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Voltage Dependence of Open Channel Blockade: Onset and Offset Rates

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Introduction

It is well known that open ion channels can bind different drugs at the pore, and this kind of interaction impedes the ion flux through the channel. In many cases, the block is dependent on the transmembrane potential difference. This article discusses the practical application and limits of models used for describing such block.

Any voltage dependence of block must be due to the movement of some charge(s) in the membrane electrical field. Since prominent voltage dependence has been found for the blocking effect of many charged drugs, the widely used Woodhull model (Woodhull, 1973) considers the effect of the field on the blocking particle itself. The field draws the charged drug in and out of the blocking site. However, some other factors may affect or determine the appearance of the voltage dependence of blockade. For instance, a blocker occluding the channel necessarily interacts with the permeant ions and must sense the influence of the electric field on the ion fluxes (Armstrong, 1971; MacKinnon & Miller, 1988). Moreover, the conformation of the ion channel itself can be changed by voltage. There are voltage-activated sodium, potassium, calcium, etc, channels. Voltage also affects the nicotinic acetylcholine receptor desensitization (Magazanik & Vyskocyl, 1970) and gating, the latter depending on the number of agonist molecules bound (Auerbach et al., 1996). Probably, the membrane field induces conformational changes of any channel that in turn can affect the interaction with blocking particles. For instance, the blockade of mechanosensitive channels by amiloride depends on voltage, which is negligible at positive potentials. It has been found that the permeant ions do not change the voltage dependence in this case (Lane, McBride & Hamill, 1993). Nevertheless, the experimental curve of voltage dependence cannot be described by the Woodhull model and is consistent with a voltage-independent binding of the drug to a site that is accessible at hyperpolarized but not at depolarized potentials (Rusch, Kros & Richardson, 1994). The opposite influence of membrane field was observed when polyamine containing neutrotoxin—argiopine (organic cation), isolated from spider venom—blocked the closed state of glutamate-activated channel at low concentrations in insect or crayfish muscle; the blockade was found at depolarizing voltage but ceased after hyperpolarization (Magazanik et al., 1987; Antonov et al., 1989). This may be related to conformational changes of glutamate receptors induced by voltage.

Several drugs reveal complex blocking effects both on the open channel and on some other receptor sites (transmitter recognition site or closed channel). Investigation of the voltage dependence in these cases requires separate analysis of each mode of action. Argiopine blocks both open and close glutamate channels in insect larvae muscle in a voltage-dependent manner but with opposite sign. This favorable situation allowed the distinction between one mechanism from the other (Magazanik et al., 1987). Chlorpromazine may be used to photolabel the amino acids of the inner wall of acetylcholine receptor channels, which suggest its effect on the open channel (Revah et al., 1990). On the other hand, there was also evidence that chlorpromazine might act as a closed-channel blocker (Magleby & Palotta, 1981). This apparent contradiction was resolved by Benoit and Chan-

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geux (1993) in single-channel current experiments. Both effects were found but only the blockade of the open channel was voltage dependent.

Thus, there is a variety of molecular mechanisms that can cause the voltage dependence of open channel blockade. The relative contribution of each factor differs in any particular case. This makes the quantitative analysis of the voltage dependence more complicated, since such analysis should begin with the choice or elaboration of an appropriate model. However, it provides a new insight into the mechanisms of channel function and blockade.

The Woodhull Model and Its Use

Woodhull supposed in 1973 that the voltage dependence of blockade was due to transition of charged blocking particles through the membrane field. She suggested that the blocker binding and permeation may be described by a two-barrier model and analyzed the properties of the model (Woodhull, 1973).

