Mode of action of drugs acting on ion channels: Affinity and accessibility.

Ph.D. Thesis

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Budapest
2016

1. Introduction

Mathematical modeling is a powerful tool for the understanding of mechanisms at single molecule, single cell, or cellular network level. Markov models are commonly used for the understanding of ion channels both at single channel and at population level. The topic of my doctoral thesis is the application of Markov models in the pharmacology of ion channels.

The essence of ion channel function is transition between different conformational states such as cosed, open, desensitized or inactivated. One of the most important attributes of drugs acting on ion channels is their state-dependence: different conformations of the protein cause ligand-protein interactions to be different also. These differences are typically quantified by giving affinity values for different states of the channel. One of the major points in my thesis is that state-dependent affinity alone is often insufficient to explain the mode of action of drugs, and it is preferable to study both affinity and accessibility. I illustrate this general principle by two examples, one is the interaction between a positive allosteric modulator and an agonist on the α7 nicotinic acetylcholine receptor (α7 nAChR), the other is

the unexpected diversity of sodium channel inhibitor mechanisms of action. In both cases we interpreted our electrophysiology data using kinetic modeling.

The treatment of cognitive deficits is an emerging area in current drug development. One of the most promising targets is the α7 subtype of the nicotinic acetylcholine receptor (α7 nAChR). Positive allosteric modulators (PAMs) of this receptor has only recently been discovered. Some of these compounds have proven to be effective in preclinical and clinical trials, and are hoped to be useful against cognitive decline, for example in Alzheimer's disease and schizophrenia. The advantage of PAMs over agonists is that they cannot in themselves activate and desensitize recpeotors, they only augment agonist-induced responses. This way the spatio-temporal activity pattern is preserved. Up to this day little is known about the mode of action of PAMs of the α7 nAChR. Even in the case of one of the most studied PAMs, *PNU-120596*, some of the fundamental questions have not been clarified. In the last few years much has been learned about its single channel kinetics, stoichiometry and temperature-dependence, but its state-dependence and the nature of its interaction with the agonist are still not understood. We therefore endeavored to study these questions.

Overexcitability due to the disfunction of voltage-gated sodium channels can be a cause of various diseases in different tissues, and sodium channels are drug targets in a number of diseases such as epilepsy, neurodegenerative diseases, muscle spasms, different pain syndromes, arrhythmia, etc. The drug binding region of the nine sodium channel isoforms is highly homologous, thus isoform-selectivity of commonly used sodium channel inhibitor drugs is minimal. The therapeutic selectivity of these drugs is therefore somewhat inexplicable; we suppose that their mode of action must be a major factor in determining their therapeutic profile. This, however, is a rather underrepresented issue in the literature of sodium channel inhibitors, where there seems to be a consensus that most sodium channel inhibitors act by essentially the same mechanism. For the explanation of the characteristic properties of inhibition (voltage-dependence, use-dependence, frequency-dependence, etc.) there are two classic theories: The "modulated receptor hypothesis" supposes conformational state-dependent differences in affinity, while the "guarded receptor hypothesis" explains the same properties with state-dependent differences in accessibility. We recognized that the two hypotheses are not necessarily alternatives but could both contribute to the mode of action of individual drugs, therefore

we aimed to create a theoretical framework where both can be described in terms of free energy changes, and in which their respective contribution can be quantified for individual drugs.

2. Aims

We investigated the mode of action for the modulator PNU-120596 on the α7 nAChR, and for five sodium channel inhibitors. We used electrophysiology experiments and kinetic modeling. Our approach in was to investigate the role of both affinity and accessibility in the mechanisms; in most investigations the latter is not studied quantitatively. We propose that separation of the two can provide important insight into mechanisms of action, which can be essential for successful drug development.

2.1. Investigation of affinity and accessibility in the interaction of PNU-120596 with agonists of the α7 nAChR

We aimed to answer the following questions:

- What mechanism lies behind the biphasic current occurring at the co-application of the modulator and the agonist?
- Is there a state-preference in binding of the modulator?

