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Neuroscientist 2001; 7; 95

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Fast Kinetic Analysis of Ligand-Gated Ion Channels

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Institut für Biochemie
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Germany

Ligand-gated ion channels mediate fast synaptic transmission in the central and peripheral nervous system and the neuromuscular junction. Their common principle of function is the conversion of a chemical signal—neurotransmitter binding—into an electrical signal, i.e., an ion influx into the postsynaptic cell. The transient nature of this signal requires experimental setups that provide adequate temporal resolution and the use of transient kinetic analysis rather than equilibrium methods for a correct description of receptor function. Although the highly specialized geometry of a synapse that allows very rapid delivery of neurotransmitter is difficult to mimic in an experimental system, a variety of techniques for rapid kinetic analysis are available, making it possible to determine at least some steps of receptor function with sufficient accuracy. This article provides an overview of strategies and methods of fast ligand application and kinetic analysis using whole-cell and single channel patch clamp. NEUROSCIENTIST 7(2):95–103, 2001

KEY WORDS Ligand-gated ion channels, Kinetics, Function, Patch-clamp, Rapid ligand application

Fast synaptic transmission in the nervous system is effected through binding of a chemical signal (neurotransmitter) to postsynaptic ion channel receptors. Upon neurotransmitter binding, the intrinsic ion pore of these receptors opens, allowing a flux of ions across the cell membrane. This ion translocation results in depolarization and, thus, facilitates or suppresses the generation of an action potential in the postsynaptic cell. Several ligand-gated ion channel superfamilies have been identified to date, including those related to the nicotinic acetylcholine receptor (Betz 1990; Stroud and others 1990) and the family of ionotropic glutamate receptors (Seeberg 1993; Wo and Oswald 1995). Although structurally different (Wo and Oswald 1995), kinetic principles of channel activation are similar for both receptor families. Here, techniques for the measurement of ion channel receptor kinetics on a single cell level are briefly introduced.

A Minimum Mechanism of Receptor Activation and Inactivation

Ion channel receptor function is a complex series of elementary steps of ligand binding and subsequent conformational changes. This discussion is limited to a minimum mechanism of receptor function (Fig. 1), which was introduced by Katz and Thesleff (1957) to describe the function of muscle nicotinic acetylcholine receptors and was subsequently found to also apply to other ligand-gated ion channel receptors (Hess 1993). In many situations, the true mechanism is more complex, yet this simple model incorporates the elementary reaction steps that are

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**Fig. 1.** Minimum mechanism for the activation of ligand-gated ion channel receptors. $K_d$ is the dissociation constant of the activating ligand from the receptor, two independent binding sites of identical affinity are assumed; binding is assumed to be noncooperative, i.e., ligand affinity is the same for the states $R$ and $RL$. $K_{op}$ and $K_{cl}$ are the rate constants for channel opening and closing respectively, also referred to as $\beta$ and $\alpha$ in the literature. The equilibrium constant for these transitions, referred to as gating, has been defined as $\Phi = \frac{K_{op}}{K_{cl}}$, the inverse form, $E = \frac{K_{cl}}{K_{op}}$, is also in use. Desensitization is the transition of the liganded receptor to the inactive state $I$. Of the rate constants shown, $K_{op}$ denotes the most important transition, which is usually sufficient to describe desensitization.

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### Table 1. Kinetic parameters of ligand-gated ion channels

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<sup>a</sup> Hess (1993).
<sup>b</sup> Two-step mechanism.
required to describe receptor function.

The individual steps in receptor activation follow:

1. Ligand binding—normally taken to be controlled by diffusion (Jackson 1989), the limit for a first-order association constant in water is on the order of $10^{-8} \text{ M}^{-1} \text{s}^{-1}$ (Fersht 1985). However, slower association has been observed for the inhibitory glycine receptor with an association rate of $\sim 10^{-7} \text{ M}^{-1} \text{s}^{-1}$ and a dissociation rate constant of 1900 s$^{-1}$ (Grewer 1999). The various states involving ligand binding are considered to be in rapid equilibrium and are usually not resolved in studies of receptor kinetics. Slower agonist dissociation from GABA$\alpha$ and glutamate receptors is being studied extensively (see, e.g., Lester and Jahr 1992; Jones and others 1998).

