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Interaction of sulfhydryl reagents with A-type channels of *Lymnaea* neurons

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The effect of sulfhydryl reagents on macroscopic inactivation of A-current in internally perfused *Lymnaea* neurons under voltage-clamp conditions was investigated. It was found that the binding of Hg^{2+} rather than PHMB with channel proteins resulted in a strong decrease of the peak current and the inactivation rate. Hg^{2+} markedly influenced the steady-state inactivation but did not change the rate of recovery from inactivation. It was found that both reagents reacted with the same groups of the channel protein and that those are most likely sulfhydryl groups. These groups seemed not to be involved in the gating charge movement. Hg^{2+} ions can immobilize some part of the gating charge thereby resulting in strong changes of the steady-state inactivation.

Introduction

The identification of reactive groups closely associated with the gating systems of ionic channels is important as a initial step of maping a functional information on to the primary structure of the channels. Several potassium channel proteins have recently been sequenced [1–7]. Their topology as well as their amino acid sequences are broadly similar to those of voltage-dependent sodium channels [8,9]. It may be expected that the A-channels of *Lymnaea* neurons belong to the same class of proteins. The sodium channel structure is believed to contain external cysteine residues located in the polypeptide chain connecting S5 and S6 segments. In contrast to sodium channels, the proposed structure of potassium channels does not reveal external cysteine residues. A number of studies have shown that sulfhydryl reagents like *N*-ethylmaleimide (NEM) or *p*-chloromercuriphenylsulfonic acid are effective on sodium currents but not on potassium currents [10–12]. However, as shown by Gilly and Armstrong [13], potassium channels in squid axon have specific binding sites for Hg^{2+} ions which may be expected to be SH-groups. NEM also altered selectively the K^+ current in nodes of Ranvier [14].

The aim of this study was to examine the effect of sulfhydryl reagents on the inactivation kinetics of A-current. It was found that Hg^{2+} ions rather than PHMB strongly influence the inactivation kinetics. There is evidence that Hg^{2+} ions and PHMB bind to the same sites in the channel protein which are most likely SH-groups. But these groups seem not to play an important role in inactivation.

Materials and Methods

In this study unidentified neurons of 80–100 μm in diameter from the right and left parietal ganglia of *Lymnaea stagnalis* were used. The isolation of neurons was performed as follows. The circumoesophageal nerve ring was dissected out of an adult mollusc and soaked in a 0.35% pronase (Sigma, USA) solution for 25–30 min at room temperature. The nerve ring was then replaced into the external physiological solution for next 30–40 min to wash out pronase. Here each ganglion was opened and neurons were isolated mechanically with sharpened tungsten wires and glass micropipette.

The method of intracellular dialysis was applied to the isolated neurons in a version of Kostyuk et al. [15] with some modifications. A polythene pipette with a pore of 40–50 μm in diameter on its tip was served as a suction electrode through which the internal solution flowed at a rate of about 1 ml/min. The outlet of a tube of the suction electrode was usually set up at a lower level than the pore to create a necessary negative

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Abbreviations: PHMB, sodium *p*-hydroxymercuribenzoate; TEA, tetraethylammonium chloride; Tris, tris(hydroxymethyl)aminomethane.

pressure difference (relative to the chamber). The neuron chosen was sucked into the pipette. A small area of neuronal membrane embedded into the pore was moved aside to permit internal perfusion.

The cells were voltage clamped using Dagan-8500 system. Currents were measured in a virtual ground configuration. They were sampled at intervals of 100–500 μs with a 12-bit analog/digital converter Dash-16 (MetraByte, USA) controlled by an IBM PC/AT computer.

The ohmic resistance of the pores was 100–120 kΩ. It was partly compensated electronically by means of Dagan-8500 facilities. Under such conditions a new level of the membrane potential could be achieved occasionally in 100 μs, but typically in 150–200 μs (estimated from the potential settling time).

To isolate I_a from other ionic currents, NaCl was omitted from the external solutions to abolish Na^+-current and 30 mM TEA was added to depress both the delayed rectifier (I_k) and Ca^{2+}-activated K^+ currents. Separation of I_a from the remaining currents, including capacitive transient currents and leakage current, was obtained by subtracting traces recorded without prepulses from the corresponding ones recorded with conditioning prepulses within the range of -80 to -180 mV according to Belluzzi et al. [16]. As a recovery of I_k from inactivation occurs slowly the next current measurement was performed 3 min after the last one to avoid an unequal subtracting of I_k. This procedure tested on the cells with very small I_a (< 1 nA at $V = 0$ mV) and upon amplifier gain adjusted to record of 10–50 nA current yields after subtracting a trace close to the base line.