A mutual cooperativity between orthosteric and allosteric binding sites has been assumed, but the nature of the interaction is under debate. It has been shown that the presence of the modulator increases agonist affinity, but this might be a virtual increase only. The gating of the modulator-free channel is so fast that it has not yet been resolved, not even with submillisecond drug exchange methods. Because of the insufficient solution exchange rate, the current is distorted, and the higher the agonist concentration, the larger the distortion is. It has been argued that by slowing down current kinetics positive modulators only unmask the affinity of agonists. For this reason we aimed to address the following questions:

- Is there a genuine cooperativity between orthosteric and allosteric binding sites?
- If there is, is it mutual? Does agonist binding also affect the modulator binding site? Does the modulator affect the affinity or the accessibility of the agonist?

2.2. Investigation of affinity and accessibility in the case of sodium channel inhibitors

We investigated state-dependent inhibition by five sodium channel inhibitor drugs (*lidocaine*, *bupivacaine*,

phenytoin, *riluzole* és *flecainide*). We addressed the following questions:

- How should the problem of affinity and accessibility be best addressed in experiments? Is it possible to quantify the contribution of "modulatedness" (state-dependent affinity) and "guardedness" (state-dependent accessibility) for individual drugs?
- Are there different types of sodium channel inhibitors based on the contribution of affinity and accessibility to different states?
- Can we determine affinity and accessibility to all states? This would be equivalent to a full characterization of the mode of action.

3. Methods

We performed patch-clamp experiments in whole-cell or outside-out patch configuration on rat α7 nAChR expressing GH4C1 cells, or on rNav1.2 expressing HEK-293 cells, using an Axoclamp 200B amplifier and the pClamp software (Molecular Devices). Currents were digitized at 20 kHz and filtered at 10 kHz. In the case of α7 nAChR experiments the holding potential was -70 mV. In the case of sodium channel

experiments we used depolarizations from -150 mV holding potential to 0 mV to evoke currents. Depoarizations were repeated every 55 ms, altogether 600 times; drugs were applied between from the 200th to the 400th depolarization. We used five different voltage protocols, which only differed in the fraction of time spent at depolarized membrane potential: It was 5, 19, 50, 81 and 95% of the total time that the membrane potential was clamped at 0 mV.

For the α7 nAChR experiments solution exchange was performed by the „*liquid filament switch*" method, using theta tubes, which were piezoelectric actuator (Burleigh LSS-3200 system). Solution exchange times (10 to 90%) were determined using open-tip junction potential measurement right after the experiments, without moving the pipette; the values were between 0.5 and 2 ms.

For sodium channel experiments drugs were performed using a pressurized (DAD-12 system) dual U-tube system. Solution exchange times (10 to 90%) were between 10 and 50 ms.

Compounds were obtained from Sigma (*choline, lidocaine, bupivacaine, phenytoin, riluzole)* or from Tocris (*PNU-120596, flecainide*).

Systems of differential equations were numerically solved using *Berkeley Madonna v8.01* and *Mathematica 9.0*. Both softwares used the Runge-Kutta fourth order method. Optimization was done using *Mathematica 9.0*.

4. Results

Co-application of 10 mM choline and 10 μM PNU-120596 evoked a characteristic biphasic current, with an initial fast peak and a subsequent prolonged activation. While it was previously found that the two phases merge at high PNU-120596 concentration, they were clearly separated in our experiments even at 10 μM PNU-120596.

The amplitude, as well as the rise and decay kinetics of the first component was identical with the current evoked by choline alone, *i.e.*, this component was not affected by PNU-120596, not even after prolonged pre-incubation. This suggests that the modulator was unable to bind to the resting receptors, but readily associated to desensitzed ones. The effect of pre-incubation nevertheless was observable, the onset of the slow phase was accelerated, possibly because the allosteric site is accessible from the membrane, and modulator molecules required time to accumulate in the membrane phase. We tested

in kinetic simulations the extent of preference for the desensitized state. In order to reproduce experimental data the preference had to be at least 44-fold.

We studied the effect of PNU-120596 on different desensitized states by pre-incubation of the agonist. We observed that prolonged agonist application drives receptors into a deeper desensitized state, from which the modulator was unable to reactivate them.

