Application of kinetic modeling in drug research

Doctoral Theses

Róbert Károly

Semmelweis University
Szentágothai János Neuroscience PhD School

Supervisor:	Dr. Árpád Mike senior scientist, Ph.D
Official referees:	Dr. István Tarnawa head of department, Ph.D Dr. László Csanády associate professor, Ph.D
Szigorlati Bizottság elnöke:	Dr. Zsuzsanna Fürst professor, Ph.D, D.Sc
Szigorlati bizottság tagjai:	Dr. György Füst professor, Ph.D, D.Sc Dr. Ildikó Világi associate professor, Ph.D

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1. Introduction

One important element of drug research is understanding the behavior of target proteins (e.g. receptors, ion channels) and the change in their behavior induced by ligands (both endogenous ligands and drug molecules). Understanding the ligand-target system is often not straightforward, because of the network of interactions present: ligand binding affects gating, while gating affects binding affinity. In such complex systems it is often not possible to draw correct conclusions by mere speculation.

In such cases kinetic modeling is a valuable tool. We can use simple rules to construct systems which can portray reality (experimental results) with appropriate accuracy. One of the basic assumptions of modeling is that if the output of the model can appropriately reproduce and predict observed phenomena, then the inner structures of the model and the observed system must be similar, thus by modeling one can obtain information regarding the hidden mechanisms of the investigated system. If a model, based on a specific mechanism of action, is able to correctly and consistently reproduce and predict experimental findings, then we can conclude that this particular mechanism of action *may* be the true mechanism of the investigated system. On the other hand, if it fails to reproduce and predict experimental findings, then we can conclude that this particular mechanism *must* be incorrect.

I endeavored to study two major topics: the state-preference of sodium channel inhibitor drugs, and the mechanism of P2X3 receptor desensitization. In both topics we encountered a complex system of interacting binding and gating reactions, and we could answer the questions with the help of kinetic modeling.

1.1. State preference of sodium channel inhibitors

Voltage gated sodium channels represent one of the key elements of signal propagation in excitable cells. Sodium channel inhibitors have been in use for a long time, to block signal propagation (local anesthetics), prevent overexcitation or erroneous activation (anticonvulsants, antiarrhythmics). The generally accepted mode of action for these drugs is selective binding to non-conducting (e.g. inactivated) conformations of the channel, and stabilization of channel conformation in these states. Selective inhibition of slow inactivated state has been recently proposed to provide therapeutic advantages in certain types of epilepsy and arrhythmia, and in the treatment of neuropathic pain.

Slow inactivated conformation is a specific state of sodium channels which develops upon prolonged depolarization. The recovery from

it is also slow: it requires prolonged hyperpolarization. Physiologically, the fraction of available channels is dynamically modulated by slow inactivation. It can be investigated experimentally by protocols containing three subsequent depolarizing pulses: a **control pulse** (short depolarization) followed by a prolonged **depolarizing pre-pulse** which is separated from the consequent **test pulse** (another short depolarization) by a short **hyperpolarizing gap** that allows fast inactivated channels to recover. The decrease in the ratio of available channels (test pulse / control pulse) reflects the slow inactivated fraction. Three major variations of this protocol are commonly used in the literature for the study of slow inactivation:

- The voltage dependence of slow inactivation is studied by modifying the voltage of the pre-pulse in the type of protocols termed **„SInact_V"**.
- The dynamics of the onset of slow inactivation is studied by modifying the duration of the pre-pulse in the protocols termed **„SInact_t"**.
- The dynamics of the recovery from slow inactivation is studied by modifying the duration of the hyperpolarizing gap in the protocols termed **„Rec_t"**.

If the difference in availability in the presence of the drug vs. control is significant, it is normally concluded that the drug binds selectively to the slow inactivated state. However, because binding kinetics may be in itself slow, and because reciprocal interactions between binding and gating processes may result in altered gating rates, the credibility of these conclusions is questionable. Furthermore, these conclusions regarding the state preference of individual drugs often contradict each other; therefore the reliability of these protocols should be investigated.

