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Biophysical Chemistry 100 (2003) 493–506

Biophysical Chemistry

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Rapid chemical reaction techniques developed for use in investigations of membrane-bound proteins (neurotransmitter receptors)

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Received 19 January 2002; received in revised form 1 June 2002; accepted 1 June 2002

Abstract

New techniques for investigating chemical reactions on cell surfaces in the microsecond-to-millisecond time region are described. Reactions mediated by membrane-bound neurotransmitter receptors that control signal transmission between $\sim 10^{12}$ cells of the nervous system are taken as an example. Cells with receptors on their plasma membrane are equilibrated with photolabile, biologically inactive precursors of the neurotransmitters. Photolysis of these compounds releases free neurotransmitter that interacts with the receptors, leading to the transient opening of transmembrane receptor-formed channels that are permeant to small inorganic ions. The current thus induced can be measured. The technique can be used to measure the elementary steps of the receptor-mediated reactions. To illustrate the approach it was shown that an understanding of the mechanism of inhibition of the nicotinic acetylcholine receptor by the drug cocaine was obtained and led to the first proof that compounds exist that alleviate the inhibition.

Keywords: Transient kinetic investigations of membrane-bound proteins; Caged neurotransmitters; Photolabile precursors; Prevention of cocaine inhibition; Neurotransmitter receptors

Professor John T. Edsall's lectures and publications [1–3] on the application of physical techniques to the solution of important biological problems have had an important influence on my education and subsequent research. I am honored to dedicate this article to his memory.

Transient (pre-steady state) techniques in which membrane-bound proteins can be equilibrated with their respective ligands within microseconds, and the ensuing reaction can be investigated in this time region, were developed only recently [4]. These techniques and some of their applications are the subject of this paper.

1. Historical perspective

In contrast to investigations of fast chemical reactions on cell surfaces, a range of well established methods have been available for many years to investigate rapid chemical reactions in solutions [5–8]. These techniques have been invaluable in elucidating complex sequences of biological reactions and the specific elementary steps in such

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0301-4622/03/$ - see front matter
PII: S0301-4622(02)00301-0

reactions. They have provided essential insights into the mechanism by which enzymes accelerate and regulate the metabolism and catabolism of essential cellular components, modify proteins involved in intracellular signaling, and translate the nucleic acid code [9–11].

2. Reactions mediated by membrane-bound proteins

What is known about the many biological reactions mediated by membrane-bound proteins that are activated by extracellular signals, such as light, sound, and hormones and other extracellular chemicals? Many of these reactions must be studied with the protein of interest in a membrane separating solutions of different ionic composition, for instance the extracellular solution and the cytoplasm. Here I shall concentrate on one of the most thoroughly investigated of these membrane-bound proteins, the nicotinic acetylcholine receptor (nAChR) [12]; reviewed in Kandel et al. [13], which participates in reactions leading to signal transmission between nerve cells and nerve and muscle cells. This receptor belongs to a class of structurally related membrane proteins, the neurotransmitter receptors, that mediate chemical reactions controlling signal transmission between some 10^{12} neurons, reactions that are believed to be basic to brain function [13,14].

The function of neurotransmitter receptors in the nervous system is illustrated in Fig. 1a. When an electrical signal reaches a nerve terminal, chemical signals, neurotransmitters, for instance acetylcholine, glutamate, γ-aminobutyric acid, glycine, are released from vesicles in the nerve terminal [20,21]. The neurotransmitter released by one cell diffuses across a gap (synapse) of approximately 20–40 nm between two cells and binds to membrane-bound neurotransmitter receptors on the second cell. Thus the signal transmission process is continued. The nAChR, one of the neurotransmitter receptors, consists of five transmembrane subunits of approximately 55 000 MW each [22–24]. On binding the neurotransmitter acetylcholine the transmembrane receptor-channel transiently opens and allows the passage of sodium and potassium ions through the channel. Other neuro-