2. Gating—rate constants for channel opening and closing are on the order of $10^3 - 10^4 \text{ s}^{-1}$ for $k_{o}$ and $10 - 10^3 \text{ s}^{-1}$ for $k_{c}$, i.e., these processes occur on a µs to ms time scale.

3. Desensitization—rate constants vary from 0.1 s$^{-1}$ to 10 s$^{-1}$, so these transitions occur on a second time scale.

Table 1 gives an overview of some constants pertinent to neurotransmitter receptor function. In view of the large diversity of receptor subtypes plus the various biological and experimental situations under which they are investigated, only a highly selective list of examples is shown.

The Electrical Signal: Ion Translocation

The physiologically most relevant ionic species are Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, Cl$^-$, and HCO$_3^-$). Various ion pumps and exchangers actively (i.e., under expenditure of energy) create an imbalance of ions inside and outside of the cell so that at rest neurons normally have a potential of ca. –70 mV relative to the surrounding medium (Kandel and others 2000).

The influx of ions through the channel pore is controlled by two gradients: (i) the ionic concentration gradient, and (ii) the electrochemical gradient, i.e., the potential difference across the cell membrane.

Starting from theoretical considerations of a cylindrical pore attached to a hemispherical ion sink, a maximal translocation of $2 \times 10^7 \text{ s}^{-1}$ can be calculated. Applying the boundary conditions given in a physiological system, one can estimate an upper limit of ion flux through the pore of ca. $3 \times 10^7 \text{ s}^{-1}$ (Hille 1992). This is based on the assumption that Ohm’s law of electric resistance and Fick’s law of diffusion along a concentration gradient apply to the microscopic scale of the ion channel. In reality, there is no free diffusion in the pore; hydration and dehydration processes as well as movement through bulk water and motion of water molecules inhibit ion permeation. Finally, the interplay of diffusion and electrostatics has to be considered (Lauger 1976), and specific binding sites within the pore also affect the travel of various ions through the channel differently as shown, e.g., for glycine receptors (Bormann and others 1987). For nicotinic acetylcholine receptors, an ion translocation rate of $\sim 1 \times 10^8 \text{ s}^{-1}$ was found (Stroud and others 1990).

Kinetic Analysis: Experimental Techniques

Setups and Recording Configurations

Development of the patch-clamp technique by Neher and Sakmann (Hamill and others 1981) made it possible to measure the minute cur-

Fig. 2. Minimum electrophysiology setup. The components required for experiments are shown; micromanipulators for the precise positioning and movement of recording pipettes and application devices are not included.
rants (pA scale) through individual receptor ion channels. A sketch of a basic electrophysiology setup is shown in Figure 2. The focus of this article is on ligand application, electrophysiological instrumentation is not further discussed.

The most common configurations used for the study of neurotransmitter receptors are shown in Figure 3A. In the whole-cell configuration (left panel) an entire cell is held by the recording pipette so that the interior of cell and pipette are in contact. Currents across the cell membrane reflect the response of the entire ensemble of receptors (usually on the order of one to several thousands) on the cell surface. Inside-out or outside-out patches (Fig. 3A, middle panel) use the same principle, but only a small membrane patch is at the pipette tip with a diameter of ~2 µm. Although easier to equilibrate, the patch has a smaller number of receptors available for study. Depending on receptor density, ensemble recordings or single-channel measurements can be performed. In the cell-attached mode (Fig. 3A, right panel) the recording pipette only touches the cell, isolating a small membrane patch suitable for stationary single-channel recordings.

