International Journal of Biological Macromolecules 70 (2014) 320–326

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Purification and functional characterization of a truncated human α4β2 nicotinic acetylcholine receptor

Nikolaos Kouvatsos[a,*,1], Athanasios Niarchos[b,1], Paraskevi Zisimopoulou[a], Elias Eliopoulos[c], Konstantinos Poulas[b,*], Socrates Tzartos[a,b,**]

[a] Department of Neurobiology, Hellenic Pasteur Institute, 127 Vass. Sofias Avenue, GR11521 Athens, Greece
[b] Department of Pharmacy, University of Patras, GR26500 Patras, Greece
[c] Department of Agricultural Biotechnology, Agricultural University of Athens, Holy Street 75, GR11855 Athens, Greece

ARTICLE INFO

Article history:
Received 27 April 2014
Received in revised form 18 June 2014
Accepted 19 June 2014
Available online 8 July 2014

Keywords:
α4β2 nAChR
Ion channel receptor
Membrane protein
Protein engineering
Baculovirus expression system

ABSTRACT

Nicotinic acetylcholine receptors (nAChR) are abundant in the brain and are essential in cognitive function, learning and memory. Previous efforts on α4β2 nAChR had been focused on functional and pharmacological characterization, where high expression yield is not essential. For structural studies though, large amounts of pure protein is important but heterologous overexpression of membrane proteins can be a burdensome task, especially if high amounts are required. In the current study, a truncated mutant of the human α4β2 nAChR was designed in order to improve expression and solubility and to obtain material suitable for high resolution structural studies. We showed that the wild type α4β2 nAChR presented low expression and solubilization yield both of which were improved with the truncated construct. The truncated nAChR showed similar binding profile to the wild type, was purified by a two-step chromatography and isolated in high purity and adequate quantity.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are pentameric ion channels, controlled by acetylcholine and formed from a pool of seventeen homologous subunits (α1–10, β1–4, γ, δ and ε). nAChRs are members of the superfamily of the ligand gated ion channels (Cys-loop receptors), which also contains the $GABA_A$, $GABA_C$, glycine and serotonin receptors. Each Cys-loop receptor subunit consists of an N-terminal extracellular domain (ECD), 210–220 amino acids long, bearing the ligand binding pocket and the distinctive Cys-loop, four transmembrane hydrophobic α-helices (M1–M4) 15–20 amino acids long, an intracellular loop (100–150 amino acids) linking the M3 and M4 α-helices, and a C-terminal extracellular tail, 4–28 amino acids long [1].

Neuronal nAChRs are located in the central nervous system and ganglia and transmit signals from one neuron to the other, but they are also found in non-neuron related cells too [2]. They are composed of α (α2 to α10) and β (β2 to β4) subunits, creating homomeric or heteromeric nAChRs, with α4β2 and α7 being the most predominant [3]. α4β2 has been strongly implicated in smoking addiction [4], in Alzheimer's disease [5], in cognition [6], in Parkinson's disease [7] and in epilepsy [8]. Besides, α4β2 nAChR has been suggested as a potential therapeutic target for novel anti-inflammation drugs [9,10] and as a target of local [11] and general anesthetics [12].

Despite the fact that ligand binding, expression and electrophysiological studies have provided details about the α4β2 nAChR, regarding locality, functionality and role of the channel in health and disease [7,10,13,14], data for its exact tertiary and quaternary structure is limited. Still, most of the structural details of the α4β2 nAChR can come from homologous studies [15] using as models, the crystal structures of mollusk acetylcholine binding proteins (AChBPs) [16,17], the crystal structures of the mouse α1 ECD [18] and the human α7 ECD-AChBP chimera [19,20].