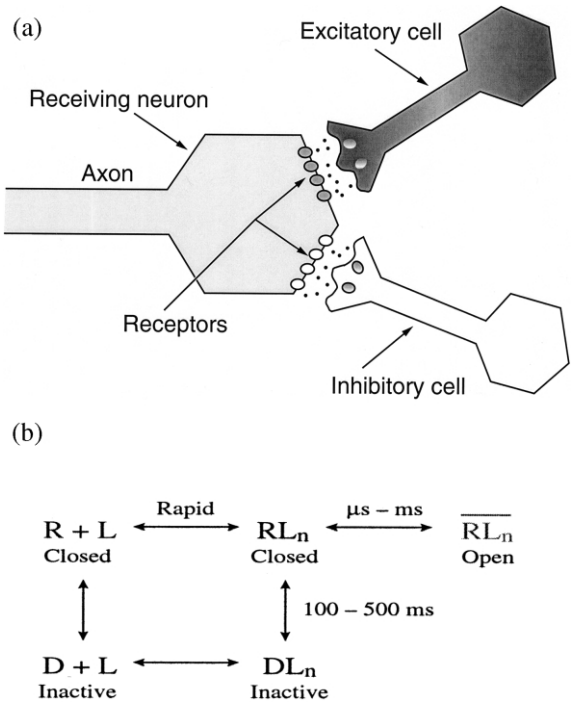


Fig. 1. (a) The connection between three neurons are shown. When the upper cell on the right is activated, it releases an excitatory neurotransmitter (e.g. acetylcholine, glutamate) from vesicles in the nerve terminal. When the lower cell on the right is activated, it releases an inhibitory neurotransmitter (e.g. γ-aminobutyric acid, glycine) from vesicles. When excitatory neurotransmitters bind to their respective receptors on the receiving neuron, cation-conducting transmembrane channels transiently open. When inhibitory neurotransmitters bind to their respective receptors on the receiving neuron, anion-conducting transmembrane channels transiently open. (b) The reaction scheme of the nicotinic acetylcholine receptor is consistent with available structural [12] and kinetic information [15] and was first suggested by Katz and Thesleff [16]. *R* represents the unliganded receptor, RL_n the liganded receptor in the closed-channel conformation, $\overline{RL}_n$ the open-channel form of the receptor, and the subscript *n* the number of neurotransmitter (*L*) molecules bound to the receptor. *D* represents the inactive, desensitized receptor form. The rate of receptor desensitization was believed to occur in the second time region [16]. Rapid kinetic investigations indicated that rapid receptor desensitization of the muscle nAChR occurs in the millisecond time region [17]. The channel-opening rate constant for the muscle nAChR was investigated by use of the laser-pulse technique [18] by Matsubara et al. [19].

transmitter receptors, for instance the one activated by γ-aminobutyric acid (GABA), allows the passage of chloride ions. The charge of the ions, as well as their concentration, determines the sign and magnitude of the change in the transmembrane voltage [25–27].

When the potential across the cell membrane reaches a critical value of approximately −40 mV, voltage-activated Na^+ channels in the axon of the cell are opened [28,29]. This leads to propagation of an electrical signal along the axon of the cell, with a rate of up to 1000 m s^{-1}. When the electrical signal reaches the nerve terminal neurotransmitters are secreted adjacent to the next cell [20] and the process can be repeated.

3. Techniques in which ligands are applied slowly (seconds) in investigations of receptor function

What is the chemical reaction mediated by these receptors? Fig. 1b shows a mechanism of the reaction mediated by the nAChR proposed by Katz and Thesleff in 1957 [20]. It is based on experiments in which the cell surface receptors are slowly (seconds) equilibrated with the neurotransmitter (acetylcholine). According to this mechanism, upon binding neurotransmitter the receptor rapidly opens a transmembrane channel permeable to Na^+ and K^+. The channel closes again when acetylcholine is removed (by diffusion or enzymatic breakdown). Upon prolonged exposure to acetylcholine the channel becomes reversibly inactivated (desensitized), in the second time region. Katz and Miledi [30] observed electrical noise when the neurotransmitter is applied to the receptor and interpreted this as due to the opening of receptor-formed transmembrane channels.

Subseqently two techniques were developed in which the cell surface receptors were equilibrated with the neurotransmitter within seconds but subsequent steps of the reaction could be observed in the sub-millisecond time domain. In one technique, the quasi-equilibrium of the reaction steps is perturbed by a voltage jump. Assigning the observed changes in membrane conductance to a definite step in the channel-opening process proved difficult [31,32]. The other technique developed to

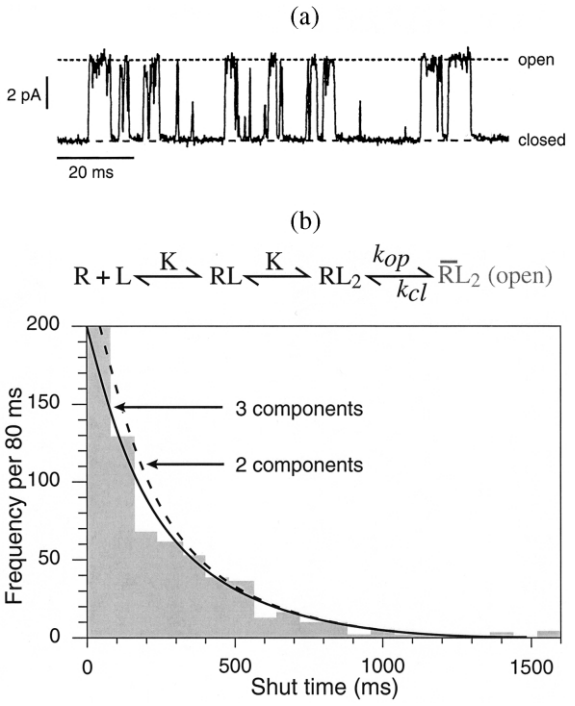


Fig. 2. (a) Recording of the opening and closing of a single transmembrane channel, low pass filtered at 1 kHz and recorded at a rate of 4 kHz (from Heinemann [34]). (b). Example of the distribution of shut times of the nAChR at ~130 mV activated by 100 μM suberyldicholine. The graph shown summarizes all observations taken at different frequencies (from Colquhoun and Sigworth [35]).

detect the current passing through a single receptor-formed channel, the single-channel current-recording technique [33] (Fig. 2a), allows one to determine the life time (related to the time the channel remains open), the conductance (measured by the current amplitude of the open channel), and the ion specificity of a single channel. This elegant technique was perfected for easy and convenient use and is now used by a large international group of scientists. It has revolutionized electrophysiology [36], and is providing a wealth of information about the single-channel properties of many different channels and their isoforms and how those properties change under different conditions [36].

