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Biochimica et Biophysica Acta 1778 (2008) 864–871
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

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

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Received 7 June 2007; received in revised form 4 January 2008; accepted 10 January 2008
Available online 16 January 2008

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

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doi:10.1016/j.bbamem.2008.01.010

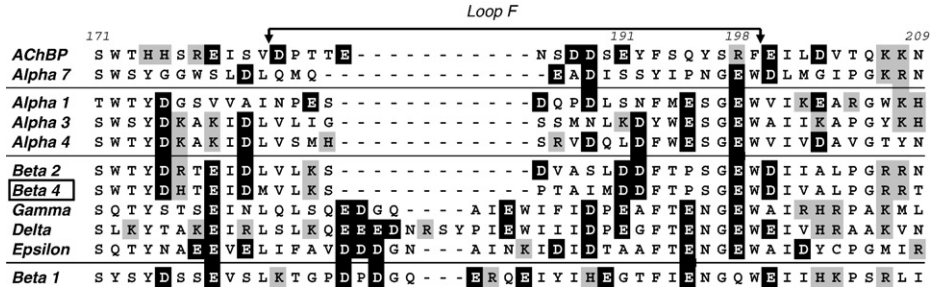

Fig. 1. Section of amino-acid sequence alignment of rat (*Rattus sp.*) nicotinic receptor subunits and *Lymnaea stagnalis* acetylcholine-binding protein (AChBP). Horizontal lines separate four functionally distinct groups of subunits. From top to bottom: subunits participating in the formation of both 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