Previous studies have expressed full length or truncated forms of the human and rat α4β2 nAChRs, in insect [21], in mammalian cells [13] and *Xenopus* oocytes [22] in quantities enough for ligand binding and electrophysiological studies. Yet for high resolution structural studies, much higher amounts of protein are needed. The baculovirus-infected insect cells expression system often achieves relatively high protein expression levels and has the ability to carry

* Corresponding author at: Department of Pharmacy, University of Patras, GR26500, Patras, Greece. Tel.: +30 2610969953; fax: +30 2610969954.
** Corresponding author at: Hellenic Pasteur Institute, Biochemistry, Leoforos Vasillisis Sofias 127, GR11521, Athens, Greece. Tel.: +30 2106478844; fax: +30 2106478842.
E-mail addresses: kpoulas@upatras.gr (K. Poulas), tzartos@pasteur.gr (S. Tzartos).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ijbiomac.2014.06.058
0141-8130/

out post-translational modification analogous to mammalian cells and therefore, has been used for expressing mammalian membrane proteins in large amounts [23–25].

In the present study, wild type (WT) and truncated (T) constructs of the human α4 and β2 nAChR subunits were coexpressed in Sf9 (*Spodoptera frugiperda*) insect cells, using baculovirus expression system. The truncated constructs are lacking the intracellular loop, in an attempt to increase the expression and the solubilization of the expressed receptor [26,27]. More importantly, though, the removal of flexible domains, would be beneficial for crystallization as there are numerous analogous examples leading to crystallization of membrane proteins [28–31]. The expression and solubilization efficiency of the wild type receptor was limited, whereas the truncated form presented higher expression levels and solubilization yield. Furthermore, the α4β2 T nAChR was purified by a two-step procedure using Ni–NTA (nickel-nitrilotriacetic acid) resin and size exclusion chromatography in an effort to obtain material proper for high resolution structural studies, such as x-ray crystallography.

2. Materials and methods

2.1. Materials

Molecular biology enzymes were obtained by New England Biolabs Inc. Detergents, anti-6xHis antibody, nicotine, imidazole, UB-165 ((1R,6R)-5-(6-chloropyridin-3-yl)-9-azabicyclo [4.2.1]non-4-ene), TC-2559 (4-(5-ethoxy-3-pyridinyl)-*N*-methyl-(-3E)-3-buten-1-amine) and all the other reagents were obtained from Sigma-Aldrich Co. LLC. unless otherwise stated. Detergents Big CHAPS (*N*,*N*′-bis(3-D-gluconamidopropyl) deoxycholamide), CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), DDM (*n*-dodecyl β-D-maltoside), DM (*n*-decyl-β-D-maltopyranoside), NaCholate (sodium cholate), NG (neopentyl glycol), OG (*n*-octyl-β-D-glucoside) and TX-100 (Triton X-100) were purchased from Anatrace Affymetrix, Inc. PageRuler Prestained Protein Ladder used in SDS-PAGE was obtained from ThermoScientific Inc. ^{125}I-α-bungarotoxin labeled human muscle nAChR was obtained from RSR Limited, UK. Ni–NTA agarose resin was obtained from Qiagen and size exclusion chromatography column Superose 6 was obtained from GE Healthcare.