The critical voltage change across the membrane of a cell at a synapse (Fig. 1a) that determines whether the signal is transmitted or not does not

depend just on the conductance or life time of the single receptor-channel. Whether an electrical signal is transmitted or not depends also on the number of receptor channels that open within a definite period of time and how long the channels remain open. What fraction of the receptor-channels present open at a particular concentration of neurotransmitter? How long does it take them to open? And how long do they remain open? To answer some of these questions one needs to know the rate constants associated with individual steps of the mechanism (ligand binding, channel opening, channel closing and receptor desensitization) and the equilibrium constants (the neurotransmitter–receptor dissociation constants and the channel-opening equilibrium constant). Can one obtain additional information about the receptor mechanism in Fig. 1b by using the single-channel current-recording technique? In principle, more information about the receptor mechanism can be obtained by analyzing the very short closed times between the opening of single channels (Fig. 2a). For the mechanism shown in Fig. 1b, these closed times are expected to contain information about the life times of three forms of the receptor, *R*, *RL* and RL_2 (Fig. 1b). The closed-time distribution of the nAChR is, therefore, expected to have three components, each component corresponding to the lifetime of one of the receptor forms. The closed-time distribution of the nAChR in the presence of 100 nM suberyldicholine (which acts as a neurotransmitter) is shown in Fig. 2b (Fig. 15 on p. 517 of Colquhoun and Sigworth [35]). For the mechanism shown in Fig. 2, each lifetime has several components. The lifetime of the unliganded receptor form *R* consists of the rate constant leading away from the *R* state multiplied by the neurotransmitter concentration. The lifetime of *RL* is the sum of the rate constant leading to *R* and the rate constant leading to RL_2 multiplied by neurotransmitter concentration. The lifetime of RL_2 is independent of neurotransmitter concentration and consists of the sum of the rate constant leading to *RL* and the rate constant leading to the open-channel form $\overline{22}RL_2$. Additional information about the life times and rate constants of interest can in theory be obtained if one can make the measurements over a wide range of neurotransmitter concentrations. The single-channel recording technique is, however, limited in this regard. It involves equilibration of receptors with neurotransmitter for some length of time (seconds to minutes) so that enough data can be collected for the statistical analysis of the life times and current amplitudes of single channels. Because of receptor desensitization leading to closure of receptor-channels, the neurotransmitter concentration must be kept low enough so that one can still obtain a current signal.

It has been well established in investigations of reactions in solution that when slow equilibration of reactants prevents evaluation of rapidly equilibrating reaction steps, much additional information can be obtained by use of rapid reaction techniques [5,6,9–11]. I shall now demonstrate that rapid reaction techniques developed for investigations of reactions on cell surfaces are capable of producing similar useful information.

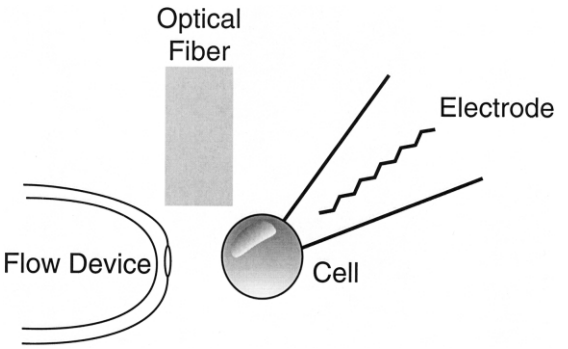


Fig. 3. Schematic drawing of the experimental arrangement used in the laser-pulse photolysis technique. A BC_3H1 cell of ~20 μm diameter carrying muscle nAChRs and attached to an electrode for whole-cell current recording [37] was equilibrated with caged carbamoylcholine The side view of a cell–flow device [38] used to equilibrate the cell surface with ligands is shown on the left. A laser is used to produce a pulse of light at a wavelength in the region of 308–337 μm. The laser beam is introduced from an optical fiber of 200-μm diameter. The fiber is adjusted to be ~400 μm away from the cell so that the area illuminated around the cell has a diameter of ~300–400 μm. The energy of the pulse of light emerging from the fiber is ~500 mJ [4].

4. Transient kinetic techniques developed for investigations of neurotransmitter receptor function

Of all the techniques that have been developed to investigate reactions in solutions [5,6], the flow technique of Hartridge and Roughton [5] appeared most promising for use in investigations of cell surface receptors. In this technique two solutions of reactants flow through separate tubes, meet and mix in the millisecond time region, and the product is analyzed. In the case of receptors on the surface of a cell, they can be equilibrated with a neurotransmitter solution flowing over the cell. The current due to opening of receptor-channels can then be determined by the whole-cell current-recording technique, which allows one to accurately determine, in the sub-millisecond time region, the current coming from all open receptor-channels on the cell surface [37]. There are, however, several problems with flow techniques when applied to cell surface receptors that have not been appreciated. These problems are best illustrated by examples that I shall give after I explain the rapid reaction technique we developed to overcome them, namely the laser-pulse photolysis (LaPP) technique [18]; reviewed in Hess and Grewer [4].

In the laser-pulse photolysis technique (Fig. 3), the cell is equilibrated with a biologically inactive, photolabile precursor of a neurotransmitter. A solution of the precursor flows over the cell surface. The precursor equilibrates with the receptors on the cell surface. It is then photolyzed by a single laser pulse to give rise to the free neurotransmitter in the microsecond-to-millisecond time region [18]. The free neurotransmitter binds to the receptors and causes the channels to open. One can then measure the ensuing current (Fig. 4a).