In determination of kinetic constants pertaining to receptor activation, the ligand application system is a crucial member of the experimental setup, because it must be guaranteed that receptor function is in fact measured, and not mixing kinetics or some other dead-time of the system. In other words, the time resolution of the entire recording system has to be faster than the time scale on which the process under investigation occurs. With some neurotransmitter receptors, current amplitude reduction through desensitization can be on the same time scale as ligand application. In such a case, properties of a mixture of active and desensitized receptors are observed and rapid processes will be missed if appropriate time resolution of the setup is not achieved (Hess 1993). Generally, kinetic resolution is higher when membrane patches rather than whole cells are used, because flow equilibration around smaller objects is faster. This often neglected fact was demonstrated by Jones and others (1998), who found a solution exchange time of < 1 ms at a open pipette tip (ca. 2 µm diameter), whereas for a whole cell, solution exchange took ca. 100 ms.

Methods

Uptake of tracer ions into vesicles. This method involves mixing of receptor-rich vesicles with a radioisotope of a permeant ion (36Rb, 36Cl, etc.). Modified stopped-flow techniques were used to rapidly equilibrate vesicles, tracer ions, and ligand (Fig. 3B). The time-dependent uptake of labeled ions is then measured. The pulsed quench-flow method (Fersht and Jakes 1975) has a time resolution of 5 ms and allowed the determination of rapidly desensitizing current phases in acetylcholine receptors (Hess and others 1979). Recently, inhibitory glycine receptors were studied using 36Cl as tracer ion (Blednov and others 1996). Because this technique is restricted to receptor-rich vesicles, requires use of radioactive ions, and does not allow to continuously monitor receptor-mediated currents, patch-clamp techniques are usually preferred.

Whole bath perfusion. In small recording chambers, the entire solution surrounding the cell may be exchanged and replaced by solution containing ligand. The time required to completely replace the chamber solution is usually too long to do rapid kinetics; bath perfusion is, however, used for preequilibration of cells with inhibitors or modulating agents (Le Foll and others 1997; Niittykoski and others 2000), or for perfusion of slice or tissue preparations (Inglefield and Schwartz-Bloom 1997), and is combined with faster perfusion systems for receptor activation.

Iontophoresis. Ligand solution is held in a delivery pipette with the help of an applied potential. For release, a brief voltage pulse is passed through the pipette forcing some of its content out onto the target cell or area (Fig. 3C) (del Castillo and Katz 1955, 1957). Although quite rapid, the time resolution for open-tip pipette solution exchange is on the order of ms; the major problem is that a pulse of turbulent ligand flow is produced. Mixing with the surrounding medium is inevitable and thus the target cell is not exposed to a homogenous well-defined concentration of ligand (del Castillo and Katz 1955, 1957; Katz and Thesleff 1957). Attempts have been made to correct for dilution due to turbulent mixing (Yakel and others 1991), but the dimensions of an open pipette tip (2–3 µm) and a whole cell (20–50 µm) are quite different, making such calculations hazardous. Iontophoresis was among the first rapid ligand application methods and is still widely used with tissue and slice preparations.

U-tube systems. This is the most common technique of ligand application, existing in many variations. Originally developed by Krishtal and Pidoplichko (1980), it allows for delivery of a laminar flow over the cell (Fig. 3D). The design of the U-tube can be adapted, so for neurons, which are large, irregularly shaped, and difficult to lift from the culture dish, a glass U-tube has been used to deliver neurotransmitter to the neuron. The time resolution of this system is on the order of milliseconds (10–50 ms), which is sufficient to analyze desensitization and determine the gating equilibrium constant, but not the individual rates k± and kobs. Some neurotransmitter receptors have current phases that desensitize in the ms time domain. Thus, current rise in these cases not only reflects ligand application and channel opening, but also desensitization that takes place during equilibration of the cell with ligand solution. A method to correct for this desensitization during ligand application was developed in the laboratory of George P. Hess.
Fig. 3. Configurations for electrophysiological recordings and ligand application. A, Patch-clamp configurations: 1 patch pipette, 2 recording electrode. B, Quench-flow apparatus: 1, 2 reactant chambers; 3 mixing chamber; 4 quench chamber. In a typical experiment, 1 would contain acetylcholine and Rb⁺, 2 receptor vesicles, 4 a large excess of nonradioactive Rb⁺. Time resolution for mixing is ~5 ms. C, Iontophoresis: 1 electrode; 2 delivery pipette; + ligand molecules. At rest, a negative potential at the electrode holds ligand in, release is triggered by a brief positive pulse to the electrode. D, U-tube devices: 1 solenoid valve; 2 opening, ca. 200 µm diameter; 3 suspended whole-cell. Agonist is delivered through the lower tube, suction is applied via the upper tube. Before the experiment, the applied suction produces a net flow of solution into the tube. For release, the valve is shut, and ligand solution flows over the cell. E, Y-tube system: 1, 2 solution delivery tubes; 3 switching valve; 4 clamped cell. The cell is suspended in the flow tube, and through tube 1 control solution is applied. Upon switching (dashed line in 3), solution from tube 2 flows over the cell. F, Flow-tube systems: either theta tubing (upper panel) or an array of flow tubes (lower panel) is used and solution switching is achieved by movement of the array using a stepper motor.
1999). As pointed out above, these low concentrations of ligand, the current rise to maximum was as rapid as 200 µs (Liu and Dilger 1991). At low concentrations of ligand, the current rise time was governed by concentration-dependent diffusion. High (supersaturating) ligand concentrations, the concentration gradient is such that the receptor is saturated at a time faster than the solution exchange time (Liu and Dilger 1991), because diffusion rate is dependent on the concentration gradient. High agonist concentration ranges can be explored for rapid kinetic studies using this technique.