In the case of an impermeant ion, the kinetic scheme of blockade is the simplest:

$$R^* + B \underset{k_-}{\overset{k_+}{\leftrightarrow}} R^*B \qquad \text{(scheme 1)}$$

where R^* is the open channel; B is the blocking particle; R^*B is the blocked channel, $k+$ and $k-$ are association and dissociation rate constants, respectively. According to the model, the free energy of the system has a minimum (U2) when the blocking particle is bound to its site. To pass from the external solution into the site, the blocking particle should overcome an energy barrier (U1) (*see* Fig. 1). Thus, $k+$ is proportional to $\exp(-U1)$, and $k-$ is proportional to $\exp(U2-U1)$. Woodhull assumed that the energy of the membrane field determined as $z\delta FV/RT$ is added to the energy minimum, which resulted in the single-exponential equation describing the voltage dependence:

$$k_d(V) = K_d(0)(\exp(z\delta FV/RT) \qquad (1)$$

where K_d is the equilibrium dissociation constant; V is the transmembrane potential; z is the valence of the blocking particle; δ is the fraction of the membrane field transversed by the blocking particle moving from outside solution to binding site into the channel. F, R and T are the Faraday constant, gas constant, and absolute temperature, respectively. The model is widely used for analysis and interpretation of blocking actions of a number of drugs on different types of channels.

The model was initially proposed for the open-channel blockade, and in subsequent studies the presence of voltage dependence was often used as decisive evidence of blocker binding in the open channel. However, there are some examples demonstrating prominent voltage dependence of the closed channel blockade (Aguayo & Albuquerque, 1986) (when the binding site is located in the depth of the membrane electric field) as well as a weak voltage dependence of the open channel blockade (Zamponi & French, 1994*a*) (when the binding site is located shallow in the pore). Certainly, Eq. (1) is applicable for closed channel blockade also. The δ value gives information about location of the binding site in the electric field but not the absolute location in the membrane (or channel), since the width of the electric field may not be equal to the width of the membrane. Several attempts to estimate the membrane field width were made using an elegant method suggested by Miller (1982) who studied the voltage-dependent channel blockade produced by bis-cation compounds of varying chain length (Subramaniam, Donevan & Rogawski, 1994; Tinker & Williams, 1995). The results obtained with the help of this approach varied greatly, from 5 Å in sodium channels according to Zamponi and French (1994*a*) to 23 Å in glutamate channels reported by Subramaniam et al. (1994), which may indicate a difference of electric field geometry in different channels.

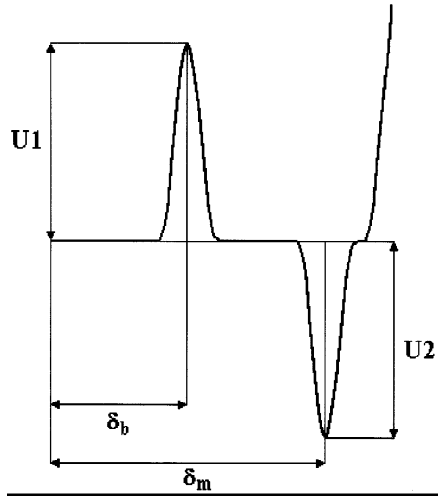

Fig. 1. Energy profile for impermeant blocker. $\delta_b = 0.5\delta_m$. Onset and offset rate constants depend on voltage equally.

It should be noted that the results of such measurements can be substantially distorted by different flexibility of polymethylene bisammonium compounds. Clearly, the more methylene groups between the ammonium ''heads,'' the higher flexibility of the chain. The flexibility of these compounds was calculated with the help of a theoretical conformational analysis (Rozengart & Zhorov, 1983). The calculations have predicted that the short-chain molecules (a number of methylene groups, $n < 6$) are practically rigid. From $n = 6$, the probability of fully extended conformations decreases

progressively, and for $n = 12$, it equals only to 4% (D.B. Tikhonov, *unpublished results*). Correspondingly, the average internitrogen distance calculated from the whole set of conformations with the weights equal to the probability of each becomes significantly less than the internitrogen distance of the fully extended conformation. A more accurate analysis of voltage dependence may be done when these average values are used. The longer-chain polymethylene bisammonium compounds ($n > 10$) are not so useful for distance probing, since the full set of their conformations is too far from the extended one. This can modify the blocking mechanisms as has been found experimentally by Miller (1982). Miller (1982) and Subramaniam et al. (1994) suggested that both cationic heads may simultaneously reach the binding site(s) located deep in the membrane field; however, these hypotheses are hard to reconcile with low calculated probability of hairpin-shape conformations, which remains negligible (less than 1%) even for $n = 10$–12.