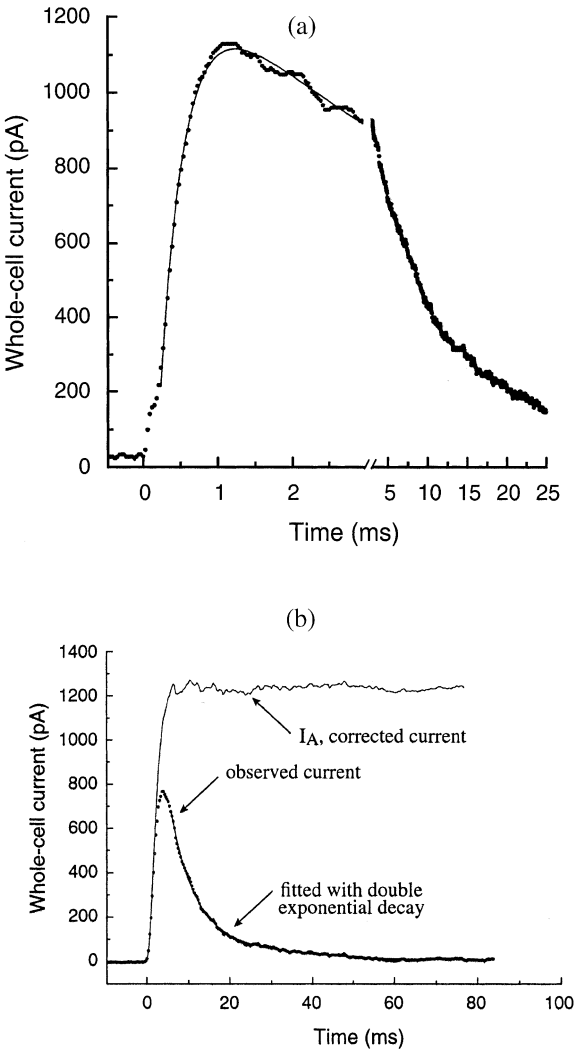
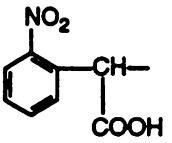
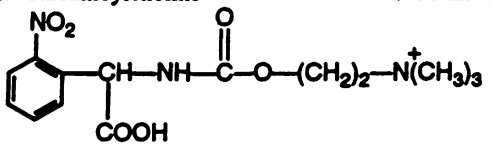
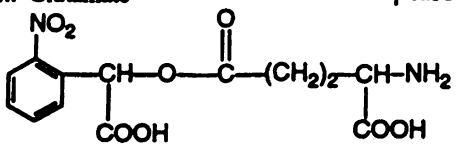
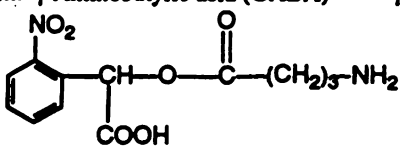
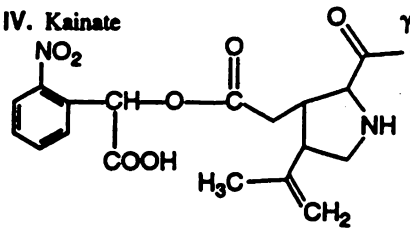


Fig. 4.

Fig. 4. (a) Laser-pulse photolysis experiment. The whole-cell current (dotted line) was recorded from an isolated rat hippocampal neuron at −60 mV, ∼22 °C, and pH 7.4. The current was induced by laser-pulse photolysis of 5 mM of αCNB-caged glutamate at time 0. The current rises very rapidly, reaching its maximum within 1 ms (the formation of open channels) and then, in a slower time region, the current decreases as the channels close (desensitization). In this trace, the concentration of glutamate released was estimated to be 0.4 mM, by comparing the maximum amplitude of the corrected whole-cell current in the presence of 0.5 mM of glutamate applied using the cell–flow technique before and after the laser-pulse trace was obtained. The rising phase of the current can be fitted by a single exponential and in this trace the observed rate coefficient, k_{obs}, was determined to be ∼3000 s^{-1} [39]. (b) Cell–flow experiment. The whole-cell current was recorded (dotted line) from an isolated rat hippocampal neuron at −60 mV, ∼22 °C, and pH 7.4 when 0.5 mM of glutamate solution was applied at time 0 [39] using the cell–flow technique [38,47]. The current corrected for receptor desensitization [47] is indicated by the thin line.

Table 1
Photolytic properties of biologically inert, photolabile derivatives of neurotransmitters caged with α–carboxy-2-nitrobenzyl group (pH 7.5, 22 °C)

α-carboxy-2-nitrobenzyl group 

Caged Neurotransmitter	Group Caged	Photolysis $t_{1/2}$ (μs)	Product Quantum Yield	Target Receptor	Reference
I. Carbamoylcholine 	carbamate	45	0.8	Acetylcholine	Milburn *et al.* 1989 [18]
II. Glutamate 	γ-carboxyl	21	0.14	Glutamate	Wieboldt *et al.* 1994 [50]
III. γ-Aminobutyric acid (GABA) 	γ-carboxyl	19	0.16	GABA	Gee *et al.* 1994 [51]
IV. Kainate 	γ-carboxyl	45	0.37	Kainate	Niu *et al.* 1996 [52]

Photolabile protecting groups for functional groups commonly found in amino acids and nucleic acids have been used by organic chemists for the last 40 years [40–44]. These compounds, in biology called caged compounds, were first used for the solution of biological problems by Kaplan et al. [45] for the purpose of rapidly generating significant and known concentrations of substrates or inhibitors inside a cell. These authors used variations of the *o*-nitrobenzyl group [42,46] to synthesize photolabile derivatives of ATP. Another nitrobenzyl derivative of ATP was synthesized by Trentham and co-workers [48] and shown to liberate ATP on photolysis with a rate of 220 s^{-1} at pH 7.0. The first attempt to synthesize a compound suitable for pre-steady state kinetic investigations of neurotransmitter receptors (the nAChR) on cell surfaces, *N*-[1-(2-nitrophenyl)-ethyl] carbamoyl-

