Mechanism of action of alpha7 nicotinic acetylcholine receptor positive allosteric modulators

Dissertation

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1. Introduction

Loss of intellectual ability may be caused by the natural process of aging, illness, or brain injury. It is characterized by mental disability, memory impairment, and personality change, which occur due to loss of neuronal function or cell death. Several neurodegenerative and psychiatric disorders are accompanied by dementia, therefore it is a serious global health problem. In recent years many attempts have been made to mitigate the symptoms of the disease.

Cognitive decline cannot be reversed, but early diagnosis and treatment can delay its progression. Agents that positively affect memory, promote alertness, focus attention, and stimulate learning and memory processes are called nootropic or cognitive enhancer compounds. There is a search for nootropic drug targets, the most likely candidates are receptors and enzymes which are known to play a key role in cognitive function.

Receptor proteins have *orthosteric binding sites*, where *agonists* bind, and initiate a physiological response. Ligands which are able to bind to this site, but do not evoke the physiological response, are called *antagonists*. These molecules interfere with the action of agonists, and inhibit their action. Some ligands bind to topographically distinct (non-overlapping), *allosteric binding sites* and alter the activity of the receptor in a specific way; these molecules are called *modulators*. Modulator binding causes a discrete, reversible, conformational change in the protein structure referred to as the *allosteric transition*.

Modulators in general cannot activate receptors in themselves, but can alter (inhibit or potentiate) agonist-evoked responses[1].

My thesis describes the homopentameric α7 subtype of nicotinic acetylcholine receptors (α7nAChR), a potential therapeutic target for neurological disorders with cognitive symptoms. Selective agonists and positive allosteric modulators (PAMs) of this receptor have procognitive effects. Downregulation of the α7 nAChR has been observed in patients with stroke and other central nervous system disorders like Alzheimer's, Parkinson's disease, schizophrenia and nicotine addiction. Its role is known in memory and learning processes, therefore it has become an important target in dementia research. The focus of my thesis is PAM compounds acting on the α7 nAChR. PAM compounds have several advantages over agonists: they do not cause receptor desensitization or agonist induced upregulation; they preserve the physiological neuronal activity pattern, and it is generally easier to find subtype-specific compounds for the less conserved allosteric binding site. Besides cognition enhancers, α7 nAChR PAMs may also be useful in the treatment of inflammation, acute and chronic neurodegenerative disorders, and various pain syndromes.

[1] Some of the compounds which bind to the allosteric site are able to evoke activation, these are called allosteric activators or Ago-PAMs.

1. Objectives

In pharmaceutical research high throughput has the priority over a comprehensive understanding of mechanisms of action. HTS methods are best suited to identify hits (compounds that bind to a certain target), and promising compounds are tested in animal models of diseases. The exact mechanism of action is of secondary importance, and it is often only examined after the compound has been proven effective (*e.g.*, whether it is an agonist, PAM, silent agonist, or Ago-PAM; and if it is a PAM, which type it belongs to). We propose that if drug developers had the means to screen from the beginning for a specific mechanism of action, this approach could be more cost-effective. In order to achieve this, one needs to investigate the physiological behavior (*i.e.*, gating kinetics) of the receptor, and then the way it is altered by ligand binding, together with association/dissociation dynamics of specific ligands. To investigate this at sufficient throughput on an automated system, one needs to perform first manual patch clamp experiments with adequate time resolution in solution exchange and data acquisition.

In this study, we focused on the kinetic analysis of two α7nAChR PAMs: PNU-120596 and A-867744. Both compounds belong to "type II" PAMs, *i.e.*, they do not only increase the amplitude of currents (like "type I" PAMs), but also fundamentally alter its kinetics. There were conflicting data in the literature regarding their binding site, whether

they share the same allosteric site; we intended to investigate this as well.

We aimed to address to the following questions:

1) How fast is the native (unmodulated) kinetics of the α7 nAChR?

2) During agonist-evoked activation what fraction of the receptor population opens simultaneously? What fraction desensitizes without ever opening?

3) What processes are reflected in the rise and the decay of currents?

4) How do modulators change gating kinetics?

4) Do the two modulators act by the same mechanism?

5) Do they compete for the same binding site?

6) Can we adopt our protocols for automated patch clamp systems?

We believe that the classification of PAMs needs to be reconsidered. We hope that our results will contribute to a better understanding of promising molecules with beneficial therapeutic effects and to more effective drug research in the treatment of cognitive decline.