In general, we cannot calculate directly the linear distance from the electrical distance δ. In any particular case, for estimation of the depth of a binding site in the channel (membrane), analysis of the voltage dependence should be accompanied by independent approaches such as affinity labeling or point mutations. In such situations, the comparative measurements of δ are more informative. For instance, a comparison of δ values obtained from the action of different agents on a single channel type allows us to recognize whether the blockers bind to the same site in the channel. Comparison of the voltage-dependent blockade produced by certain drugs in different channels, which belong to the common subfamily, may reveal details of the channel structure. A different voltage dependence of the open-channel blockade produced by two local anesthetics, procaine and QX-222, has been found in experiments on neuronal nicotinic ACh receptor-channels. Blockade was more sensitive to the membrane voltage in $\alpha_4\beta_2$ neuronal nicotinic receptors and frog extrajunctional muscle receptors than in the neuronal and muscle receptors of rat or frog junctional muscle receptors (Cuevas & Adams, 1994). These aspects of the structure-activity relationships are very important since the Woodhull model is widely used in channel pharmacology.

Model with Asymmetrical Barrier Location

The direct use of the Woodhull model for analysis of the voltage dependence of rate constants is difficult and sometimes leads to incorrect interpretation. Woodhull assumed for simplicity, that the barrier is located strictly at the distance equal to half of δ. As a result, the onset and offset rate constants have an equal and opposite voltage dependence (Fig. 1). In fact, this situation is rarely observed in practice. A number of examples demonstrate different voltage dependence of onset and offset rate constants (Yellen, 1984a; Lansman, Hess & Tsien, 1986; MacDonald et al., 1991; Zhorov et al., 1991; Donevan & Rogawsky, 1992; Zamponi & French, 1994b). When the voltage dependence of rate constants is not under investigation, in particular, in the original Woodhull paper, such simplification is quite admissible. Otherwise, the investigation of the voltage dependence of rate constants requires a more detailed analysis.

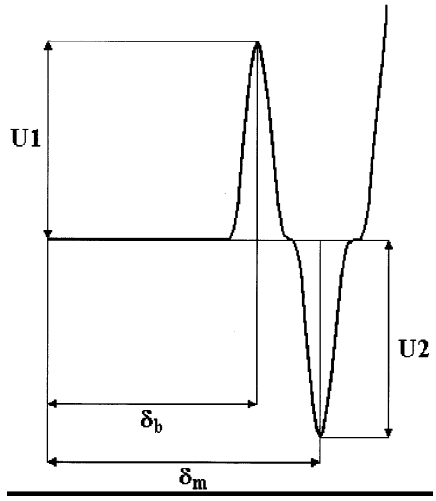

Fig. 2. Energy profile for impermeant blocker with $\delta_b \approx \delta_m$. Only the onset rate of blockade is voltage dependent.

Taking the Woodhull model as a starting point, i.e., accepting its main postulate that the voltage dependence of blockade is mainly due to the movement of the charged blocker in the electric field, it is possible to modify the model for explanation of the different voltage dependence of rate constants. In general, both barrier and minimum should have their own voltage dependence determined by δ_b and δ_m—fractions of the membrane field which correspond to the location of the barrier and minimum (Marchais & Marty, 1979). Thus,

$$k_+(V) = \exp(-U1 - z\delta_b FV/RT) \quad (2)$$

$$k_-(V) = \exp(-U + U2 + z(\delta_m - \delta_b)FV/RT) \quad (3)$$

$$K_d(V) = \exp(U2 + z\delta_m FV/RT) \quad (4)$$

The voltage dependence of the equilibrium dissociation constant remains unchanged since δ_m (minimum location in Eq. (3,4)) equals to δ (minimum location in the Woodhull model, Eq. (1)). Two extreme situations are now possible: $\delta_b = \delta_m$ and $\delta_b = 0$. In the first case, only the association rate constant is voltage dependent (Fig. 2). On the other hand, in the second case, only the unblocking rate constant is voltage dependent (Fig. 3). The Woodhull model corresponds to the situation when $\delta_b = 0.5\delta_m$ (Fig. 1). The notion that the voltage depen-