Table 2
Equations for reaction scheme shown in Fig. 1b

$$I_t = I_{max}[1-\exp(-k_{obs}t)] \quad (1)$$

$$k_{obs} = k_{cl} + k_{op}\left(\frac{L}{L+K_1}\right)^2$$

I_t, I_{max} represent the whole-cell current at time t and at the current maximum, respectively. k_{obs} is the observed first-order rate constant for the current rise. k_{cl}, k_{op} and K_1 represent the rate constants for channel closing and channel opening, and the receptor–ligand (L) dissociation constant, respectively (Fig. 1b).

$$(I_A)_{tn} = (e^{\alpha\Delta t}-1)\sum\left((I_{obs})_{\Delta ti} + (I_{obs})\right)_{\Delta tn} \quad (2)$$

In cell–flow experiments the current observed during the current rise time is corrected for receptor desensitization occurring during each time interval Δt of approximately 1 ms. After n constant time intervals ($n\Delta t = t_n$) during each of which the current $(I_{obs})_{\Delta t}$ is measured, the corrected current I_A is given by Eq. (2), where $(I_{obs})_{\Delta ti}$ is the observed current during the ith time interval and t_n is equal to or greater than the current rise time, the time it takes the current to reach a maximum value. Under conditions of laminar flow of the neurotransmitter solution over the cell, the value of I_A was found to be independent of the solution velocities used and could be determined with good precision ($\pm 10\%$) [47].

$$I_A = I_M R_M(\overline{RL}_2)_o; \quad (\overline{RL}_2)_o = L^2/\left[(L+K_1)^2\Phi + L^2\right] \quad (3)$$

$$[I_M R_M (I_A)^{-1} - 1]^{1/2} = \Phi^{1/2} + \Phi^{1/2} K_1 [L_1]^{-2}$$

I_A is the current due to open receptor-channels in the cell membrane corrected for receptor desensitization, I_M the current due to 1 mol of open receptor-channels. R_M represents the number of moles of receptors in the cell membrane and $\overline{RL}_2$ represents the fraction of receptor molecules in the open-channel form. Φ represents the channel-closing equilibrium constant and all other symbols are defined in Fig. 6.

$$k_{obs} = k_{cl}\left[\bar{K}_I/(\bar{K}_I + I_o)\right] + k_{op}[L/(L+K_1)]^2 \quad (4a)$$

$$k_{obs} = k_{cl} + k_{op}[L/(L+K_1)^2][K_I/(K_I+I_o)] \quad (4b)$$

k_{obs} is the observed first order rate constant for the current in presence of an allosteric inhibitor. K_I represents the dissociation constant of the inhibitor binding to the closed-channel form of the receptor (Eq. (4b)) and $\bar{K}_I$ the dissociation constant of the inhibitor binding to the open-channel form of the receptor (Eq. (4b)). All the other symbols are defined in Fig. 6.

choline, was made by Walker et al. [49]. Carbamoylcholine activates the nAChR and is more stable than acetylcholine. The compound was photolyzed in the microsecond time domain but it was not biologically inert and both inhibited and inactivated the nAChR. The first successful compound for such investigations of this membrane-bound protein was synthesized by Milburn et al. [18], who introduced a new *o*-nitrobenzyl derivative, the α-carboxy-2-nitrobenzyl group, in order to 'cage' carbamoylcholine. At room temperature and neutral pH, the compound is photolyzed with a rate of 17 000 s^{-1} ($t_{1/2}$=41 μs) and a quantum yield of 0.8. It was demonstrated that neither the caged compound nor the photolyzed caging group affect the measured properties of the nAChR in BC_3H1 cells [18]. Since then, the α-carboxy-2-nitrobenzyl group has been successfully used in the synthesis of caged neurotransmitters suitable for pre-steady-state kinetic investigations of the glutamate [50], γ-aminobutyric acid (GABA) [51], kainate [52], and serotonin [53] receptors (Table 1). Rapid chemical kinetic investigations using the laser-pulse photolysis technique and caged neurotransmitters have now been carried out with the nicotinic acetylcholine [19]; reviewed in Hess and Grewer [4], γ-aminobutyric acid [54],

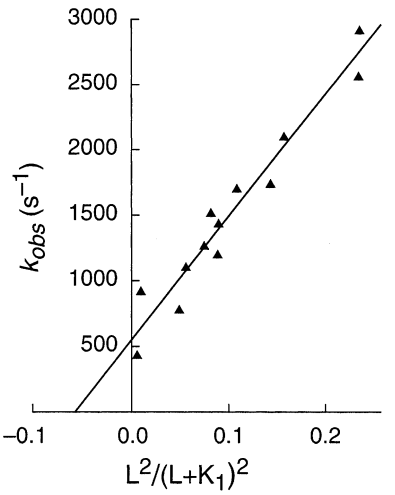


Fig. 5. Determination of k_{op} and k_{cl} and K_1 for the opening of nAChR-channels in BC_3H1 cells at pH 7.4, 23 °C, and −60 mV. The values of k_{obs} determined from an experiment similar to the one shown in Fig. 4a plotted according to Eq. (1) (Table 2). The values of k_{op}, k_{cl}, and K_1 are 12 000 s^{-1}, 500 s^{-1}, and 210 μM respectively [19].

glycine [55], and glutamate [39] receptors. The results obtained with the LaPP technique and with other methods are compared in the references cited.

