decrease reflects a very slow time constant of recovery from the desensitized state, which must be substantially longer than the 90 s gap between the responses. The decrease in the peak values during repetitive stimulation of the D192N (Fig. 5C), E198Q and D200N (not shown) mutants was slower and less complete, however the reproducibility of successive responses remained unsatisfactory. In contrast to these receptors, D191N receptor responses decreased only slightly during repetitive application of Epi and it was only in D191N+D192N receptors that responses to repetitive stimulation were stable.

Table 4
Inhibition of Epi responses by TC and partial agonists

Inhibitor – receptor	pK_i	K_i (μM)	n
TC – WT	5.2±0.2	6	12
TC – D191N	5.16±0.2	6.9	6
TC – D192N	5.24±0.1	5.8	4
TC – D191N+D192N	4.78±0.1	16.7	10
ACh – D191N+D192N	2.7±0.1	1864	5
Nic – D191N+D192N	3.57±0.1	271	6
CCh – D191N+D192N	2.35±0.2	4410	5

$pK_i = -\log(K_i)$, and n is the number of cells.

A higher plateau together with a faster response recovery indicates that the time constant of leaving the desensitized state, and therefore Epi unbinding from the desensitized double-mutated receptors, is substantially faster than in the WT.

3.3. Inhibition

The competitive antagonist (+)tubocurarine (TC) was used to test whether mutated amino acids are also involved in the interaction with molecules which do not induce the open channel conformation. TC was applied at varying concentrations as a 3 s prepulse followed by coapplication with Epi at the concentration of its EC_{50} for the given receptor variant. Epi was chosen as the prototype agonist because responses to other agonists in mutated receptors were small and often undetectable. Concentration–inhibition dependence was used to calculate K_i for the WT and mutated receptors using the modified Cheng–Prusoff formula [17–19]. Only double-mutated receptors had a K_i value that was significantly higher than that of the WT (Table 4) (Fig. 6). It indicates that receptor interaction with TC is affected by each single mutation to a much lower degree than the interaction with agonists.

The classical agonists Nic, ACh and CCh are almost unable to activate the double-mutated receptor. We tested their ability

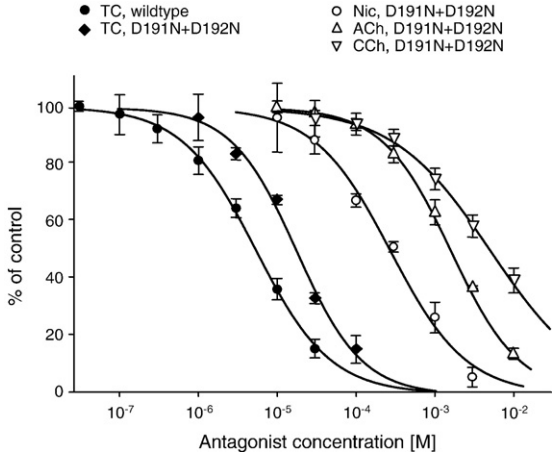

Fig. 6. Inhibition of wild-type and mutated α3β4 receptors. Concentration–inhibition curves of Epi responses blocked by antagonist TC in the wild-type (Epi 0.6 μM) and by TC, Nic, ACh and CCh in D191N+D192N double-mutated receptors (Epi 10 μM). Experimental data points were fitted to the Hill equation (nH=1) with the exception of CCh points.

to bind to this receptor via competition with Epi using the same protocol as in experiments with TC (data shown in Table 4.). All compounds lowered responses to 10 μM Epi (Fig. 6), Nic being the most effective inhibitor with K_i almost 300 μM. The order of K_i of Nic, ACh and CCh is identical with the order of their EC_{50} in the WT.

4. Discussion

Our characterization of mutated α3β4 receptors showed that both negatively charged amino acids D191 and D192 in the F-loop of the β4 subunit participate in channel activation and the desensitization mechanism. When tested separately, the D191N mutation was generally more effective.