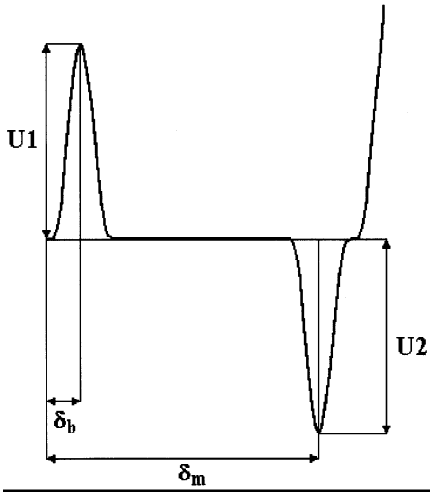

Fig. 3. Energy profile for impermeant blocker with $\delta_b \approx 0$. Only the offset rate of blockade is voltage dependent.

dence of rate constants reflects the relative positions of barriers and wells has been discussed and used to interpret experimental data by some authors (Lansman et al., 1986; Li-Smerin & Johnson, 1996). However, this valuable approach had not yet become common.

We have included an additional parameter in the voltage dependence model for a more realistic interpretation of the experimental data. It seems reasonable to equate δ_m with the location of structural groups in the channel that bind the blocking particle, and δ_b with the location of groups in the channel determining access to the binding site (barrier U1 at Figs. 1–3). The hindrances preventing the entry of blocker into the channel and its binding, in particular, the shedding of some water molecules from the hydration shell, determine the barrier. Among the numerous factors controlling this barrier position, the geometrical properties of the channel vestibule should be mentioned. The above considerations show that the voltage dependence of the association rate constant can not be used as a reliable measure of binding site location.

Open Channel Blockade of NMDA-type Glutamate Receptors

The most impressive example demonstrating different types of voltage dependence is the open channel blockade of the NMDA receptor. It may be blocked by cations of various structure. Blockade by Mg^{2+} ions demonstrates voltage dependence mainly of the association rate constant (Ascher & Nowak, 1988). Dissociative anesthetics (MK-801, PCP, ketamine, etc.) also produce an open channel block, but only the dissociation rate constant is responsible for the voltage dependence (MacDonald et al., 1991). Another class of blockers, adamantane derivatives, represents the intermediate situation, where both rates depend on the membrane potential (Antonov et al., 1995).

The NMDA receptor behaves as a singly occupied channel, as if there is only one permeant cation at a time occupying the site that is in the narrow region of the permeation pathway of the pore (Zarei & Dani, 1994). Point mutations of NMDA receptor subunits alter the blocking potency of Mg^{2+} ions and MK-801 (Mori et al., 1992). The δ_m values for the blockers vary from 0.7 to 0.9. These observations are consistent with the hypothesis that the binding site(s) are identical or close. However, a different voltage sensitivity of individual rate constants as determined for Mg^{2+} ions and MK-801 (MacDonald & Nowak, 1990) or bis-acridine compounds (Nelson & Albuquerque, 1994) suggests a different view. This contradiction can be overcome by the usage of the asymmetrical barrier model.