1.2. The mechanism of $P2X_3$ receptor desensitization

$P2X_3$ receptors are ATP sensitive receptor ion channels, predominantly located on nociceptive neurons. Their assumed role is to sense tissue damage-associated elevation of extracellular ATP concentration. Furthermore they probably are involved in neuropathic pain sensation, since antagonists of $P2X_3$ receptors have been proven effective in animal models of neuropathic pain.

Besides ATP, the receptor is sensitive to several other ATP-derivatives, such as α,β-methyleneATP, or β,γ-methyleneATP. It is desensitized rapidly (~100 ms), but requires several minutes to recover from desensitization, and the rate of recovery is dependent on the identity of the agonist. In the presence of low concentration of agonist it is able to desensitize without activation, which phenomenon is termed High Affinity Desensitization (HAD). HAD is assumed to be important in receptor regulation, and the phenomenon can possibly be exploited to design novel

analgesics; therefore its investigation is potentially important.

A puzzling experimental observation was made during the study of HAD: Low concentration (3nM) of ATP was found to be completely unable to cause desensitization when applied alone, but caused an almost full inhibition when it was perfused during repetitive activation of the receptor using the rapidly dissociating agonist CTP (cytidine triphosphate). The explanation that was offered was, that upon activation and rapid desensitization, a novel, high affinity binding site is formed, to which ATP could bind even at low concentration. Additional experiments suggested that the novel high affinity binding site is physically not separable from the classic binding site, but undergoes some special structural conversion. This high affinity site appears to have a 10 000 fold higher affinity then the conventional binding site, which is exceptional among receptors. It would be interesting to investigate whether this extraordinary binding site could be exploited as a therapeutic target.

2. Goals

2.1. Investigation of the reliability of protocols used for investigating state preference of sodium channel inhibitors

1. Construction of a kinetic model of sodium channels that can simulate drug effects, and incorporates both fast- and slow inactivated states.

While in experiments one observes the effects of individual drugs in different protocols, and constructs hypotheses regarding their modes of action, in simulation experiments the experimenter can define the mode of action, and observe how drugs of specific modes of action behave in different experimental protocols. In order to use the latter approach, we aimed to construct a sodium channel model which can simulate drug effects, and involves both fast and slow inactivation.

2. Investigation of the reliability of generally used protocol types („SInact_V", „SInact_t", „Rec_t")

Using the constructed model we aimed to investigate whether the mode of action of drugs is indeed reliably revealed by using the three major types of protocols which are currently in use to study slow inactivation. To this end, we aimed to test simulated drugs with pre-defined modes of action.

2.2. Investigation of the mechanism of $P2X_3$ receptor desensitization

1. Exploration of desensitization mechanisms of the A $P2X_3$ receptor.

Our primary question was whether HAD indeed requires previous desensitization. If so, is it because upon desensitization a new, high affinity binding site is indeed created? Do we need to suppose a unique mechanism?

2. Elucidation of the mechanisms behind conflicting experimental data from

the literature. Can we propose a mechanism which is able to explain all apparently contradicting experimental results of the literature?

If we cannot confirm formation of a new binding site we need an alternative explanation. Our aim was to find out if we can construct a kinetic model that can appropriately reproduce receptor behavior. Then we can propose an explanation for the unusual behavior of the receptor based on the model.

3. Methods

3.1. Electrophysiology

Transmembrane currents were recorded by standard whole-cell patch-clamp electrophysiology using an Axopatch 200B amplifier and pClamp software. Borosilicate pipette resistances ranged between 1.4 and 3.8 MΩ. Series resistance (3.5 to 9.0 MΩ) was compensated to 60-80%. Experiments were done on room temperature (22°C). Electronic artefacts were corrected off-line. Sodium currents were sampled at 100 kHz frequency, and filtered at 10 kHz; $P2X_3$ receptor mediated currents were sampled at 5 kHz and filtered at 2 kHz. The holding potential for $P2X_3$ receptor mediated currents was -70 mV.