The kinetics of development of inactivation were studied by two different methods. The first one was to measure the A-current decay under maintained depolarization. In the potential range from -100 to -60 mV a double-pulse method was used. The decay of the peak current was measured in a test pulse as a function of preceding depolarization pulse duration. The removal of inactivation was studied in double-pulse experiments, in which conditioning prepulses of variable duration and at different potential levels are applied immediately before the test depolarization. Peak I_a currents are plotted as a function of the conditioning pulse duration.

The time course of recovery from inactivation was approximated by single exponential. The inactivation kinetics were approximated by a two-exponential function:

$$H(t) = C_0 + C_f \exp(-\gamma_f t) + C_s \exp(-\gamma_s t) \quad (1)$$

Here C_0, C_f, C_s are time independent pre-exponential coefficients, γ_f and γ_s are rate constants of the fast and slow phases of inactivation ($\gamma_f \gg \gamma_s$).

Fitting various functions to kinetics was done by linearization of the function with respect to its parameters and finding the values of these parameters for which the sum of the squares of the deviations was a minimum [17]. The current traces used in the fitting procedure were normalized to their peak amplitudes.

The internal solution contained 80 mM KCl and 10 mM Tris (pH 7.3). The external solution contained (mM): KCl, 1.6; $CaCl_2$, 2; $MgCl_2$, 4; TEA, 30 and Tris, 45 (pH 7.5). Sodium *p*-hydroxymercuribenzoate (PHMB) was obtained from Fluka (Switzerland). Sulfhydryl reagents $HgCl_2$ and PHMB were added into the external solution. The neurons were treated with reagents for 6–10 min. Then the reagent containing solution was replaced by a fresh one without reagent and the current traces were recorded. The time course of the Hg^{2+} effect on the peak current and inactivation rate was obtained in the presence of $HgCl_2$ in external solution throughout the experiment. Experiments were performed at 18–20°C.

Results

Mercury ions, at low concentrations and in the presence of chlor anions, as well as organic mercury compounds, e.g., PHMB, are along the most specific reagents for the determination of SH-groups in proteins. The dissociation constants of mercaptides of organic mercury compounds are much greater than those formed by Hg^{2+} ions [18]. As a result, the mercury ions are able to displace the organic mercury reagents from the binding sites in proteins. PHMB is commonly used for a quantitative determination of monosulfhydryl groups.

Interaction with mercury ions

Treatment of a neuron with mercury ions led to a considerable decrease of both peak current and inactivation rate of I_a (Fig. 1). Current-voltage relationships before and after treatment with mercury ions are presented in Fig. 2. The peak currents recorded in the presence fo mercury ions and multiplied by a factor of 2.9 can be seen to be in reasonable agreement with the control current-voltage relationship. It can thus be concluded that the degree of the current fall in the poisoned neuron does not depend on the potential. Hence, the steepness of the steady-state activation curve appears unchanged as well. This result is very similar to that of Gilly and Armstrong [13] found for the potassium current in squid axons.

Fig. 3 demonstrates the effect of mercury ions on the steady-state inactivation of I_a. The steady-state curve in the control and experiment with Hg^{2+} is of a symmetric shape and is described by a Boltzmann distribution:

$$h(\infty) = 1/(1 + \exp((V - V_0)/d)) \quad (2)$$

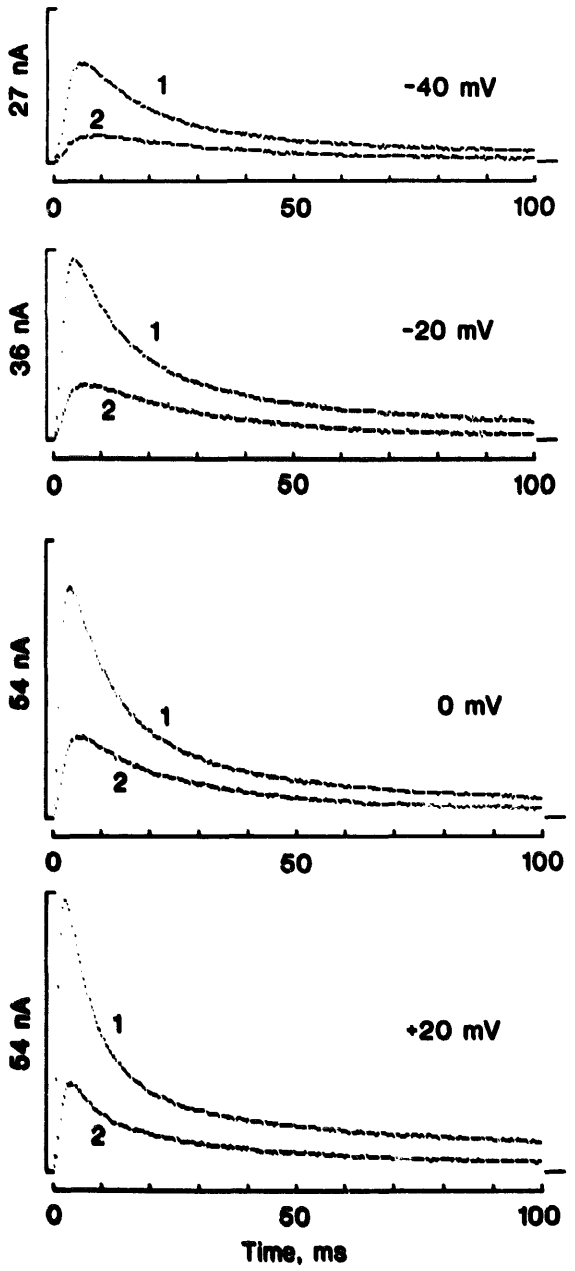

Fig. 1. Effect of Hg^{2+} ions on *A*-current. Superimposed records of I_a activated by depolarization from a holding potential of −60 mV to −40, −20, 0 and +20 mV with preceding hyperpolarizing pulses of 200 ms duration to −150 mV and obtained from neuron under control conditions (1) and after preincubation for 6 min in 0.01 mM $HgCl_2$ (2).

where V_0 is membrane potential, at which $h(\infty) = 0.5$, d is a slope factor. Mercury ions can clearly be seen to cause (i) a drastic change in steepness of the steady-state curve (d increases from 6.2 (±0.2) mV in the control to 11 (±1) mV in the experiment with $HgCl_2$, almost 1.8 times) and (ii) its shift along the potential axis by 12 (±2) mV to the right.

Mercury ions strongly influenced the inactivation kinetics as well. In control, the rate constants of the fast phase of inactivation γ_f rose steeply at potentials

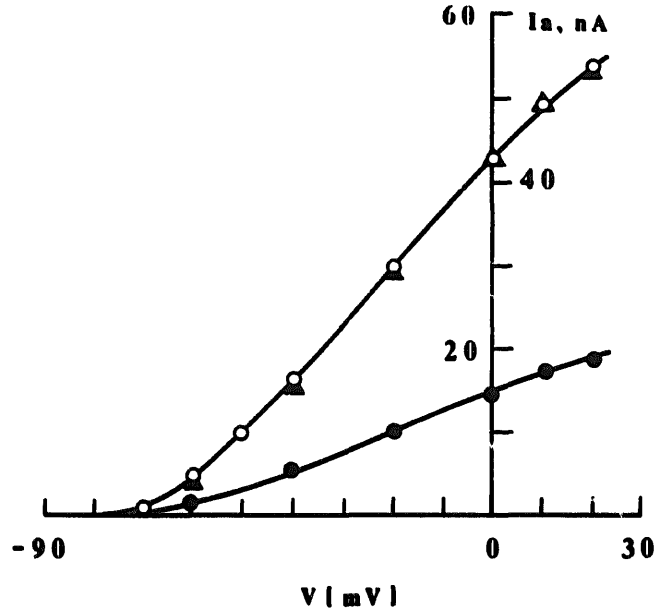

Fig. 2. Current-voltage relationships for peak A-current obtained from neuron before and after treatment with 0.01 mM $HgCl_2$ for 6 min. The data are indicated by open and full circles, respectively. Triangles: Hg data scaled up by a factor of 2.9.

