B'SYS GmbH

nAChR $\alpha_4\beta_2$ CHO Cell Line

Cell Culture Conditions

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1 BACKGROUND

1.1 Human Nicotinic Acetylcholine Receptors

The nicotinic acetylcholine receptors (nAChRs) are members of a superfamily of ligand-gated ion channels that mediate fast signal transmission at synapses. The nAChRs are thought to be hetero-pentamers composed of homologous subunits. The proposed structure for each subunit is a conserved N-terminal extracellular domain followed by three conserved transmembrane domains, a variable cytoplasmic loop, a fourth conserved transmembrane domain, and a short C-terminal extracellular region. The protein encoded by this gene forms a homo-oligomeric channel, displays marked permeability to calcium ions and is a major component of brain nicotinic receptors that are blocked by, and highly sensitive to, alpha-bungarotoxin. Once this receptor binds acetylcholine, it undergoes an extensive change in conformation that affects all subunits and leads to opening of an ion-conducting channel across the plasma membrane.

1.2 B'SYS' HEK-293 $\alpha_4\beta_2$ nAChR Assay

B'SYS has designed an assay on a CHO cell line with constitutive coexpression of human $\alpha_4\beta_2$ nicotinic Acetylcholine Receptor. The $\alpha_4\beta_2$ nAChR cDNA were cloned and transfected into CHO cells and then the functional properties of the $\alpha_4\beta_2$ nAChR validated by means of the patch-clamp technique. The assay is suitable to study the effect of agonists, antagonists and positive as well as negative modulators.

2 VALIDATION OF $\alpha_4\beta_2$ nAChR

CHO cells stably expressing $\alpha_4\beta_2$ nAChR were tested by means of the manual and automated patch-clamp technique in the whole-cell configuration. The bath solution contained (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10 and Glucose 10. The pH was adjusted to 7.4 with NaOH. The pipette solution consisted of (in mM) KCl 130, $MgCl_2$ 1, MgATP 5, HEPES 10, EGTA 5. The pH was adjusted to 7.2 with KOH. After formation of a GΩ seal between the patch electrodes and individual $\alpha_4\beta_2$ nAChR stably transfected HEK-293 cells, the cell membrane was ruptured to assure electrical access to the cell interior. All solutions applied to cells were continuously perfused and maintained at room temperature. As soon as a stable seal could be established, an holding potential of -80 mV was applied. Test solutions were applied by a fast application system. $\alpha_4\beta_2$ nAChR channels were activated for 4 s in intervals of at least 45 s. Test items were applied at increasing concentrations.

2.1 Agonist Screen

As an example for the agonistic screen the concentration dependence of Acetylcholine was investigated. The EC_{50} value was determined. For Acetylcholine an EC_{50} of 0.95 ± 0.34 µM, Hill coefficient: 1.25 ± 0.16 was calculated.