**Y-tube systems.** In this technique, the cell is positioned in a flow tube (Fig. 3E); solution exchange is effected by a switching valve (Brett and others 1986). An optimized setup had an exchange time at the open tip of a pipette of ca. 25 µs, and the observed current rise to maximum was as rapid as 200 µs (Liu and Dilger 1991). At low concentrations of ligand, the current rise time was governed by concentration-dependent diffusion. At high (supersaturating) ligand concentrations, the concentration gradient is such that the receptor is saturated at a time faster than the solution exchange time (Liu and Dilger 1991), because diffusion rate is dependent on the concentration gradient. High agonist concentration ranges can be explored for rapid kinetic studies using this technique.

**Flow-tube application systems.** Here, solutions are fed constantly either through the individual half-barrels of a theta tube ([Colquhoun and others 1992; Jonas 1995], Fig. 3F, upper panel) or through an array of tubings (Fig. 3F, lower panel [Franke and others 1987; Maconochie and Knight 1989; Jonas 1995]). The cell or patch is immersed in the flow of control solution and solution switching is achieved by rapid stepwise movements of either the tubing (more common) or the pipette using a step motor (Jonas 1995). Patches and whole cells can be used. For patches, time resolutions of 100 µs can be achieved (Jonas 1995). A continuous flow of solutions is required, and at the same time the entire chamber should be perfused at the same flow rate to avoid turbulences. The practical limit of solution exchange using this method was estimated at ca. 20 µs and imposed by the barrel switching movement (Sachs 1999). As pointed out above, these rapid exchanges can only be attained with patches, not with whole cells (Jones and others 1998).

**Laser-pulse photolysis.** A new, entirely different approach to rapid agonist delivery was developed in the laboratory of George Hess (Milburn and others 1989; Matsubara and Hess 1992; Matsubara and others 1992). This involved the use of caged neurotransmitters bearing an attached photolabile group. To date, caged derivatives for most neurotransmitters are available (Hess and Grewer 1998). The principle is shown in Figure 4A. A light pulse from a laser (~10 ns duration) cleaves the photolabile caging group from the neurotransmitter. Given the right chemistry, this reaction occurs on the µs time scale. Ligand binding is a priori assumed to be in rapid pre-equilibrium (if not, time constants for association and dissociation can also be measured). Figure 4B shows the experimental setup. The cell is equilibrated with a solution of caged ligand; neurotransmitter release is effected by a laser pulse applied via an optical fibre. Figure 4C shows an experimental trace (gray line) together with the best fit using single-exponential rise and decay functions. The whole-cell current due to photoliberated ligand can be treated using jump kinetics to determine $k_{on}$ and $k_{off}$ from the time constant of current rise, $t_{on}$. There is no mechanical stress due to solution flow. More important, with this technique the ligand concentration jump is truly on a much faster time scale than the reaction under study. Therefore, time-dependent diffusion—critical in all perfusion methods—is no limiting factor, and a wide range of ligand concentrations is accessible for study. Practical and theoretical aspects of the technique have recently been reviewed (Hess and Grewer 1998).