from −90 to −30 mV and more positive than about 0 mV (Fig. 4). In the range from −30 to 0 mV the $\gamma_f(V)$ curve had a pronounced plateau. The rate constants of the slow phase $\gamma_s(V)$ showed little dependence on the voltage [19]. The rate constants of the fast phase of inactivation undergo considerable changes upon treating the neuron with mercury ions. Formally, the rising branches of the $\gamma_f(V)$ curve were found to be proportional to $\exp(\pm V/d)$ where d is a slope factor. Let us highlight the main changes in the $\gamma_f(V)$ curve. Firstly, the mercury ions induce a decrease in the steepness of $\gamma_f(V)$ curve branch on the left of the plateau (d alters from 27 (±3) mV in the control to 52 (±3) mV in the experiment with $HgCl_2$, i.e., 1.9 times) and do not

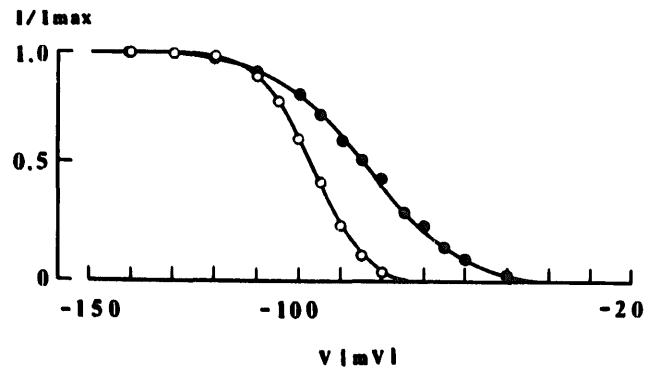

Fig. 3. Voltage dependence of the steady-state inactivation of I_a obtained under control conditions (open circles) and after preincubation of neuron for 6 min in 0.01 mM $HgCl_2$ (full circles). The steady-state inactivation curves through the data points are fitted by Eqn. 2: control values: $V_0 = -97$ mV, $d = 6.2$ mV vs. $V_0 = -85$ mV and $d = 11$ mV for the Hg curve.

affect the steepness of the right-hand branch. Secondly, the action of mercury ions results in a shift of the branch on the right of the plateau along the potentials axis by 8 (±3) mV to the right. Thus the decrease of the rate constants of the fast phase of inactivation with potential occurs in an irregular manner. In contrast, the rate constants of recovery from inactivation after treatment with Hg^{2+} were not altered at all (Fig. 4).

The slow phase of the inactivation kinetics was essentially unaffected by mercury ions. There is a trend toward a slight increase in the rate constants. However, these changes lie within the accuracy of the γ_s determination. On the whole, γ_s values are similar to those presented in paper [19]. The contribution of the slow phase of inactivation, C_s, rises by 10–15% (at $V = -20$ mV), while that of the fast phase, C_f, falls in the same proportion.

Fig. 5 shows the time course of the changes in current amplitude and γ_f value for the first 10 min of the reaction. As can be seen, along with a rather fast phase of reaction between mercury ions and the channel protein there was a slower reaction phase in the course of which (for about 40–50 min) an entire irreversible inactivation of A-channels occurs. The fast phase of the kinetics is approximated by the expression:

$$D = D_1 \cdot \exp(-t/\tau) + D_0 \qquad (3)$$

with τ being about 61 s for both curves, $D_1 = 0.65$ and $D_0 = 0.35$ for curve $I(t)/I_{max}$; $D_1 = 0.52$ and $D_0 = 0.48$ for curve $\gamma_f(t)/\gamma_f max$. Thus, the rates of change in current amplitude and inactivation kinetics turned out to be practically equal. This result suggests that the