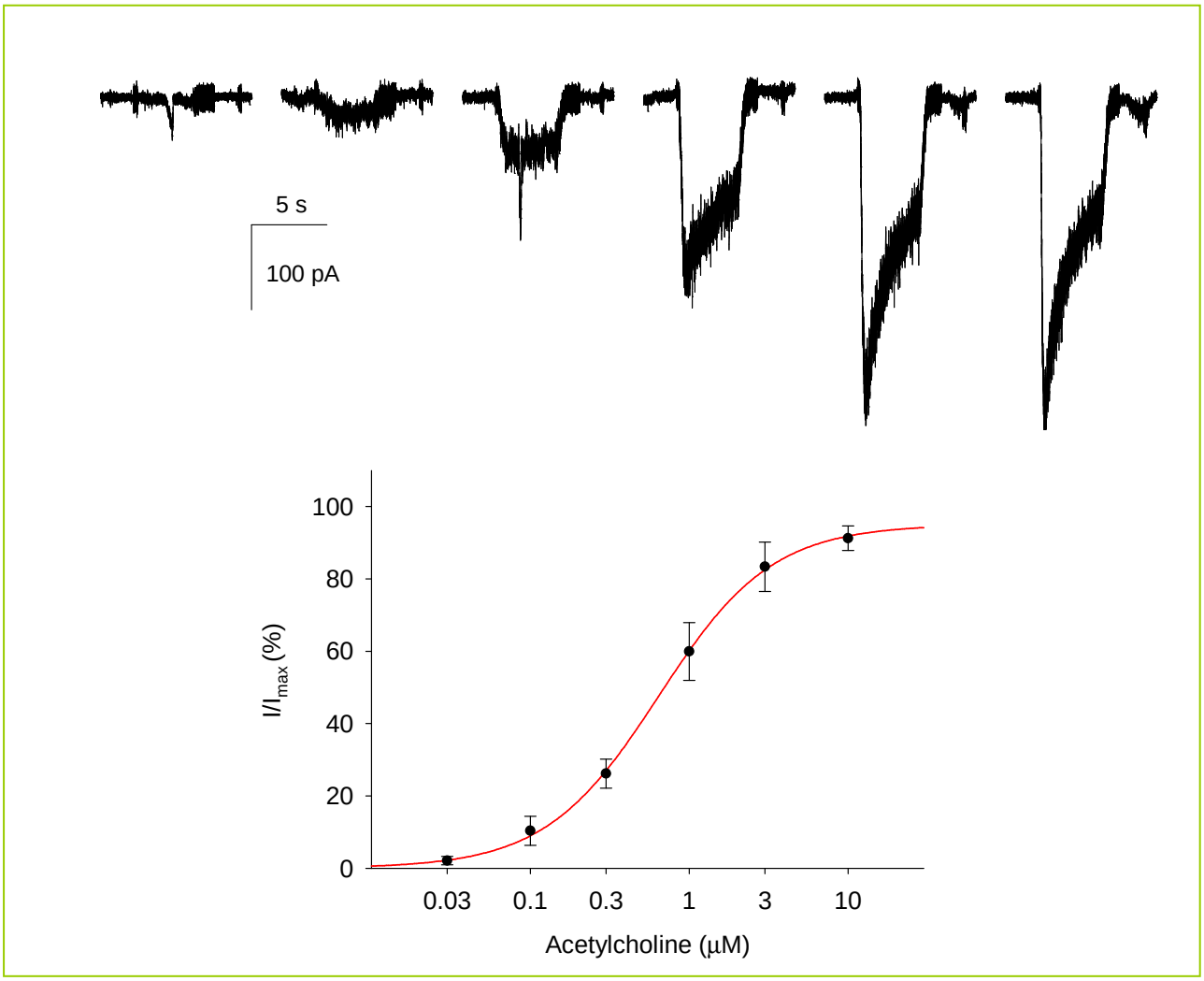

Figure 1: A) representative current recordings from $\alpha_4\beta_2$ nAChR HEK-293 cells stimulated with 0.03, 0.1, 0.3, 1, 3 and 10 µM Acetylcholine (upper trace), dose response curve for Acetylcholine, EC_{50}:0.95 ± 0.34 µM.

2.2 Antagonist Screen

As example for the antagonistic screen the concentration dependence of Tubocurarine was investigated at EC_{50} concentration of Acetylcholine (1.0 µM). The IC_{50} value was determined. For Tuboburarine an IC_{50} of 1.67 µM, Hill coefficient: 1.24 was calculated.