4.1. Activation

The parameters of activation of α3β4 WT receptor are in good agreement with similar studies [20,21]. The finding that maximal responses to ACh, Cyt, Nic and CCh hardly reached 65% of maximal response to Epi can be further analysed using a simplified model of receptor activation, described in detail elsewhere [22,23]. During this analysis the maximal relative response to an agonist is given as $100\% \cdot L/(L+1)$ where L is the equilibrium constant of channel gating [24]. It can be shown that if the maximal response to the agonist does not exceed 65% of maximal response to Epi, then L for this agonist is below approximately 1.9, regardless of L for Epi. The Hill coefficient being between 1.5 and 2 indicates that the occupation of both binding sites by an agonist is necessary to induce effective channel opening. Moreover, the Hill coefficient values together with low L values imply high degree (10–100) of positive cooperativity between binding sites for ACh, Nic, Cyt and CCh in this model.

Generally, the increase in EC_{50} for Epi and Nic is similar, which is in contrast with the 3.5–5-fold diminished responses to Nic relative to those to Epi. In the WT, the maximal Nic response is around 65% of the maximal Epi responses (Fig. 2), whereas in the D191N and D192N receptors responses to Nic are only 14% and 17% of the Epi responses, respectively (Fig. 3). This striking difference could indicate that the changes in EC_{50} for Epi and Nic are caused by different mechanisms. With Nic it might be a substantial decrease in L, indicating a decreased efficacy of channel opening and increase in EC_{50}. Only this kind of change could explain the decrease in Nic maximal response relative to Epi. That a decreased cooperativity between both binding sites or direct change in microscopic equilibrium constant for Nic binding to the first binding site of the closed receptor might contribute to this increase in EC_{50} is unlikely, since they are not connected to any change in maximal response amplitude. Also, analysis of the competitive inhibition of Epi responses in the double-mutated receptor by Nic indicates that binding is not substantially diminished, as will be discussed later. In terms of the allosteric model of channel activation — the affinity of the open state to Nic is decreased by mutations more than the affinity of open state to Epi. The affinity of the closed state to Nic was perhaps even enhanced.

The mechanism underlying the increased EC_{50} of mutated receptors to Epi could be explained by a comparison with the role of ε D175 in human muscle receptor, which corresponds to position 189 in the F-loop of the β4 subunit. Sine et al. [25] found that the D175N mutation decreases channel open probability and reduces closed state affinity 17-fold in one case of congenital myasthenic syndrome.

The complete loss of the ability of ACh, Cyt and CCh to activate the double-mutated receptor is in contrast with antagonistic behavior of ACh and CCh (Fig. 6). We could conclude that it is the efficacy of those agonists rather than ligand binding that is substantially affected.

4.2. Desensitization

Most reports concerning amino acids identified as important for the desensitization process focus on the M2 transmembrane domain and especially on α7 canonical L247 [6,26]. Mutations in α7 L247 make the channel's desensitized state permeable to ions. The α7 E172 residue is connected to channel potentiation by Ca^{2+} ions [14,27]. However, its neutralization also partially compensates the changes induced by the L247K mutation in α7, indicating a role in the interaction of the desensitized receptor with the agonist [28].

The influence of calcium cations on α3β4 receptor activation and desensitization is less understood than in α7 receptors. Measurements of channel activation at various Ca^{2+} concentrations revealed a significant decrease in responses at increased Ca^{2+} on α3β4 [21]. It could not be excluded by analogy with the α7 receptor that the role of negatively charged amino acids in the F-loop is also mediated by the binding of the Ca^{2+} ion.

The change in the degree of desensitization could also be compared with the role of ε D175 in one of the congenital myasthenic syndromes [25], which is also important in channel activation as mentioned above. The neutralization of this negative charge leads to a progressive decrease in the affinity of the desensitized state to the agonist (800-fold).

These two groups of data could indicate the possible importance of the F-loop in connecting binding site occupation by an agonist with conformational changes leading to channel opening and desensitization.