According to this model, the main difference in the blocking mechanism of Mg^{2+} ions and organic blockers is due to different locations of barriers. The energy barrier determining the onset rate of magnesium blockade could be caused mainly by the destruction of the hydration shell. This relatively small ion enters the channel pore probably without significant energy losses, and shedding of water molecules takes place just before binding. This type of interaction corresponds to the situation that $\delta_b \approx \delta_m$. On the other hand, the larger molecules of dissociative anesthetics may meet significant hindrances just upon entering the channel pore. Therefore, the energy barrier for such blockers should be located at the initial portion or even out of the membrane field. It is noteworthy that the offset rate constant of MK-801 blockade of nicotinic acetylcholine receptor is also more sensitive to membrane voltage than onset rate constant (Amador & Dani, 1991). The dimensions of adamantane derivatives are intermediate: less than MK-801 and more than magnesium ions that correspond to the prominent voltage dependence of both rate constants estimated from analysis of NMDA channel blockade. Detailed investigation of the voltage-dependent blockade produced by bis-cationic adamantane derivatives revealed an interesting effect. For compounds with the smallest variable head ($-N^+H_3$), the dependence of k_- on membrane potential is not described by a single-exponential function. This was much more pronounced at more negative potentials (Antonov & Johnson, 1995). The authors suggest that at negative potentials this drug may reach a deeper binding site that is not accessible for derivatives possessing bulky cationic groups.

Blockade by Permeant Particles

Until now we have discussed the blocking action of impermeant ions only. The blockade acquires some additional properties if the blocker can pass through the chan-

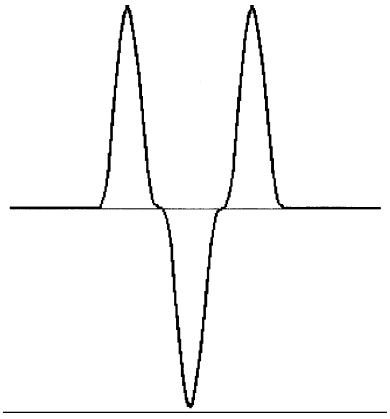

Fig. 4. Energy profile for permeant blocker. Both barriers locate near the minimum. Only the onset rate constant is voltage dependent.

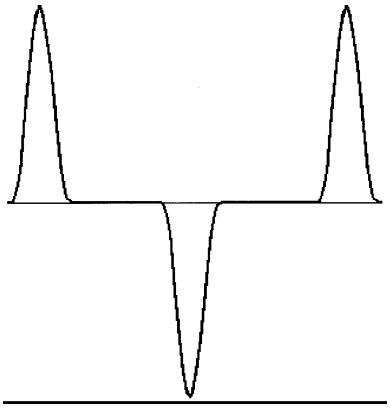

Fig. 5. Energy profile for permeant blocker. Both barriers are distant from the minimum. Only the offset rate constant is voltage dependent.

nel. This situation corresponds to the classical two barrier model discussed by Woodhull:

$$R^* + B_{\text{out}} \underset{k_{-1}}{\overset{k_+}{\leftrightarrow}} R^*B \underset{k_{-2}}{\rightarrow} R^* + B_{in} \qquad \text{(scheme 2)}$$

where the inner concentration of blocker is assumed to be negligible. In this scheme, the position of the second barrier determining k_{-2} should also be analyzed. If the barrier is located close to the minimum, the leak of blocking particles into the cytoplasm is voltage independent. When the second barrier is much deeper in the channel than the energy minimum, hyperpolarization will potentiate passage of the blocking particle (if its charge is positive) into the cell. The voltage dependence of the dissociation rate constant should then be determined by the height of both barriers and their positions relative to the minimum.

The complexity of rate constant voltage dependence was found experimentally when permeant blocking ions were used. In the block by Mg^{2+} ions of NMDA channels, only the association rate constant was found to be voltage dependent (Ascher & Nowak, 1988). There is some evidence that Mg^{2+} ions may both block and pass through the NMDA channel (Ascher & Nowak, 1988; Li-Smerin & Johnson, 1996; Stout et al., 1996). These results indicate that both barriers are located near the minimum (Fig. 4). No voltage dependence of the association rate constant was observed for the blockade of calcium channels in ventricular heart cells by Ca^{2+}, Cd^{2+} and La^{3+} ions (Lansman et al., 1986). However, this situation differs significantly from the blockade of NMDA channels by impermeant dissociative anesthetics, since in the former case the dissociation rate constant decreases by hyperpolarization, whereas it increases in the blockade of Ca^{2+} channel by inorganic cations (Lansman et al., 1986; Lansman, 1990; MacDonald et al., 1991). This should be due to a distant location of the outer barrier in reference to the minimum. Independence of the association rate constant on voltage indicates the location of the first barrier in the initial portion of membrane field (Fig. 5). The behavior of Mg^{2+} ions as a blocker of Ca^{2+} channel is dualistic (Lansman et al., 1986): at depolarized potentials (from −10 to +20 mV), Mg^{2+} seems to be a poorly permeable ion and the dissociation rate constant decreases with hyperpolarization; a greater hyperpolarization enhances the probability of Mg^{2+} ions passage through the channel and reverses the voltage dependence of the offset rate constant