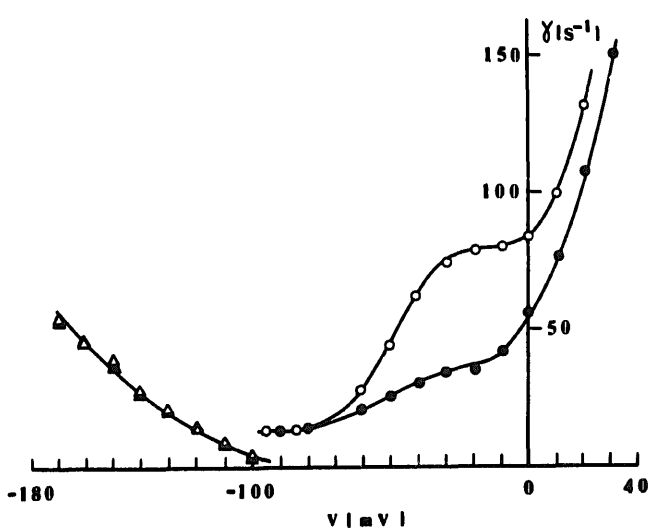

Fig. 4. Rate constants of the fast phase of inactivation and recovery from inactivation of I_a as a function of voltage under control conditions (open circles and triangles, respectively) and after preincubation of neuron for 6 min in 0.01 mM $HgCl_2$ (full symbols).

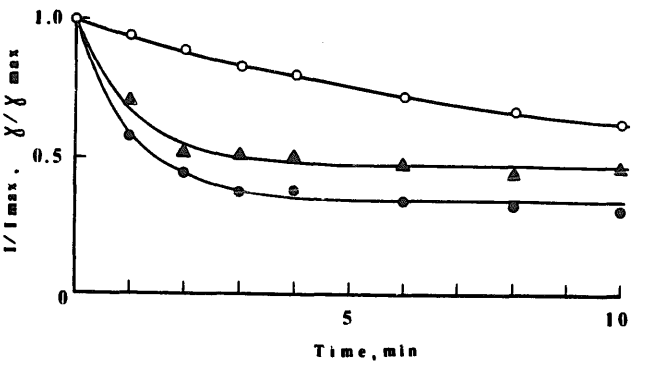

Fig. 5. Decrease in peak current (full circles) and rate constants of the fast phase of inactivation of I_a (triangles), referred to their primary values, during repeated steps to −20 mV in the solution containing 0.01 mM $HgCl_2$. Open circles: time course of peak current decrease in the presence of 0.01 mM $HgCl_2$ after preincubation of neuron for 6 min in 1 mM PHMB. Curves through full circles and triangles are fitted by Eqn. 3 at $\tau = 61$ s.

reaction of mercury ions with a single functional group of the channel protein underlies the changes observed both in current amplitude and γ_f. If so, to study the kinetics of these reactions, it will be sufficient to measure the changes in one of the parameters mentioned below: either those in the current amplitude or in the rate constant of the fast phase of inactivation. It is noteworthy that further changes in current and kinetics cease as soon as mercury ions have been washed out of the solution.

Interaction with PHMB

Mercury ions are generally known to be capable of reacting also with sites other than sulfhydryl groups of proteins. In the experiments with Hg^{2+} ions, therefore, an additional identification of the binding sites is required. For this purpose it seemed to be expedient to use the reaction of competing replacement of PHMB from binding sites by mercury ions. PHMB binding to reactive groups should slow down the reaction rate of mercury ions with these groups. Besides, the effect of PHMB on inactivation kinetics is of independent interest as well.

Fig. 6 presents the records of current before and after treatment of neuron with PHMB. The PHMB action leads to a decrease of the current amplitude (usually by not more than 25% for 8 min incubation) without any apparent changes in the kinetics of development and removal of inactivation and the steady-state inactivation.

When the cell is preincubated in solution with 1 mM PHMB for 6 min, the rates of changes in the current amplitude and in the rate constants of the fast phase of inactivation upon adding mercury ions to the external solution are sharply slowed. As can be seen in Fig. 5, the initial reaction rate decreases about 10 times. Though slowly, the current amplitude falls to the level