A typical laser-pulse photolysis experiment illustrating the use of the technique in investigating the glutamate receptor in hippocampal neurons is shown in Fig. 4a. A cell in the whole-cell recording mode [37] was equilibrated with caged glutamate, which upon photolysis yielded 0.4 mM of glutamate at zero time. In the resulting current trace, we see two phases of the reaction. The current rises due to the formation of open receptor-channels and this phase follows a single exponential rate law [Table 2, Eq. (1)]. The maximum current amplitude is reached within approximately 1 ms. Then in a much slower time region the current decreases due to receptor desensitization and the closing of receptor-channels. It is instructive to look at an experiment (Fig. 4b) in which 0.5 mM of glutamate rather than caged glutamate flowed over the same cell as was used in the experiment in Fig. 4a. The diameter of the cell was approximately 20 μm and the rate of flow of the glutamate solution was approximately one cell diameter per 0.5 ms. When small electrodes (tip diameter ~1 μm) that can sense a change in electrolyte composition are used, this flow rate is verified. Why does it take the current ~10 times longer in the flow experiment (Fig. 4b) to reach its maximal value than it does in the laser-pulse photolysis experiment (Fig. 4a), with the same cell and essentially the same neurotransmitter concentration? The theory of solution flow over spherical objects predicts that objects as large as the cell are expected to be covered by a layer (diffusion layer) of water [56,57]. In cell–flow experiments such as the one illustrated in Fig. 4b, the observed current amplitude is expected to be determined by the rate of flow of the solution, the diffusion constant of the ligand, the time it takes to replace the diffusion layer by the solution containing the neurotransmitter, and the rate of receptor desensitization [47,58]. It should be noticed that the observed maximum current amplitude in the cell–flow experiment (dotted line Fig. 4b) is much lower, even with the same cell, than in the laser-pulse photolysis experiment (Fig. 4a). The explanation for this is that in the cell–flow experiment the receptors desensitize while the neurotransmitter equilibrates with the receptors. If one takes the theory of the solution flow over submerged objects into account [56,57], as well as the rate of receptor desensitization, the observed current amplitude can be corrected (Table 2, Eq. (2)) [47]. The wavy line parallel to the abscissa gives the current amplitude corrected for receptor desensitization in the cell–flow experiment (Fig. 4b). Agreement between the current amplitude obtained in laser-pulse photolysis experiments and the corrected current amplitude in cell-flow experiments has invariably been obtained (reviewed in Hess and Grewer [4]). In experiments with the γ-aminobutyric acid receptor, when comparison between flowing solutions over small membrane patches (rather than whole cells) [59] and the laser-pulse photolysis technique was possible [54], we observed that the time resolution with membrane patches of 2-μm diameter, although vastly improved, was still not sufficient to determine the effect of neurotransmitter concentration on the channel-opening rate. Because the number of receptors in such membrane patches is much smaller than in whole cells the fact that two different receptor forms with different desensitization rates and different rate constants for channel opening exist in the cells [54] was not observed in experiments with membrane patches [54,59]. The information that can be extracted from laser-pulse photolysis experiments such as that illustrated in Fig. 4a is shown in Fig. 5. Under conditions where equilibration of the receptors with neurotransmitter is rapid compared to channel opening, the channel-opening rate is determined by a first-order rate constant (Table 2, Eq. (1)).

In Fig. 5 the observed first-order rate constant for opening of the nAChR channel is plotted as a function of the ligand concentration. The slope of the line gives the channel-opening rate constant. The intercept gives the rate constant for channel closing. From the effect of neurotransmitter concentration on the maximum current amplitude we obtain information about the observed dissociation constant of the neurotransmitter–receptor complexes (Table 2, Eq. (3)). Additionally, if an inhibitor that affects channel opening or closing is

applied, either the intercept or the slope, or both, change. From this change one can calculate the dissociation constant of the inhibitor from both the closed- and open-channel forms (Table 2, Eq. (4)).

Investigation of the nAChR [19], and similar experiments carried out with the γ-aminobutyric acid [54], glycine [60] and glutamate [39] receptors, indicate that many of the constants of the mechanism shown in Fig. 1b can now be evaluated. These are the rate constants for channel opening and closing, the rate constant for receptor desensitization, the channel-opening equilibrium constant, and the observed dissociation constant of the receptor–neurotransmitter complex. One can determine whether an inhibitor is competitive or allosteric (not competitive). One can evaluate the apparent dissociation constant of competitive inhibitors and the apparent dissociation constants of allosteric inhibitors binding to the closed- or open-channel forms (Table 2, (Eq. (4).

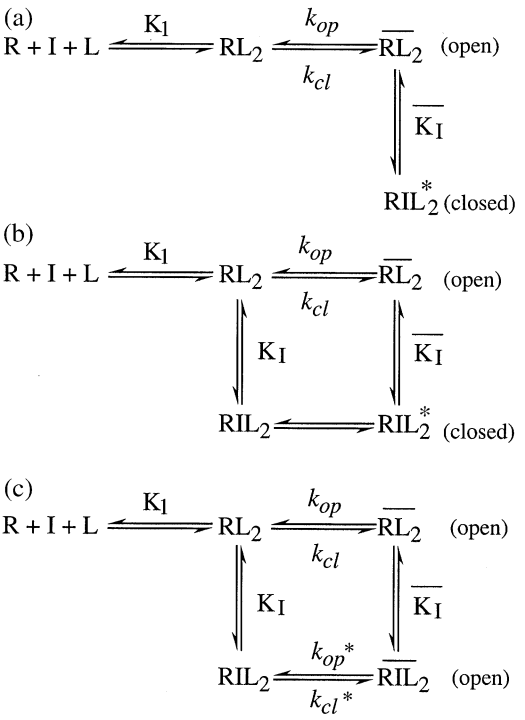


Fig. 6.