1. Methods

Manual patch clamp

GH4C1 cells stably expressing α7nAChRs were used for experiments in whole-cell or outside-out patch configurations, using an Axopatch 200B amplifier, Digidata 1322A interface, and the pClamp software (Molecular Devices, Sunnyvale, CA). Agonist-evoked currents were recorded at -70 mV holding potential, digitized at 100 or 20 kHz and filtered at 10 kHz. Experiments were carried out at room temperature (~25°C). Borosilicate glass pipettes (World Precision Instruments) were pulled with a P-87 micropipette puller (Sutter Instruments) and filled with pipette solution (50 mM CsCl, 60 mM CsF, 10 mM NaCl, 10 mM HEPES, and 20 mM EGTA, pH adjusted to 7.2 using CsOH). Pipette resistances ranged between 1.7 and 4.0 MΩ, series resistance values between 2.1 and 9.1 MΩ. Cells were transferred to the recording chamber and culture medium was exchanged to a HEPES containing extracellular solution (140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM HEPES-Na, 10 mM D-glucose, pH adjusted to 7.3 using NaOH). During experiments, the control extracellular solution was perfused continuously (flow rate ~1.66 ml/min). Osmolality of all experimental solutions were set to ~330 mOsm. For fast drug application, we used piezoelectric-driven theta tubes (Burleigh LSS-3200 ultrafast solution switching system). Solution flow (~0.2-0.3 ml/min) in the theta tubes was pressure controlled (DAD-12, ALA Instruments). For data analysis we used Microsoft Excel, the Solver function was used for curve fitting.

Automated Patch Clamp

For automated patch clamp measurements we used the same cell line. For the last day before the experiment, cells were transferred to a 30 °C incubator for increased channel expression. Cells were dissociated from the dish with Accutase (Corning), shaken in serum free medium for 60 minutes at room temperature, then centrifuged, and resuspended into the extracellular solution to a concentration of 3-5x10^6 cells/ml. Cells and solutions were pipetted into microfluidic 384 well plates. Solution composition and osmolality was the same as in manual patch clamp experiments. Currents were evoked with 1-10 mM choline, sampled at 10 kHz, filtered at 2 kHz. Holding potential was -70 mV. Protocol design, data acquisition, and initial analysis were done with the software of the instrument. Further analysis was done in pClamp Clampfit 10.7 and Microsoft Excel.

1. Results

Manual patch camp experiments

We found that α7 nAChR receptor has uniquely low open probability. After agonist binding, the majority (75-80%) of receptors desensitize without opening. At the peak of the current only ~3% of the receptor population is in open state simultaneously. From the low open probability it follows that the opening rate constant does not affect current kinetics, only current amplitude. Both current onset and decay are determined by closing and desensitization rate constants. Because of the high closing and desensitization rates, the kinetics of

experimentally measured currents does not reflect the intrinsic kinetics of the channel, but rather the rate of the solution exchange. The higher the agonist concentration, the larger the distortion of currents by insufficiently fast solution exchange. We investigated the extent of distortion by using different solution exchange rates. We concluded, that for example in the case of 10 mM choline-evoked currents, instantaneous solution exchange would result in a current that has twice the amplitude and 2-3 times faster kinetics, than currents evoked by 1 ms (10 to 90%) solution exchange time.

Positive modulators (PAMs) of the α7 nAChR are structurally diverse compounds which are able to radically alter gating kinetics and open probability. Their mechanism of action and their interactions with the receptor are not fully understood yet. PAMs are currently classified into two types based on their effect: compounds that increase the peak current amplitude without changing its transient nature (type I) and compounds that also change current kinetics, causing prolonged activation (type II). It is becoming increasingly clear, that this classification is an oversimplification originating from insufficient time resolution of recorded currents and a lack of detailed studies of mechanisms of action.

Different mechanisms of action may be preferable for different therapeutic effects. For example in the treatment of inflammatory processes a prolonged, moderate increase in activity is required, therefore type II PAMs are expected to be more beneficial. However, in conditions, where preservation of the original neural activity pattern

is important (such as in preservation of cognitive abilities) type I PAMs are likely to be more effective.

We have found the following differences between the two modulators:

1) A-867744 readily associated to resting receptors, while PNU-120596 could not bind to resting conformation.

2) Bound PNU-120596 radically prolonged openings and induced long bursts. At the whole-cell level this is reflected by the slow onset ($\tau \approx 100$ ms) and prolonged deactivation ($\tau \approx 300$ ms when both the agonist and the modulator were removed, $\tau \approx 2000$ ms in the continuing presence of the modulator). In contrast, the onset of A-867744-modulated current was about ten times faster ($\tau \approx 10$ ms) than the onset of the PNU-12056-modulated current. The difference was even larger in the process of deactivation ($\tau < 10$ ms, regardless of the continuing presence of A-867744). This indicates that A-867744 caused much shorter single-channel openings or bursts than PNU-120596.