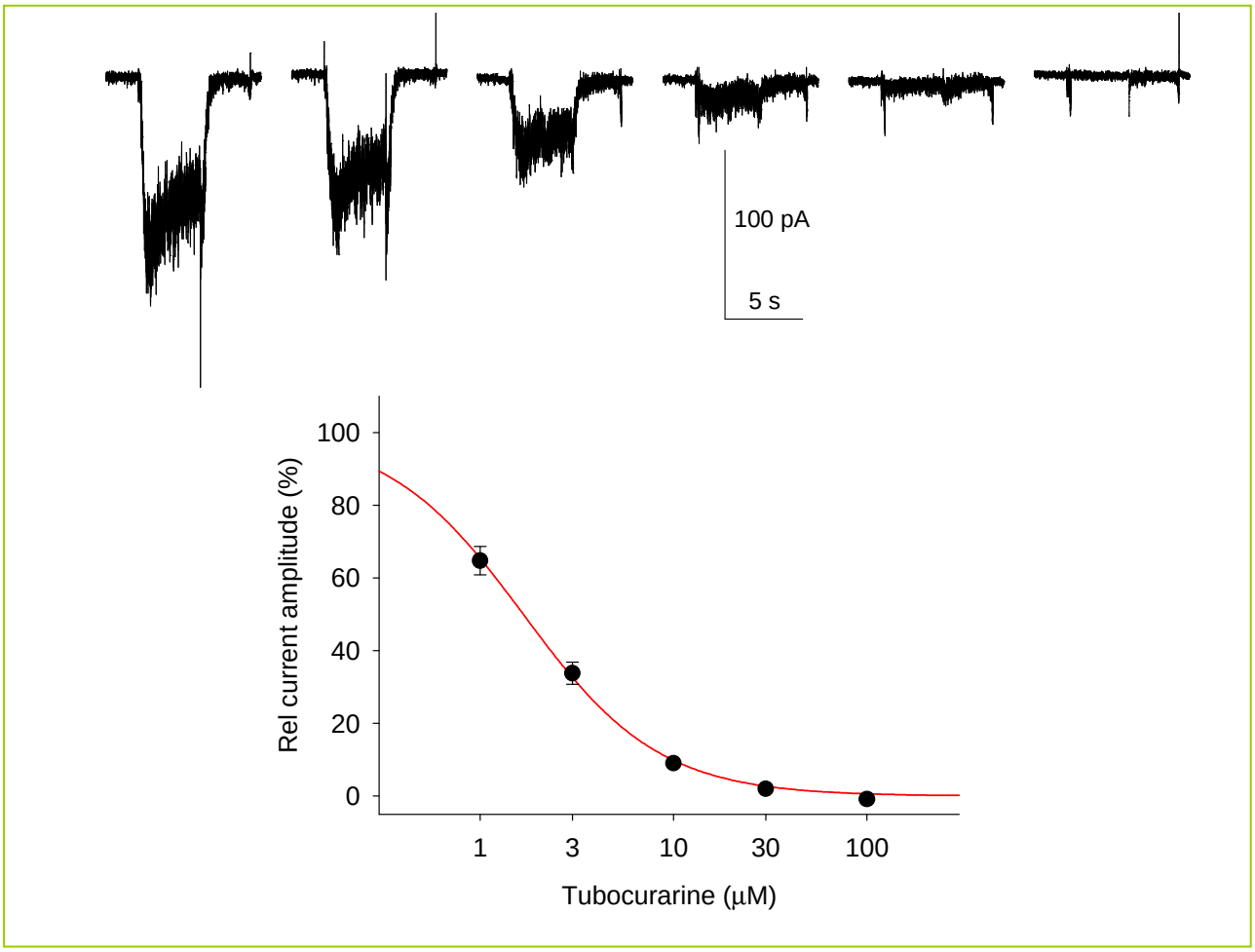

Figure 2: Antagonist screening. A) representative current recordings from $\alpha_4\beta_2$ nAChR HEK-293 cells stimulated with 1.0 µM Acetylcholine. Increasing concentrations of Tubocurarine were applied: 0, 1.0, 3.0, 10, 30 and 100 µM (upper trace), dose response curve for Tubocurarine, IC_{50}:1.67 µM.

3 CELL CULTURE CONDITIONS

3.1 General

HEK $\alpha_4\beta_2$ nAChR cells are incubated at 37°C in a humidified atmosphere with 5% CO_2 (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing F12 (HAM) medium supplemented with 9% fetal bovine serum, 0.9% Penicillin/Streptomycin solution and 100 µg/mL Hygromycin, 2.5 µg/mL Puromycin as selection antibiotic. The $\alpha_4\beta_2$ nAChR cells are passaged at a confluence of about 80%.

- All solutions and equipment coming in contact with the cells must be sterile.
- Use proper sterile technique and work in a laminar flow hood.
- Be sure to have frozen cell stocks at hand before starting experiments.
- Cells should be split every 2-3 days at 70% - 80% confluence at 1:3 to 1:5 ratio.

3.2 Recommended Complete Medium

- F12 (HAM) with Glutamine or GlutaMAX
- 10% FBS
- 1.0% Penicillin/Streptomycin

3.3 Antibiotics

- HEK $\alpha_4\beta_2$ nAChR clones were selected under 500 µg/mL Hygromycin and 5.0 µg/mL Puromycin antibiotic pressure.
- To cultivate HEK $\alpha_4\beta_2$ nAChR cells, also a reduced antibiotic pressure (100 µg/mL Hygromycin and 5.0 µg/mL Puromycin) can be used.
- To separate HEK $\alpha_4\beta_2$ nAChR cells from untransfected cells, use 500 µg/mL Hygromycin and 5.0 µg/mL Puromycin.

Remark: The permanent application of high antibiotic pressure has no effect on current density.

3.4 Thawing Cells

- Remove vial of cells from liquid nitrogen and thaw quickly at 37°C.
- Decontaminate outside of vial with 70% ethanol.
- Transfer cells to a T-25 culture flask containing 5 mL complete medium.
- Incubate cells at 37°C for 4-6 h to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 5 mL complete medium & antibiotics.
- Antibiotics: 100 µg/mL Hygromycin and 5.0 µg/mL Puromycin.
- Incubate cells and check them daily until 70% - 80% confluence is reached.

3.5 Splitting Cells

- When cells are 70% - 80% confluent remove medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Passage cells into new flask with complete medium and antibiotics at 1:3 to 1:5 ratio.
- Use remaining suspension for counting the cells.

3.6 Freezing Medium

- Mix 0.9 mL fresh complete medium and 0.1 mL DMSO for every 1 mL freezing medium.
- Sterilize freezing medium by means of appropriate micro filter (0.1 µm – 0.2 µm). Alternatively sterile DMSO can be used.

3.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluence prior to freezing.
- Remove the complete medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells with centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately 2.0 E+06 cells per mL with fresh freezing medium.
- Aliquot 0.5 mL of cell suspension into each cryovial.
- Overnight incubate cells in a polystyrene box at –80°C.
- The next morning transfer cryovial in liquid nitrogen tank for long-term storage.

4 $\alpha_4\beta_2$ nAChR SEQUENCE

4.1 Alpha 4 subunit

Cloned cDNA sequence of human α_4 nAChR subunit was error-free and encodes for NP_000735.1 sequence:

4.2 Beta 2 subunit

Cloned cDNA sequence of human β_2 nAChR subunit was error-free and encodes for NP_000739.1 sequence:

5 CONTACT INFORMATION

5.1 Contact Address

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