5. Use of transient kinetic techniques to solve biological problems not soluble by techniques in which the application of ligands is slow

In conclusion, I will illustrate one practical aspect of rapid chemical kinetic investigations of a membrane-bound protein. The inhibition of the nAChR by the abused drug cocaine is of interest. This receptor is one of several proteins important in nervous system function that is inhibited by cocaine. Understanding the mechanism of inhibition of the nAChR has been a major goal of this field for over two decades [36,61,62]. Despite

Fig. 6. Proposed mechanisms for the inhibition of nAChR by MK-801 and cocaine. In each case, the upper line represents the minimum mechanism for the opening of the receptor-channel [16]. Receptor *R* binds the neurotransmitter *L* (or another activating ligand, for instance carbamoylcholine). RL_2 represents the closed-channel conformations. $\overline{RL}_2$ represents the open-channel conformation of the receptor that allows inorganic cations to cross the cell membrane. K_1 is the observed dissociation constant for the activating ligand. k_{op} and k_{cl} are the rate constants for channel opening and closing, respectively. Φ^{-1} $(=k_{op}/k_{cl})$ is the channel-opening equilibrium constant [4]. The reactions shown occur in the microsecond to millisecond time region [4]. For clarity, the desensitization reaction, which in the case of the nAChR occurs in the 100–500-ms time region [4,17] and the binding of the inhibitor I to the unliganded receptor form are not shown. The relatively slow transitions of receptor/inhibitor complexes to non-conductive forms [67] are also not shown. (a) Channel-blocking mechanism in which the inhibitor binds in the open channel and blocks it [61]. (b) Extended channel-blocking mechanism. The inhibitor binds to the closed- and open-channel forms giving the non-conductive receptor forms IRL_2 and IRL_2. K_1 and $\bar{K}_1$ are the observed inhibitor dissociation constants pertaining to the closed- and open-channel form, respectively. (c) Proposed cyclic inhibition mechanism involving a complex of the inhibitor with the open-channel conformation in which the open channel is not blocked by the inhibitor (i.e. it conducts ions). This minimum mechanism is based on chemical kinetic measurements [67,68]. The principle of microscopic reversibility requires that the ration $K_1/\bar{K}_1 = \Phi^{-1}/\Phi_{IO}^{-1}$ where $\Phi_{IO}^{-1} = k_{op}^*/k_{cl}^*$. Therefore, compounds that bind to a regulatory site with higher affinity for the closed-channel conformation than the open-channel form will shift the equilibrium toward the closed-channel form and inhibit the receptor. Compounds that bind to the open-channel conformation with equal or higher affinity than to the closed-channel form are not expected to change the channel-opening equilibrium constant unfavorably. These compounds are, therefore, expected to displace inhibitors from the regulatory sites without inhibiting receptor activity [69].

intensive efforts, no specific drug that prevents cocaine inhibition of any of the important proteins in the nervous system has yet been found (reviewed in Carroll et al. [63]). There are more than 5 million cocaine users in the United States alone at an estimated cost to society of ~60 billion dollars [64]. As I shall demonstrate, the use of pre-steady-state kinetic investigations led to the proposal of a new mechanism for inhibition of the nAChR [65–69] and suggested another chemical approach to test this mechanism [70]. This approach led to the first proof that compounds exist that can prevent cocaine inhibition [69].

Fig. 6 shows several possible mechanisms of inhibition of the neurotransmitter receptors. The receptor *R* reacts with a neurotransmitter *L* to form a complex, which is transiently converted to the open-channel form. Mechanism A is the one that is generally accepted [36,61,62,72]. It suggests that inhibitors bind in the open channel and block it. This mechanism for the nAChR is based mainly on results obtained using the single-channel current-recording technique of Neher and Sakmann [33] in which the receptor is exposed to low concentrations of neurotransmitter for considerable periods of time. But other mechanisms are not excluded by these measurements [32,71–74].

In the second mechanism, Mechanism B, an inhibitor binds both before and after the channel opens. Once the inhibitor binds the receptor does not form an open channel.

A third possible mechanism, Mechanism C, is at the bottom of Fig. 6. Mechanism C entails a cyclic equilibrium between the open- and closed-channel forms. In this mechanism, receptor inhibition is the result of an unfavorable change in the channel-opening equilibrium constant as a result of the inhibitor binding with higher affinity to the closed- than the open-channel form [69].

The use of transient kinetic techniques revealed new information about the inhibition mechanism of the nAChR by the anticonvulsant MK801 [67] (Fig. 7). The experiments showed that the inhibition occurs in two phases, a very rapid phase with a half time of approximately 12 ms, and a much slower phase with a half time of 300 ms. In fact, the slow phase is seen whether the inhibitor is applied from inside or outside the cell membrane.

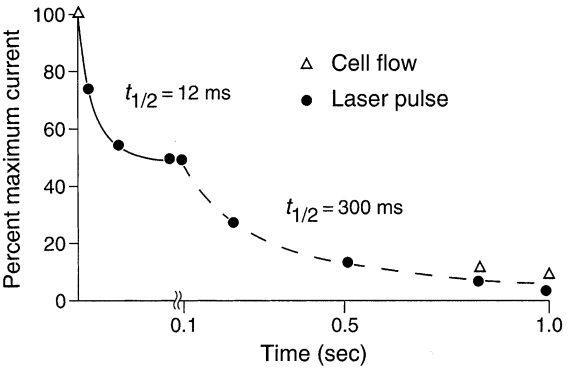


Fig. 7. Laser-pulse and cell–flow experiments of the inhibition of the nAChR by 200 μM MK-801 in the presence of 100 μM OF carbamoylcholine, 22 °C, −60 mV, pH 7.4. Two phases of inhibition are shown. A fast process with a $t_{1/2}$ of 12 ms and a second slower process with a $t_{1/2}$ of 300 ms.