2.2. Cloning and expression of wild type and truncated human α4β2 nAChRs in Sf9 insect cells

The α4 and β2 wild type and truncated subunits of the human nAChR coexpressed in Sf9 insect cells using the Bac-to-Bac® Baculovirus Expression System (Invitrogen). The α4 and β2 cDNA constructs, were flanked at the N-terminal end, by their own signal peptides and at the C-terminal end by a polyhistidine (8xHis) tag. Particularly, using as templates the full-length cDNAs coding for the human α4 (ACHA4_HUMAN, P43681) and β2 (ACHB2_HUMAN, P17787) nAChR subunits, kindly provided by Prof. J.M. Lindstrom (Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, PA), the wild type α4 and β2 cDNA constructs were enzymatically amplified by PCR using a primer combination where an EcoRV restriction site was incorporated in both ends and an 8xHis tag in the 3′ end of the PCR product and subsequently subcloned to pBluescript II SK (+) plasmid (Agilent). Additionally, in order to remove the cDNA sequence that corresponds to the intracellular loop of α4 and β2 subunits and generate the truncated α4 and β2 cDNAs in pBluescript II SK plasmids, the GeneArt® Site-Directed Mutagenesis System (Invitrogen) was used. For this reason, the previously made wild type α4 and β2 pBluescript plasmids were used as templates along with a pair of primers for each subunit. Particularly, on the wild type α4 pBluescript plasmid, the pair of primers used was A4SFOR: 5′GCTCGCCACGCACGCACATGAAGCGGATGGTCATCGACCG3′ and A4SREV: 5′GTGCGTGCGTGGCGAGCGGTGGTGCACGTTGAG3′. Similarly, on the β2 pBluescript plasmid, the pair of primers used was B2SFOR: 5′GCTCGCCCACCACGCACATGCAGCAGATGGTGATCGACCG3′ and B2SREV: 5′GTGCGTGGTGGGCGAGCGGTGGTGCACG3′. Subsequently, the wild type and truncated α4 and β2 cDNA constructs were subcloned into the pFastBac1 vector (Invitrogen). The recombinant pFastBac1 plasmids were used for generation of recombinant baculoviruses according to the manufacturer's protocols [32]. Recombinant viral stocks were prepared, tittered, and stored in SF900 II medium supplemented with 5% heat-inactivated fetal bovine serum (Gibco BRL, USA) at 4 °C.

The expression of recombinant α4β2 nAChRs was performed in suspension culture of Sf9 insect cells adapted in SF900 II serum free medium (Invitrogen) at 27 °C. When the culture reached cell density of 2.0–2.5 × 10^6 cells/ml was double-infected with α4 and β2 recombinant baculoviruses at multiplicity of infection (MOI) of 5. The cells were harvested after 3 days post infection and the cell pellet stored at −80 °C until used.

2.3. SDS PAGE and Western blot analysis

Samples were analyzed by 12% SDS-PAGE (Laemmli method) under reducing conditions. For visualization, gels were stained with Coomassie blue. In Western blot analysis protein bands were transferred to a PVDF membrane (Macherey-Nagel), in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3 buffer, applying 350 mA for 45 min, and after blocking in PBS/2% BSA (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH2PO4, 2% BSA, pH 7.4) for 1 h at 4 °C, were probed with anti-polyhistidine peroxidase conjugated mAb (Sigma) (1:1,000 in PBS/0.2% BSA) overnight at 4 °C. Finally the PVDF was developed by incubation in DAB (3,3′-diaminobenzidine) staining buffer (PBS, 0.5 mg/ml DAB, 2 mM $NiCl_2$, 0.02% H_2O_2).

2.4. ^{3}H-epibatidine binding saturation studies

Sf9 cells (1 × 10^5) were incubated with increasing concentrations of ^{3}H-epibatidine (PerkinElmer, Inc.) at room temperature for 1 h in PBS. The samples were then analyzed by filter binding assay. In detail, samples were diluted with 1 ml of PBS and passed under vacuum in a Millipore multiport filtration apparatus through Whatman GF/B (1.0 μm pore size) 2.5 cm diameter glass microfiber filters. After sample passing, filters washed thrice with 1 ml of PBS, dried and the bound radioactivity was counted after overnight incubation in 5 ml of Quicksafe A scintillation liquid (Zinsser Analytic GmbH, Frankfurt, Germany), under constant agitation, at room temperature. Non-specific binding was determined by performing the assay using non-infected cells. Results were expressed as the specifically bound radioactivity (Δcpm), after subtracting the non-specific bound radioactivity. The concentrations of total and the amounts of bound ^{3}H-epibatidine were calculated using the specific radioactivity of the stock solution. ^{3}H-epibatidine saturation curves were fitted using non-linear regression according to the model: $Y = B_{max} \times X/(K_d + X)$, where X is the radioligand concentration, and Y is the amount of specifically bound radioligand. Equilibrium dissociation constant (K_d) and maximum binding capacity (B_{max}) were calculated in the fitting process using the program Prism 5.0 (GraphPad Software, Inc., San Diego, USA).

2.5. Competition experiments

Specific binding of imidazole, nicotine, TC-2559 and UB-165, was studied in co-incubation competition experiments. Increasing concentrations of the unlabeled ligands, or PBS as control,