3.2. Cell culturing for sodium channel experiments

Experiments were done on primary cultures of rat embryonic hippocampal neurons, prepared on the 18^{th} day after gestation. Experiments were performed on neurons cultured for 7 to 24 days. Composition of solutions was the following: Intracellular solution (in mM): CsCl - 70, CsF - 70, NaCl - 10, HEPES - 10, Cs-EGTA -10, pH = 7.3. Extracellular solution (in mM): NaCl - 150, KCl - 5, $CaCl_2$ - 1.4, glucose - 10, HEPES - 5, pH = 7.3.

3.3. Cell culturing for $P2X_3$ receptor experiments

In neurons homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors are coexpressed. $P2X_{2/3}$ receptors are also sensitive to α,β-meATP, so they cannot be separated pharmacologically, but their activation and desensitization kinetics is different. In order to acquire data from a homogeneous population of receptors, we needed to perform experiments on $P2X_3$ receptors, ectopically expressed in HEK239 cells. Experiments were performed 2 to 6 days after transfection. Composition of solutions was the following: Intracellular solution (in mM): CsCl - 135, $MgCl_2$ - 20, $CaCl_2$ - 1, HEPES - 20, Cs-EGTA -11, Mg-ATP - 1.5, Li-GTP - 0.3, pH = 7.3. Extracellular solution (in mM): NaCl - 140, KCl - 5, $MgCl_2$ - 2, $CaCl_2$ - 2, glucose - 11, HEPES - 10, pH = 7.4.

3.4. Construction of kinetic models

We constructed compartment models, in which distinct states correspond with conformational states of the ion channel (e.g., closed vacant, open tri-liganded, etc.). States are connected by transitions, which correspond with biological processes (e.g., association, dissociation, desensitization, etc.). Transition rates may be controlled by external factors (e.g. agonist concentration, membrane potential, etc.). Once the transition rate constants are known, the changes in the occupancy of specific states can be written as differential equations for each state. The system of differential equations can be solved numerically, thus the behavior of the system can be followed. We can execute experimental protocols by changing external parameters such as membrane potential or ligand concentration, the sum of occupancy values for conducting states gives the evoked "current", thus experiments can be simulated.

4. Results

4.1. State preference of sodium channels

4.1.1. Kinetic model of sodium channels

Our model, similarly to the classic Hodgkin Huxley model, is a phenomenological one. We assumed that the three gating processes (activation, fast- and slow-inactivation) are independent and voltage-dependent. We regarded them as single-step transitions between two states. This way each state could be represented by open or closed status of the three gates, thus we could define eight possible distinct states, and from each individual state, three distinct transitions was possible. Using this model we could reproduce voltage dependence of activation and inactivation, as well as the dynamics of inactivation. The model did not reproduce current onset, but since our goal was to investigate inactivation processes, we disregarded the shape of current onset.

Drug effects were modeled by constructing a similar eight-state structure of drug-bound states, and connecting each individual drug-bound state with its vacant counterpart by association and dissociation reactions. This structure is only possible energetically if states which had higher affinity were stabilized by drug binding.

4.1.2. Investigation of protocols using prototypical drug modes of action

We considered two aspects of drug mode of action: state preference and binding rate. We defined fast inactivated state preferring (FI) and slow inactivated state preferring (SI) drugs, as well as fast binding (fb) and slow binding (sb) ones. Using these characteristics, we created four

prototypical mechanisms: “FI_fb”, “FI_sb”, “SI_fb” and “SI_sb”.