Comparison of the hypothetical profiles obtained from the voltage dependence analysis clarifies some ideas about the channel structure. For instance, Fig. 4 (both barriers are close to the minimum) may correspond to a relatively short length of the narrow part of the NMDA channel. This suggestion is in an agreement with results obtained independently (Zarei & Dani, 1995). From this point of view, the narrowest part of calcium channels should be much longer (Fig. 5).

The Influence of Permeant Ions on the Voltage Dependence of Blockade

The behavior of permeant blocking ions in the pore resembles that of the current-carrying ones. Both kinds of ions have to interact with sites in the channel, and the only difference between them is the more stable binding of the blocking ion. For instance, Ca^{2+} itself can inhibit the current carried by Sr^{2+} or Ba^{2+} through the calcium channel (Lansman et al., 1986). Since the open-channel blockade is steric, i.e., the blocking particle hinders the passage of other ions through the channel, the interaction of blocker with permeant ions is indispensable for this kind of current inhibition. How do current-carrying ions affect the rate constants of the open-channel blockade? The theory of diffusion-limited reactions predicts that under high voltages, freely permeant ions would be depleted from the region near the channel mouth (Läuger,

1973; Andersen, 1983). This causes a decrease of the channel occupancy by the current-carrying ions and as a result the probability of the blocker binding to vacant channels rises. Simultaneously, the depletion of highly permeant ions from the channel vestibule provokes an increase of local concentration of blocking ions at the mouth of the channel. Both effects enhance the voltage dependence of the onset rate of the blockade without affecting the offset rate (Yellen, 1984*b*).

Another kind of interaction between blocking and current-carrying ions may accelerate the dissociation of blocker from the channel. If the permeant ion entering into the blocked channel has enough energy, it can knock out the blocker from its binding site. This ''kinetic'' effect on the offset rate constant depends evidently on the concentration of the relieving ion and on the voltage. The ''equilibrium'' knockout theory requires the existence of at least two binding sites. When the sites are occupied by the blocking and permeant ions simultaneously, electrostatic repulsion may facilitate the relief of blockade (Hille & Schwarz, 1978; Neyton & Miller, 1988*a*,*b*). Unfortunately, the ''kinetic'' and ''equilibrium'' schemes are not easily distinguished in the experiments. The opposite situation may be observed: an increase of permeant ion concentration retards the dissociation of blocker from the channel. The permeant ion enters the channel after the blocking ion and binds to another site. As a result the blocker can not leave the channel while the second site is occupied by the permeant ion (Neyton & Miller, 1988*a*). In some cases, both locking and relieving effects take place in the same channel. A number of variants of interactions between several binding sites makes the Woodhull model inapplicable for multi-ion channels. The theory of multi-ion channels and their blockade, which is more complicated, was presented in detail by Hille and Schwarz (1978) and by Neyton and Miller (1988*b*).

The permeant ions can also affect significantly the blockade and its voltage dependence not only in multi- but in single-ion (e.g., NMDA) channels (MacDonald et al., 1991; Zarei & Dani, 1994). We mentioned above interesting peculiarities found in the investigation of potential dependence of blockade produced by bis-cationic adamantane derivatives on the NMDA receptor channel. In a subsequent study (Antonov, Gmiro & Johnson, 1996) the authors analyzed the influence of permeant ions on the blockade. It has been found that permeant cations can bind to the voltage-independent site(s) located at the outer channel vestibule, and thereby decrease the apparent k_+ of the blockers due to competition for entering into the channel. If the blocker is in the channel, this competition induces the lock-in effect. Internal cations also prevent binding of a compound with the smallest ammonium group ($-N^+H_3$) to the deeper binding site.