The results indicate that two inhibitory sites are present. One is readily accessible to the inhibitor from the solution covering the surface of the membrane, and a second site is somewhere at the interface between the membrane and the receptor. Two sites that equilibrate in different time domains are also seen when cocaine is the inhibitor [66]. Therefore, in experiments done before the introduction of transient kinetic techniques for use with membrane-bound proteins, inhibition of the nAChR observed on a slow time scale reflects the properties of not just one but two different inhibitory sites.

The results of transient kinetic kinetic investigations of the inhibition of the muscle type nAChR expressed in BC_3H1 cells are consistent with the cyclic mechanism shown in Fig. 6 (Mechanism C) [65–67]. In this mechanism, the ratio of the channel-opening equilibrium constant in the absence and presence of an inhibitor are equal to the ratio of the dissociation constants for the inhibitor from the open- and closed-channel forms [69].

Two predictions arise from this mechanism [69]. The first is that compounds that bind with higher affinity to the closed-channel form than to the open-channel form will shift the equilibrium towards the closed-channel form. They will inhibit the receptor.

The mechanism also makes an exciting prediction [69]. This is that the compounds that bind to the open-channel form with equal or higher affinity than to the closed-channel form will not change the channel-opening constant. They will not, therefore, inhibit the receptor, but they will still displace inhibitors. We could now test this prediction.

We confirmed [70] the predictions by using combinatorial synthesis [75,76] and acetylcholine receptor-rich membranes from the *Torpedo californica* electroplax. Two classes of RNA polymers (aptamers) were isolated, Class I and Class II, each with a different consensus sequence (Table 3) [70]. We demonstrated that two types of compound do exist, as predicted by the mechanism, Class I aptamers bind with higher affinity to the closed-channel form of the nAChR in BC_3H1 cells and inhibit the receptor [70]. Class II aptamers bind with about equal affinity to the closed- and open-channel forms and prevent receptor inhibition [69] (Fig. 8).

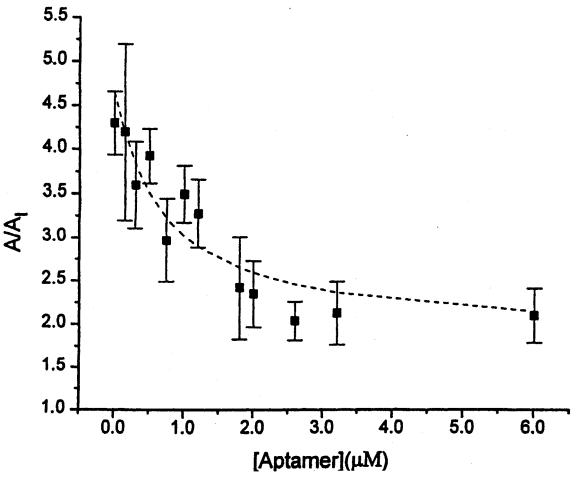


Fig. 8. Alleviation by RNA aptamer II-3 of nAChR inhibition by cocaine [69]. The whole-cell current corrected for receptor desensitization was determined by using the cell–flow technique [47]. At a constant concentration of 100 μM of carbamoylcholine, the ratio of the maximum current amplitude obtained in the absence, A, and presence, A_1, at a constant concentration (150 μM) of cocaine was determined as a function of the concentration of RNA aptamer II-3. The BC_3H1 cell was preequilibrated with aptamers II-3 for 2 s at 22 °C, −60 mV, and pH 7.4. Each data point represents the average of two to three experiments and using an average of two cells per point.

Transient kinetic techniques have previously made an enormous impact on our understanding of biological reactions in solution [5,6,9–11]. I have described the rapid reaction techniques that are now available to investigate reactions on cell surfaces (reviewed in Hess and Grewer [4] and Gee et al. [77]). I have given an example that suggests that they may be as important in investigations of rapid reactions on cell surfaces as they have been previously in investigations of rapid reactions in solution. I have given one example in which these techniques provide information that was not obtainable by previous techniques in which ligands were equilibrated with cell surface receptors slowly (seconds time domain). The application of these rapid chemical kinetic techniques to investigations of membrane-bound proteins such as the neurotransmitter receptors is in its early stages. There exists a wealth of neurotransmitter receptors and their isoforms of unknown chemical mechanisms [13]. Malfunction of neurotransmitter receptor-mediated reactions are implicated in many diseases of the nervous system (e.g. Huntington's disease, Parkinson's disease, epilepsy). Epilepsy alone affects approximately 42 million people worldwide [78]. Many clinically important compounds (e.g. tranquilizers, antidepressants) and abused drugs (e.g. cocaine) affect receptor function [79]. The results obtained so far suggest that the techniques and approaches described are capable of providing essential information about these reactions in the future.

Acknowledgments

I wish to acknowledge grants (NS08527 to G.P.H., GM04843 to G.P.H., DA 11643A to R.E. Oswald, and 1U54N539408 to V. Eterovic) from the National Institutes of Health that made it possible to do the research described here. I also thank Kevin Hodgson of Triple C for preparing the illustrations.

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