We investigated the behavior of these “drugs” in experimental protocols. In protocols “SInact_V” and “SInact_t”, not only “SI” type “drugs” but also “FI” type mechanisms proved effective. The explanation of this phenomenon is that “FI” type ligands slow down the recovery from fast inactivation. In “SInact_V” and “SInact_t” protocols the duration of hyperpolarizing gap must be chosen so that channels recover from fast inactivation, so in control case, decrease of the test-pulse-evoked current will reflect slow inactivation. This is not so in the presence of a bound ligand: the recovery from fast inactivation is delayed, therefore, the decrease will be partly caused by drug-bound channels kept in fast inactivated state, not only by slow inactivated channels. In “Rec_t” protocol “FI_fb” only caused inhibition in the initial phase of the recovery, while “FI_sb” was as effective as “SI” drugs. The explanation is slow dissociation. If ligand binding prevents opening of the channel, then recovery from inhibition will depend on both ligand dissociation and recovery from inactivation.

Our simulations showed that “SInact_V” and “SInact_t” protocols were unable to discriminate “FI” and “SI” type inhibitors, and “Rec_t” protocol was only informative in the case of fast dissociating ligands.

To answer the question whether these conclusions are true generally, or only within the context of our specific model, we performed Monte Carlo simulations to investigate parameter dependence of the conclusions. Protocols were tested on a hundred, randomly parameterized (within previously set reasonable boundaries) channel models. Our conclusions proved parameter-independent.

4.1.3. Possibilities of developing more discriminative protocols

Although “SInact_V”, “SInact_t” and “Rec_t” protocols could not reliably discriminate “FI” and “SI” type inhibitors, some tendencies were nevertheless observable. We attempted to extract more reliable information using combinations of protocols. Plotting “Rec_t” effectiveness data against “Slow_t” effectiveness of the same drug, we mapped several possible modes of action. Altogether one hundred different modes of action were simulated and plotted, including different state selectivities, different degrees of state preference, and different association and dissociation rates. Plots resulted in two areas for “FI” and “SI” drugs, which were not disjunct but partially overlapped. We tested these areas by plotting results of the Monte Carlo simulation on the same “Rec_t” effectiveness – “Slow_t” effectiveness plane. While “FI_fb” and “SI_sb” drugs were on the fast-exclusive and slow-exclusive areas, respectively, “FI_sb” and “SI_fb” drugs

were predominantly within the overlapping area. In summary our method has improved discriminatory power, but it is still does not give absolutely certain results for all drugs.

4.1.4. Comparing simulation results with biological experiments

We tested experimentally the following sodium channel inhibitors: lidocaine (local anesthetic and antiarrhythmic), carbamazepine and phenytoin (anticonvulsants), as well as desipramine and fluoxetine (antidepressants). In "SInact_t" protocol lidocaine shifted the curve similarly to the "FI_fb" prototypical mechanism, while carbamazepine and phenytoin were relatively ineffective. Fluoxetine and desipramine acted similarly to "FI_sb" and "SI" drugs. In "Rec_t" protocol, classic sodium channel inhibitors (lidocaine, carbamazepine and phenytoin) were only effective in the initial phase of recovery, similarly to "FI_fb" mechanism, while antidepressants caused a significant inhibition (like "FI_sb" and "SI" mechanisms. According to the method we proposed classic sodium channel inhibitors were classified as fast inactivated state preferring drugs, while antidepressants could not be unambiguously classified, because they fell into the overlapping area.

We found that it is most problematic to discriminate "FI_sb" and "SI_fb" type drugs, i.e. to decide whether the observed behavior is due to slow formation of the preferred state, or slow association and dissociation. It is evident therefore, that discrimination could be improved by determination of binding rate. This however is impeded by the complexity of several distinct processes detectable during both the onset and the offset of inhibition. These processes probably include aqueous phase – membrane phase partitioning, intramembrane diffusion and translocation, as well as binding itself. These thus far have proved experimentally inseparable, although their time constants differ by several orders of magnitude. Because of the lack of information regarding the rates of these processes, the complex phenomenon of drug effect onset and offset is not yet available for reliable modeling.