Moreover, the return from the desensitized state is substantially accelerated in the double-mutated receptor. The long-term desensitization of a wild-type α3β4 receptor by a higher concentration of Epi indicates that the corresponding rate constant is extremely low and the binding constant of the desensitized state for Epi is much higher than that of the resting state. The decreased extent of desensitization and acceleration of resensitization could indicate a decreased affinity of the desensitized state to Epi.

4.3. Antagonist action

In electrophysiological experiments, the antagonist activity could only be assessed as the comparative inhibition of agonist activity. The concentration of the antagonist necessary to decrease the control response to 50% depends not only on

mutation-induced changes in antagonist binding itself, but also on possible changes in the EC_{50} of the agonist. On the other hand the measure of the ability of the antagonist to bind to its binding site, the inhibition constant K_i, is independent of agonist binding and could be calculated from the IC_{50} values determined for any agonist concentration.

The IC_{50} of TC for α3β4 may depend strongly on the receptor source. In the bovine receptor K_i was 0.1 μM [29], while in the rat receptor K_i was 20 μM [30]. Both results were obtained by measuring TC competition with ^{3}H Epi binding.

The finding that the interaction of the antagonist TC with the binding site is also influenced by point mutations, but to a lower extent, could be explained in at least two ways. Binding site epitopes that were changed by the mutations interact with the agonist molecule more strongly than with the antagonist molecule. Antagonist binding is stabilized by interaction with the unchanged epitopes. Another explanation is that both ligands interact with identical epitopes, but the EC_{50} of the agonist is enhanced by allosteric conformation changes connected with the cooperativity between two binding sites, channel opening and desensitization. As the binding of the antagonist is not stabilized in this way, changes putatively weakening such allosteric phenomena cannot contribute to changes in antagonist binding. Analysis of the interaction of the mutated receptor with TC could be complicated by the fact that in neuronal receptors containing the β4 subunit, TC is able to act as not only a competitive inhibitor [31]. It was found that receptors with one binding site occupied by ACh and the second binding site occupied by TC could still open and contribute to the ionic current.

4.4. Nic, ACh and CCh as partial agonists

The ability of Nic, ACh and CCh to serve as competitive antagonists indicates that these ligands bind to the double-mutated receptor. The interpretation of the comparison of the EC_{50} of agonists (ACh, Nic or CCh) in the WT with their K_i for the double-mutated receptor depends strongly on the model of allostery between the binding sites. The ratio K_i/EC_{50} for Nic is 3.6, for ACh is 9.4 and for CCh is 2.6. To analyze this relationship we again used an approach based on a simplified model of the relationship between the macroscopic constants EC_{50} and nH with microscopic parameters such as the degree of allostery between both binding sites and equilibrium constant for channel gating described in detail elsewhere [22,23]. Since the Hill coefficient for activation of the WT by these agonists is 1.8–2 and assuming that the equilibrium constant for channel gating L is less than 2, we could estimate the necessary degree of allostery between the binding sites to be 10–100-fold. Therefore the equilibrium constant for binding to the first binding site should be 5–20 times higher than the measured EC_{50}. This means that the equilibrium binding constant of the closed wild-type receptor could be even higher than the inhibition constant K_i of the double-mutated receptor. This suggests that in the double-mutated receptor, Nic and CCh binding to the closed receptor is at least undiminished if not enhanced. The inhibition constant for ACh permits the explanation that there is a slight weakening of its binding to the double-mutated receptor. Taken together, this indicates that the loss of ability of these agonists to activate the double-mutated receptor is entirely caused by changes in the last step of the activation — in the probability of channel opening. However, the possibility remains that former agonists binding to the double-mutated receptor cannot cause channel opening but still remain cooperative.

The alternative explanation is that the possible increase in affinity of the double-mutated receptor to former agonists/blockers is a product of the allosteric mechanism [32], where the loss of activating properties is caused by a decrease in the affinity of the opened state accompanied by an increase in the affinity of the closed state. A similar pattern could also be observed in the decrease of the affinity of the desensitized state to Epi.

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