Because both compounds modulate currents to form a characteristic temporal pattern, one can discern which of the two modulators is affecting the receptor population. This could be used to monitor displacement of one modulator by the other. Modulator effects were not additive, instead, the presence of one modulator seemed to hinder the effect of the other. This does not necessarily prove a shared binding site, because the interaction may occur on the level of conformational changes. If, however, we start with biding sites fully

occupied with one modulator, and then are able to reverse its effect by increasing the concentration of the other modulator, that indicates a genuine displacement of the former compound by the latter. In the case of A-867744 and PNU-120596, when they were simultaneously applied at the same concentration, the current showed characteristics of A-867744-modulation. The reason for this may be higher affinity, or different stoichiometry. It has been shown that the effect of PNU-120596 only occurs if at least 4 of the 5 binding sites is occupied by PNU-120596 molecules. In contrast, for A-867744 1 or 2 binding sites might be enough to produce modulation. In this case mixed-occupancy receptors would exhibit A-867744-modulated characteristics. By increasing the concentration of PNU-120596, currents changed their temporal pattern, A-867744 characteristics were overcome by PNU-120596 characteristics. This suggests that the two modulators are competing for identical or overlapping binding sites.

Automated patch clamp experiments

In order to make our methods applicable in drug development, they must be compatible with high throughput automated patch clamp systems. For this reason we investigated how our protocols can be adapted on the IonFlux Mercury instrument, which is probably the best commercially available system in terms of solution exchange technology. The plate-integrated pressure driven microfluidic system, provides precise flow control, rapid and parallel liquid exchange and continuous flow of solutions throughout the whole experiment.

Voltage protocols can be combined with complex perfusion protocols. These properties provide an ideal platform for studying ligand interactions and ligand binding kinetics.

Studying α7 nAChRs with an automated patch clamp is challenging for two reasons: Firstly, the unmodulated receptor has extremely low open probability, (as we have discussed before < 3% of the receptors can be open simultaneously). Secondly, the modulators are highly lipophilic, and tend to adhere to silicone and plastic surfaces, with very low desorption rate, and leakage of modulators from these surfaces can make it impossible to record unmodulated currents.

We have introduced a number of modifications: 1) We optimized pressure settings to achieve the best solution exchange rate. This enabled detection of unmodulated agonist-evoked currents. 2) We optimized the length of "priming" (pre-experiment perfusion of the microfluidic channels) to avoid cross-contamination. 3) We perfused control extracellular solution continuously from one of the compound wells. This isolated trapped cells from the rest of the cell suspension, and provided better solution exchange rates. 4) We optimized the duration of preincubation. Some of the compounds were found to require prolonged preincubation, much longer than in manual patch clamp experiments.

5. Conclusions

One important conclusion is, that beyond knowledge about the receptor and its ligands one needs to know the limitations of the technology used for performing the experiments. The most important aspect in the case of α7 nAChRs was solution exchange rate. We explored the advantages and limitations of both the manual and the automated patch clamp systems, and combined these methods to obtain more information about the investigated molecules. Manual patch clamp provides higher resistance, but less stable pipette-cell connection, therefore the quality of the data is much better but long recordings can rarely be completed. Solution exchange using piezo-driven theta tubes is much faster, but the number of compounds is limited. In the case of an automated patch clamp, the plate-cell connection has lower resistance, but it is extremely stable, so it allows long drug application. In addition, even with one of the compound channels reserved for control extracellular solution, it allows application of seven different compounds.

In manual patch clamp experiments, using theta tube ultrafast solution exchange system, we managed to understand the unique intrinsic kinetics of α7 nAChRs, a receptor type, where the majority of receptors desensitize without activation, openings are extremely short, and open probability is extremely low. We determined how fast the receptor can react to an abrupt increase in agonist concentration (such as in the case of synaptic activity), and analyzed what determines onset and decay rates (paradoxically both are determined by closing

and desensitization rates, opening rates only affect the amplitude of the currents).

We examined two modulator compounds, both classified as type II PAMs. Interestingly, we found their mechanism of action fundamentally different. Compound A-867744 is particularly interesting because it seems to be able to act either as a type I, or as a type II PAM, depending on the temporal pattern of agonist concentration. When encounter a prolonged elevation of agonist concentration (such as in the case of elevated choline levels during brain injury), A-867744 could act as a type II PAM, helping to reactivate desensitized receptors. However, under physiological synaptic activity, it could act as a type I PAM on synaptic or perisynaptic α7 nAChRs, preserving the exact pattern of synaptic activity, because it can react rapidly to abrupt changes in agonist concentration.

We propose that *in vivo* studies of therapeutic effectiveness and *in vitro* studies of the mechanism of action should be combined for a more effective drug development strategy of α7 nAChR PAMs.