4.2. $P2X_3$ receptor desensitization

4.2.1. Conditions of HAD development

The main question regarding $P2X_3$ receptor desensitization was whether previous desensitization was indeed an absolute requirement for the formation of the high affinity binding site. If we suppose that there are no two separate binding sites, and behavior of the receptor can be described without assuming any extraordinary mechanisms, then equilibrium desensitization of the receptor will only be determined by agonist

concentration and not the initial state of receptors. In this case the distribution of states approaches a single equilibrium, whether starting from a fully vacant, resting receptor population (when perfusion is started without previous stimulation), or from a fully agonist-bound desensitized receptor population (when perfusion is started after an agonist-evoked current). If the system is tested too early, before reaching full equilibrium, then the degree of desensitization will be different depending on the initial state. We therefore, first investigated the rate of equilibration in the presence of low concentration agonist. We evoked currents in the presence of 10 nM α,β-methyleneATP after different time intervals. We observed that appropriate equilibration required 20-30 minutes. For this reason we measured equilibrium desensitization at 32 min, and found that the final degree of desensitization is not dependent on the initial state. This suggests that it is not necessary to assume formation of a separate binding site.

4.2.2. Mechanism of $P2X_3$ receptor behavior

The next question was, how we could explain then the paradoxical behavior of receptors. Because of the assumed interactions between the processes of agonist binding and gating, we had to use kinetic modeling. We aimed to construct a model which can reproduce concentration-response curve, concentration-HAD curve, and current kinetics. Based on our experimental results and data from the literature we constructed a model with three agonist binding sites, where occupancy of a single binding site could cause desensitization. We used the assumption of the allosteric receptor model, that if the agonist binding enhances desensitization, then agonists must bind to the desensitized receptor with a higher affinity. Furthermore, we supposed that binding of all three agonists is required for activation. We failed to reproduce all experimental findings with this model. However, if we assumed that binding of two bound agonist molecules can already activate the receptor, the behavior of the receptor in all protocols could be appropriately reproduced. This finding was important for two reasons: First it indicated that $P2X_3$ receptors can most probably be opened at partial agonist occupancy, second, it supported our finding that receptor behavior can be explained without supposing additional binding sites or unusual mechanisms.

We observed, that the paradoxical experimental results were always detected, when two different agonists were used during experiments (such as CTP and ATP, in the experiment mentioned in the Introduction). We repeated the experiments with different agonists, and with the same agonist used at both high and low concentration. We concluded that the paradoxical results occur only when different agonists are used. To

investigate the reason of this, we had to construct a model that was able to simulate different kinds of agonists bound to the same receptor. We assumed three identical binding sites and two agonists, states of the model were defined as the possible combinations of agonist occupancy both in resting and desensitized conformation. The extent and rate of desensitization was assumed to depend on the number of bound agonists alone, i.e., we supposed that the effect of agonists on the receptor is identical; agonists only differ in their binding kinetics. For the sake of simplicity we ignored open states, and monitored instead available fraction of receptors.

We observed that when receptors are desensitized by a rapidly dissociating agonist (such as CTP), and then perfuse with low concentration of slowly dissociating agonist, then agonists are rapidly exchanged at binding sites at a rate that is practically identical with the dissociation rate of the fast agonist. The reason of fast agonist exchange is that one bound agonist is enough to keep the receptor at desensitized, i.e., high affinity state. When one or two agonist molecules leave their binding sites, non-bound sites, therefore are high affinity sites, therefore low concentration of agonist can readily and rapidly associate. Furthermore, because during the process of agonist exchange more receptors offer a high affinity site than during the final equilibrium, there is an "overshoot" of slow agonist binding: More slow agonists bind temporarily, and the system approaches equilibrium by dissociation of the slow agonist subsequently.

This overshoot of binding during agonist exchange is exactly what happens when low concentration of ATP is perfused during repeated CTP applications. Interpulse interval was set to allow full dissociation of CTP molecules and recovery of receptors. However, in the presence of ATP, after agonist exchange ATP molecules keep the receptors in desensitized state. This way an extraordinarily strong inhibition seem to develop, if we interpret the inhibition as one caused by the low concentration agonist alone. As we have seen, however, this is not the case, because the inhibition reflects an interaction of the two agonists.