We also investigated the decay of the current after termination of agonist – modulator co-application. We compared (i) full washout with (ii) washing out the modulator only with prolonged agonist application, and (iii) washing out the agonist with prolonged modulator application. We observed a radical slowing of the decay in the continuing presence of the modulator, which suggests that modulator binding may decrease the dissociation rate of the agonist.

In kinetic simulations this pattern required only a moderate (1.25 to 2.1-fold) increase in agonist affinity, but a marked (100 to 1000-fold) decrease in agonist accessibility.

The apparent increase in agonist affinity could be reproduced in simulations without an actual effect on microscopic affinity.

In order to interpret sodium channel inhibitor experiments, we constructed a model where state-dependent changes in affinity and accessibility were adjustable parameters. This way the contribution of modulate- and guarded receptor hypotheses could be quantified.

In the case of *lidocaine* and *bupivacaine* a simple inactivated state preference could acceptably reproduce inhibition patterns. This preference was caused by an apparent acceleration of association, while the dissociation did not seem to be altered. In the light of the literature and the properties of our model this could be interpreted as a coincidental increase in accessibility and deceleration of dissociation.

In the case of *phenytoin* and *riluzole* we observed a characteristic pattern of inhibition: In the first three of the five protocols there was a similar weak inhibition, but it radically increased in the fourth and fifth protocols. This pattern was best reproduced by supposing a low-affinity, low-accessibility, low-state-dependence binding site, responsible for the weak inhibition in the first three protocols, and an additional highly state-dependent binding site, which, however did not cause channel block, but acted exclusively via modulation.

Inhibition patterns produced by these two compounds were nevertheless manifestly different, in the case of phenytoin a

great change in affinity was most apparent, while riluzole showed a radically state-dependent onset and offset rate.

Inhibition by *flecainide* could be reproduced by assuming an extreme (~1000-fold) increase in accessibility of the open state alone.

5. Conclusions

We propose the following hypothesis regarding the effect of *PNU-120596* on α7nAChRs:

i. The affinity of PNU-120596 to resting state is low, association predminantly occurs to desensitized receptors.
ii. PNU-120596 binding radically slows down dissociation of choline, probably by decreasing its accessibility.

We suppose, therefore, that in a quasi-physiological situation, where a low concentration of PNU-120596 is present, and agonist concentration is elevated in pulses the following sequence of events occurs: The modulator is unable to bound to the resting receptor in the absence of the agonist. When the agonist pulse arrives, it associates to the orthosteric site, and causes activation and desensitization, thus providing access for the modulator to the allosteric site. Modulator binding stabilizes an active conformation from which agonist

dissociation is radically slowed down, in effect the agonist is "trapped" by the modulator. This leads to a prolonged activity even after removal of the agonist, thus agonist-evoked activity is enlarged and prolonged. The agonist eventually dissociates, the receptor assumes its resting position, and the cycle of events can start again. This mechanism provides a particularly effective way to intensify a signal.

Therefore, the basis of the apparent cooperativity between the two binding sites is not a mutual effect on the affinity of each other. It is much rather an effect on accessibility, and an essential element of the interaction is the special dynamics of interacting binding and gating processes.

The most important conclusion of our results with sodium channel inhibitors is that in spite of the apparent similarity of phenomena, the underlying mode of action may be fundamentally and radically different, in the contribution of affinity *vs.* accessibility, in terms of the particular conformations preferred, and even in the topography of the binding sites. The significance of diversity among sodium channel inhibitors is that their mode of action may determine their therapeutic applicability. The discovery of a distinct "no-block-modulator-binding-site" is of special interest in this regard. Understanding the details of differences between

distinct modes of action, is important in order to learn which particular mechanism is likely to be effective against a particular disease, which will help drug development to be more effective.

6. Publications

[1] Szabo AK , Pesti K , Mike A , Vizi ES Mode of action of the positive modulator PNU-120596 on alpha7 nicotinic acetylcholine receptors. NEUROPHARMACOLOGY 81C: pp. 42-54. (2014)

[2] Pesti K , Szabo AK , Mike A , Vizi ES Kinetic properties and open probability of alpha7 nicotinic acetylcholine receptors. NEUROPHARMACOLOGY 81C: pp. 101-115. (2014)