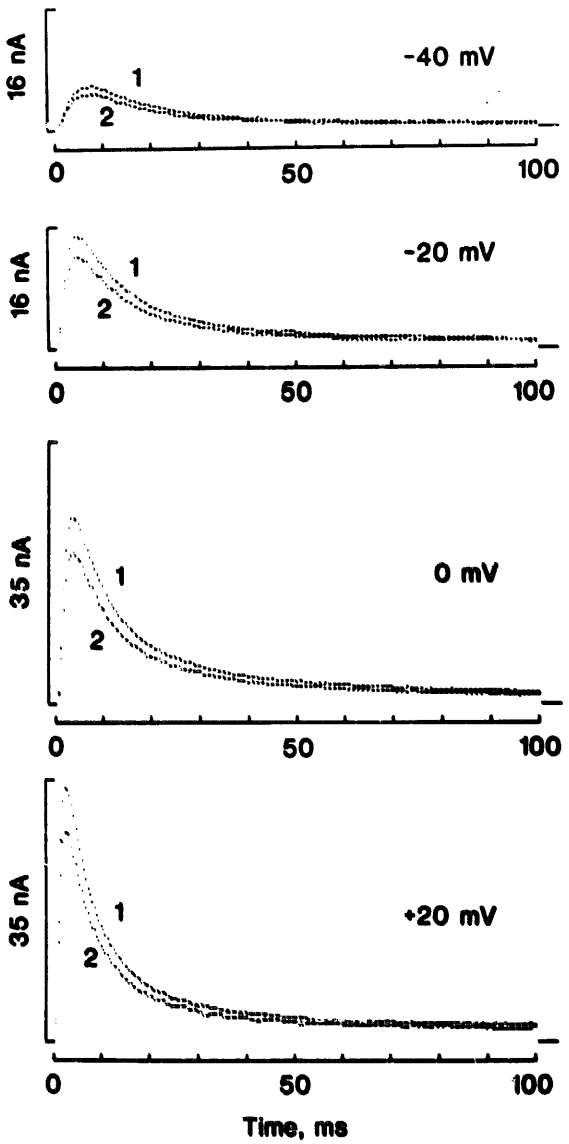

Fig. 6. Effect of PHMB on A-current. Superimposed records of I_a evoked by depolarization from a holding potential of −70 mV to potentials indicated in the figure with preceding hyperpolarizing pulses of 200 ms duration to −150 mV and obtained from neuron under control conditions (1) and after treatment with 1 mM PHMB for 6 min (2).

that it reaches in the experiments without preincubation with PHMB. It is also interesting that the component with $\tau = 61$ s, typical for reaction of mercury ions with the channel protein in the absence of PHMB, is eliminated. This suggests that all groups able to react with mercury ions, bind PHMB during preincubation and that mercury ions probably slowly displace PHMB from the binding sites.

As has been shown, it is difficult to demonstrate an interaction of pHMB with the channel protein in direct experiments. The experiments with competing replacement of PHMB by mercury ions clearly demonstrate that PHMB binds to the protein and that the time constant of this reaction is less than preincubation period, 6 min. It can be concluded that mercury ions and PHMB react with the same sites on the channel protein. Since the reaction rate of mercury ions and PHMB with the reactive groups is relatively high, these groups can be assumed to be easily accessible to the reagents. High specificity of the reagents and their equal effectiveness in binding indicate that the binding sites are most likely SH-groups.

In neutral solutions PHMB is known to carry negative charge. Therefore, its binding with the protein, to some degree, enhances the surface negative charge which should affect the surface potential of the membrane. The absence of an expected shift of the steady-state inactivation and $\gamma_f(V)$ curves for the first 6–8 min of PHMB action could be explained if the density of the reactive groups is too low to induce a marked effect.

Discussion

Recently, many details of molecular structure of potassium channels have been known. By analogy with sodium channels, potassium channels seem to be tetramers, but the sites of subunit interaction are unknown [20,21]. Variation in the N- and C-terminal sequences of the *Shaker* proteins can significantly influence the time course of the development and removal of inactivation [22–24]. The substitutions of the leucines in the heptad that overlaps S4 and S5 segments produce large effects on both activation and inactivation of K^+ channels [25]. The application of site-specific mutagenesis to the potassium channels encoded by the *Drosophila Shaker* gene has been made possible to understand some mechanisms of inactivation in detail [26–29]. It was shown that the two exponential decay of macroscopic potassium currents reflects two different types of inactivation: N- and C-type. These types of inactivation occur by different molecular mechanisms. N-type inactivation is fast and involves the amino terminus of the channel protein by a 'ball and chain' mechanism first proposed by Armstrong and Bezanilla [30]. The ball and chain mechanism predicts that the channel protein will form a receptor on a cytoplasmic side of the membrane for the inactivation ball. C-type inactivation manifests itself as a slow decay in the current remaining after the fast phase of inactivation. It does not involve a cytoplasmic ball and chain mechanism. C-type inactivation is markedly affected by a single amino acid change in the transmembrane segment S6 [29].