**Single-channel methods.** Stationary single-channel experiments (no change in concentrations and conditions during the measurement) offer an approach from an entirely different angle. Observing a single channel means that many individual opening events must be analyzed using statistical methods in contrast to the ensemble responses obtained from whole-cell recordings. Subsaturating concentrations of ligand have to be used to avoid desensitization and to ensure that only one channel is active over most of the time. In the simple model used here, channel opening can only occur from the AL$_s$ state, and because the receptor can adopt all possible states, openings will occur in groups, called bursts, which are separated by silent intervals (where A and AL states are populated). As shown in Figure 4D for one channel with only one conducting state, the openings can be analyzed (Jackson 1992; Colquhoun and Hawkes 1995; Colquhoun and Sigworth 1995) to give $k_{on}$ $= \frac{1}{t_{on}}$, where $t_{on}$ is the observed mean open time. Difficulties arise from the existence of multiple conductance states, which may overlap in records. Because usually more than one channel is present in a membrane patch, multiple openings can be observed. If channel openings are frequent, individual bursts can overlap. The determination of burst duration, inter- and intraburst openings and closings, is then difficult even if no multiple openings occur. Furthermore, receptor channels can adopt several conductance levels that may be due to interconverting conducting or nonconducting states. The determination of $k_{off}$ using this method is relatively simple, provided there is only one conductance state and only one accessible transition pathway. Determination of the many kinetic parameters that are in these data is elaborate, requiring a large quantity of good quality recordings and quite involved mathematical analysis (Jackson 1992; Colquhoun and Hawkes 1995; Colquhoun and Sigworth 1995).

**Epilog: Why Study Ion Channel Kinetics?**

It is the very nature of ligand-gated ion channels to produce a transient
Fig. 4. Nonperfusion techniques. A. Laser pulse photolysis (lpp) reaction principle: a neurotransmitter derivative (NT) bearing a light-sensitive protecting group (cage) is exposed to a laser pulse (1), the subsequent photoreaction liberates free neurotransmitter. The rate of neurotransmitter release is determined by the rate of the photoreaction, which in turn, is governed by the nature of the chemical bond between the two molecules. The free neurotransmitter then binds to the receptors (2), triggering the channel-opening reaction (3). B. Lpp experimental setup: a flow delivery tube (similar to the one used in Fig. 3D) can be used to equilibrate the suspended whole-cell with caged neurotransmitter (not drawn to scale). The laser pulse is delivered to the cell via an optical fibre. C. Typical lpp trace: a HEK293 cell transfected with a mutant glycine receptor subunit was equilibrated with caged glycine, the laser was fired at time 0, the resulting whole-cell current was recorded. D. Single-channel recording—Lower panel: receptor states, note that only the open state can be observed. Upper panel: corresponding single-channel current trace. The channel closing rate \( k_{cl} \) can be calculated from the mean open time \( t_{op} \). The mean closed time within a burst \( t_{closed/burst} \) (often difficult to define) reflects \( k_{op} \). For simplicity, only one conductance state is shown.
signal. In contrast to protein binding (e.g., antibodies), no stable adduct is formed, and other than enzymes, ion channels yield no product whose concentration could be measured offline. Therefore, neurotransmitter receptors must be studied using kinetic methods. A recording system is needed that can resolve the time scale relevant to receptor function.

Ion channel kinetics are often affected in the case of disease, where point mutations or deletions cause the formation of impaired ion channel receptors, as observed, e.g., in slow channel myasthenic syndrome (Wang and others 1999) or various hyperekplexia mutations (Saul and others 1999). Kinetic constants, therefore, define the ion channel receptor and must be targeted and controlled in the development of selective therapeutic strategies of receptor-mediated disorders.

Acknowledgments

I am grateful to Cord-Michael Becker, Matthias Herkert, and Andreas Humeny for helpful discussions and a critical reading of the manuscript.

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