Practical Approaches

Sometimes the experimental estimation of blockade rate constants is a serious problem. In principle, single-channel recording provides such possibility (Neher & Steinbach, 1978). However, this approach is not universal. The low conductance of some channels (e.g., kainate channels) can prevent the correct analysis. When the unblocking rate is slow, its direct determination from the distribution of closed times becomes technically difficult. In this case the single-channel recordings should be accompanied by the whole-cell method which allows to estimate the equilibrium dissociation constant.

For whole-cell work, the equation

$$I/I_0 = K_d/(K_d + [B]) \qquad (5)$$

where I is a steady-state current, I_0 is a current in the presence of blocker, and $[B]$ is blocker concentration, is widely used for calculation of δ (electrical distance) from the analysis of current attenuation. However, this equation is fully applicable only when the blocker binding does not affect the channel gating, as it was noted by Woodhull (1973). In the sequential scheme of the ligand-gated channel blockade

$$R + A \leftrightarrow R^*A + B \leftrightarrow R^*AB \qquad \text{(scheme 3)}$$

where R is the closed channel, R^* is the open channel, A is the agonist and B is the blocker, the channel cannot close while the blocker is bound and the blockade shifts the equilibrium between the open and closed channels towards the open state (Adams, 1977). Such connection of the channel gating and blockade requires a modification of the equation:

$$I/I_0 = K_d^* (1 + K_a/[A])/(K_d^*(1 + K_a/[A]) + [B]) \qquad (6)$$

where $[A]$ and K_a are the concentration and dissociation constant for agonist.

This calls either for determination of the agonist dissociation constant or for use of saturating agonist concentrations. When these are not experimentally available (for instance, due to fast desensitization), Eq. (1) may be used. The apparent K_d for a blocking drug should be calculated from dose-response curves obtained at different potentials. If blockade obeys the sequential model, the apparent dissociation constant estimated in the whole-cell configuration (K_d) will differ significantly from its real value (which may be measured in single-channel recordings):

$$K_d = K_d^*(1 + K_a/[A]) \qquad (7)$$

However, the voltage dependence should remain the same, irrespective of whether sequential or other blockade schemes are applicable.

Although experiments on the single-channel currents are the most favorable for determination of reaction rates, the relaxation methods (voltage jump, fast drug application and removal) are still useful, especially if the

single-channel recording does not provide the clear-cut information.

Conclusions

If the action of an inhibitor on ionic current through the membrane depends on the applied voltage, one should check if the inhibition observed is due to an open-channel blockade. Decisive evidence of the open-channel block would be an inability of the inhibitor to bind to the channel before its activation (application of agonist, voltage jump, etc.). It can be revealed by kinetic analysis of the fast activation of channels in the presence of blocker.

The mechanism(s) responsible for voltage dependence should be defined. The role of ionic fluxes may be determined by varying intra- and extracellular concentrations of the permeant ions. A prominent influence of ion concentration on voltage dependence requires substantial correction of the parameters obtained with the help of the Woodhull model. In the case of multi-ion channels, where the effect of permeant ions on the blockade is especially important, multi-ion block models should be used. The number and properties of sites (location, selectivity, cooperativity) vary in different multi-ion channels, which prevents the use of any standard model.

The predictions of the location of the binding site are productive only when all possible sources of voltage dependence have been included in the model or eliminated in experiments. It requires the extension of experimental approaches. In particular, it seems very helpful to estimate the voltage dependence of rate constants separately, because they may be used to determine and evaluate the contribution of different voltage dependence sources. Even in the simplest case the investigation of the voltage dependence of the association and dissociation rate constants provides valuable information about the energy barrier and well location, which in its turn allows to study more precisely the relationship between the structure of channel blocking drugs and their pharmacological action.

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