5. Conclusion

5.1. Conclusions regarding the state preference of sodium channel inhibitors

1. Commonly used protocols are unable to discriminate sodium channel inhibitors based on their state preference. Fast inactivated state preferring drugs slow down recovery from inactivation, therefore they will be effective in "SInact_V" and "SInact_t" type protocols. In case of slowly dissociating ligands, recovery from

slow inactivated state, and slow dissociation cannot be discriminated by "Rec_t" type protocols.

2. Fast binding, fast inactivated state preferring drugs can be identified using combined information from "SInact_t" and "Rec_t" type protocols. However, fast binding, slow inactivated preferring, and slow binding, fast inactivated state preferring drugs cannot be discriminated.
3. The classic sodium channel inhibitors: lidocaine, carbamazepine and phenytoin were shown to prefer fast inactivated state. Based on the results with the antidepressants fluoxetine and desipramine we could not determine state preference. Binding rate of fluoxetine and probably other strongly lipophilic compounds are difficult to determine because onset and offset of inhibition are complex, multi-step processes.

5.2. Conclusions regarding $P2X_3$ receptor desensitization

1. The equilibrium in the presence of low concentration agonist is independent of initial state. HAD does not require previous desensitization. All experimental results can be reproduced assuming universal mechanisms of activation and desensitization, there is no need to suppose appearance of a novel binding site upon desensitization.
2. $P2X_3$ receptors probably can open at less-than-full agonist occupancy.
3. The reason of the apparent increase in affinity upon previous desensitization is the rapid exchange of the high concentration agonist used to evoke currents to the low concentration agonist used to study HAD. Unexpected results of experiments can be explained by the following mechanism: During the dissociation of the high concentration rapidly dissociating agonist, unbound high affinity binding sites are formed on the receptors, to which the low concentration agonist can associate. During the rapid exchange of agonists the receptor temporarily binds more agonists than at equilibrium in the presence of the low concentration agonist alone.

6. List of publications

6.1. Publications related to the thesis

Karoly R, Lenkey N, Juhasz AO, Vizi ES, Mike A. (2010) Fast- or slow-inactivated state preference of Na+ channel inhibitors: a simulation and experimental study. *PLoS Comput Biol. 6(6)*:e1000818.

Karoly R, Mike A, Illes P, Gerevich Z. (2008) The unusual state-dependent affinity of P2X3 receptors can be explained by an allosteric two-open-state model. *Mol Pharmacol. 73(1)*:224-34.

6.2. Publications independent from thesis

Lenkey N, Karoly R, Epresi N, Vizi ES, Mike A. (2010) Binding of sodium channel inhibitors to hyperpolarized and depolarized conformations of the channel. *Neuropharmacology*. [Epub ahead of print]

Vizi ES, Fekete A, Karoly R, Mike A (2010) Nonsynaptic receptors and transporters involved in brain functions and targets of drug treatment. *Brit J Pharmacol;160(4)*:785-809.

Szasz BK, Mike A, Karoly R, Gerevich Z, Illes P, Vizi ES and Kiss JP (2007) Direct inhibitory effect of fluoxetine on NMDA receptors int he central nervouse system. *Biol Psychiatry.;62(11)*:1303-9

Lenkey N, Karoly R, Kiss JP, Szasz BK, Vizi ES and Mike A (2006) The mechanism of activity-dependent sodium channel inhibition by the antidepressants fluoxetine and desipramine. *Mol Pharmacol.; 70 (6)*: 2052-2063

Jobbagy A, Harcos P, Karoly R, Fazekas G. (2005) Analysis of finger-tapping movement. *J Neurosci Methods.; 141(1)*: 29-39.

Mike A, Karoly R, Vizi ES and Kiss JP (2004) A novel modulatory mechanism of sodium currents: Frequency-dependence without state-dependent binding. *Neuroscience; 125(4)*: 1019-28

Mike A, Karoly R, Vizi ES and Kiss JP (2003) Inhibitory effect of the DA uptake blocker GBR 12909 on sodium channels of hippocampal neurons. *NeuroReport; 14*: 1945-1949