On the basis of similarities in the primary structure of various ionic channels it is reasonable to expect that N- and C-type inactivation in some modification are also involved in inactivation of the *Lymnaea* A-type channels and are responsible for the fast and slow

phases of the inactivation kinetics, respectively. Both types of inactivation are considered to be coupled to activation and to be voltage-independent [29]. Inactivation gains its voltage dependence upon that of the activation gate. Hg^{2+} did not influence the steady-state activation and slowed the activation rate by 40–50% (data not shown). In terms of kinetic model proposed by Zagotta and Aldrich [31] the inactivation rate can not be affected by given changes in τ_a as the rate constants of activation are supposed to be much larger than those of inactivation. Hence, the effect of Hg^{2+} on the inactivation kinetics can be described as a reduction in the rate constants of transition to the inactivation state. For N-type inactivation it means that mercury ions either cause an allosteric change in the conformation of the receptor site slowing its interaction with the amino-terminal inactivation particle or alter functioning of other segments of the channel protein which, like S6 segment in *Shaker* potassium channels [29], take part in inactivation. The direct effect of external Hg^{2+} on the receptor site or ball is excluded.

The most prominent result of the effect of Hg^{2+} on the A-channels is the reduction of the slope of the steady-state inactivation. This indicates that a part of gating charge responsible for the potential dependence of $h(\infty)$ is immobilized by Hg^{2+}. It was found that inactivation does not involve significant movement of charge through the electric field across the membrane [26,29]. Then, if this is the case, mercury ions most probably interact with activation gating particle. Such an interaction seems to influence only the rate constants of initial steps of activation which can alter the activation rate without affecting the steady-state activation [30].

As shown by Hoshi et al. [29] in ShA channels with N- and C-type inactivation intact the rate of recovery from inactivation is mainly due to recovery from C-type inactivation, i.e. the changes in the rate of N-type inactivation have little effect on the recovery rate. In *Lymnaea* neurons the recovery rate constants are coupled to those of the slow phase of inactivation [19]. This result suggests that the same type of inactivation (probably C-type) may be responsible for both the slow phase of inactivation and recovery from inactivation. If so, the effect of Hg^{2+} on this type inactivation is very weak as the rate of recovery from inactivation and the slow phase of inactivation are not markedly affected by Hg^{2+}. Thus, supposition of the two types of inactivation allows us to describe satisfactorily the different effect of Hg^{2+} on the rates of inactivation and recovery from inactivation. But it remains to be seen whether mechanisms of inactivation in *Lymnaea* A-channels are identical to those in *Shaker* potassium channels.

Mercury ions and PHMB have been shown to interact most probably with SH-groups of the channel protein. The question is whether this group (or groups) belongs to a gating system. If the binding sites are located on the gating particles, binding of either mercury ions or PHMB to them would cause a change in the effective gating charge by $\pm ne$, where e is an electron charge, n is the number of groups involved in the reaction. Since PHMB does not affect the inactivation kinetics at all, the groups with which mercury ions and PHMB react can not belong to the gating system. Moreover, it seems that these groups do not play an important role in inactivation of I_a. Similar results was obtained by Oxford and Wagoner [12] who found that another sulfhydryl reagent, NEM, does not cause a change in inactivation kinetics of K^+ current of GH_3 cells.

After interaction with SH-groups or other reactive groups of protein mercury ions form mercaptides that still remain capable of reacting either with neighbouring SH-groups or with other functional protein groups like imidazolic, carboxyl, amino groups, etc. A possible binding of mercaptides, formed by mercury ions, to functional protein groups of the gating system should be considered the most probable mechanism of interaction of mercury ions with the gating system of I_a. This interaction would result in immobilization of a part of the gating charge. Mercury ions usually react preferentially with SH-groups because their affinity for these groups exceeds that for other functional protein groups. Therefore, the assumption that the binding of mercury ions to SH-groups constitutes the initial event in the reaction of these ions with the channel protein is quite logical. Any of the above groups could be a neighbouring functional group of the gating system reacting with mercaptide. The SH-groups, being inaccessible, under normal conditions, to PHMB for steric reasons, should not be ignored in this consideration.

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