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Cyclic Nucleotides Induce Long-Term Augmentation of Glutamate-Activated Chloride Current in Molluscan Neurons

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SUMMARY

1. Literature data indicate that serotonin induces the long-term potentiation of glutamate (Glu) response in molluscan neurons. The aim of present work was to elucidate whether cyclic nucleotides can cause the same effect.

2. Experiments were carried out on isolated neurons of the edible snail (*Helix pomatia*) using a two-microelectrode voltage-clamp method.

3. In the majority of the cells examined, the application of Glu elicited a Cl^--current. The reversal potential (E_r) of this current lied between −35 and −55 mV in different cells.

4. Picrotoxin, a blocker of Cl^--channels, suppressed this current equally on both sides of E_r. Furosemide, an antagonist of both Cl^--channels and the $Na^+/K^+/Cl^-$-cotransporter, had a dual effect on Glu-response: decrease in conductance, and shift of E_r to negative potentials.

5. A short-term (2 min) cell treatment with 8-Br-cAMP or 8-Br-cGMP caused long-term (up to 30 min) change in Glu-response. At a holding potential of −60 mV, which was close to the resting level, an increase in Glu-activated inward current was observed. This potentiation seems to be related to the right shift of E_r of Glu-activated Cl^--current rather than to the increase in conductance of Cl^--channels. The blocking effect of picrotoxin rested after 8-Br-cAMP treatment.

6. The change in the Cl^--homeostasis as a possible mechanism for the observed effect of cyclic nucleotides is discussed.

KEY WORDS: cyclic AMP; cyclic GMP; potentiation; glutamate response; chloride current, molluscan neurons.

INTRODUCTION

Long-term potentiation (LTP) of glutamate (Glu) synapses is considered by many authors as a basis for learning and memory in animals of different species (Milner *et al.*, 1998; Lewin and Walters, 1999; Lu *et al.*, 1999; Chitwood *et al.*, 2001; Antonov *et al.*, 2003; Balaban *et al.*, 2004). Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) play an important role in the mechanisms of this potentiation. It was shown that an increase in cAMP or cGMP level in postsynaptic

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neuron could cause the potentiation of Glu synapses in rat hippocampus (Milner *et al.*, 1998; Yu *et al.*, 2001; Lu and Hawkins, 2002).

The mechanism(s) of potentiation of Glu synapses in molluscan neurons evokes a lot of interest. Serotonin (5-HT), the neurotransmitter playing a critical role in behavioral sensitization, was found to cause long-term facilitation of Glu transmission in *Aplysia* and *Helix* neurons (Borisova and Skrebitsky, 1991; Milner *et al.*, 1998; Chitwood *et al.*, 2001; Antonov *et al.*, 2003; Balaban *et al.*, 2004). It was shown that mechanisms of this facilitation involve both the increase of neurotransmitter release from presynaptic terminal and the increase in sensitivity to Glu of postsynaptic membrane. At present, the explanations of the enhancement of Glu response during serotonin-induced facilitation is believed to be the insertion of additional AMPA-type Glu receptors into postsynaptic membrane (Chitwood *et al.*, 2001). The participation of cAMP and cGMP both in presynaptic (Milner *et al.*, 1998; Lewin and Walters, 1999; Antonov *et al.*, 2003) and postsynaptic (Borisova and Skrebitsky, 1991) mechanisms of facilitation is well documented. However, the precise mechanisms of the cyclic nucleotides action on Glu response is poor understood.

Glutamatergic transmission in molluscan neurons has a number of characteristic features that make it different from this process in higher animals. In molluscan neurons, Glu can activate not only cation channels, but also anion (Cl^-) channels (Bolshakov *et al.*, 1991; Dale and Kandel, 1993; Kehoe and Vulfius, 2000; Bravarenko *et al.*, 2003). Glu-induced cationic current (E_r is close to 0 mV) causes an excitatory response (Dale and Kandel, 1993; Bravarenko *et al.*, 2003), while the outward K^+-current ($E_r = -85$ mV) hyperpolarizes the membrane and inhibits spike activity (Bolshakov *et al.*, 1991). The E_r of Glu-activated Cl^--current has been found to vary in molluscan neurons from -60 to -41 mV, and, therefore, this current can hyperpolarize or depolarize cell membrane, depending on whether E_r is negative or positive, respectively, to the resting membrane potential (Eusebi *et al.*, 1978; Sawada *et al.*, 1984; Bolshakov *et al.*, 1991; Kehoe and Vulfius, 2000).

The question of possible involvement of Cl^--component of Glu response in the mechanisms of synaptic plasticity in molluscan neurons was not examined yet. The present study was undertaken to clarify whether cyclic nucleotides can induce long-term potentiation of Glu-activated Cl^--current in molluscan neurons.

METHODS

The experiments were performed on isolated neurons of the visceral ganglion and the left and right parietal ganglions of the land snail (*Helix pomatia*). Neurons were isolated with the help of perfect needles without any pretreatment of the ganglia with proteolytic enzymes. The neurons were pipetted into the recording chamber of about 1 ml volume and continuously perfused with a standard Ringer solution feeding by gravity. The flow rate of the perfusion was 0.6 ml/min.

Two microelectrodes voltage-clamp technique was used. The microelectrodes were filled with potassium citrate solution (2 M), and the microelectrode tip resistance was about 10 MΩ. The experiments were performed using a MEZ 7101 microelectrode amplifier and a CEZ 1100 voltage clamp amplifier (Nihon Kohden, Japan).

Voltages and currents were recorded using a RJG 4024 four-channel pen-recorder with a bandwidth of up to 40 kHz. The outer solution contained (in mM): NaCl 100, KCl 4, $CaCl_2$ 5, $MgCl_2$ 4, $NaHCO_3$ 3, Tris-Cl 5 (pH = 7.6). L-glutamic acid (glutamate) (Sigma), picrotoxin (Sigma), furosemide (Sigma), tetraethylammonium (TEA) (Sigma), 8-Br-cyclic adenosine monophosphate (8-Br-cAMP) (Fluka), and 8-Br-cyclic guanosine monophosphate (8-Br-cGMP) (Fluka) were diluted with control solution to the required concentrations and added to the working chamber at stopped flow. All of the drugs were applied with the help of microsyringe just above the cell (within 2 mm) under visual control. The volume of applied chemicals was 10 μL, and to apply this it took about 0.5 s. The intervals between glutamate applications were 6–7 min. The perfusion started 3 s after glutamate application and lasted during 3 min. Then the perfusion was stopped and the cell was exposed to normal solution (control) or to either chemical. Picrotoxin, TEA, 8-Br-cAMP or 8-Br-cGMP was administered 2 min before glutamate application. Furosemide was applied 4 min before glutamate application.

Statistical analysis was performed with the use of the Prism 3.0 (GraphPad) software. Group data are presented as mean ± SE. Statistical tests of drugs effects were performed using unpaired *t*-test. A *t* value producing $P < 0.05$ was considered to be significant.

RESULTS

Cl^--Nature of the Excitatory Glu-Response in the Majority of Neurons

The isolated *Helix* neurons ($n = 47$) were clamped at a holding potential of −60 mV, which was close to the resting level. The application of 100 μM-Glu elicited, in different neurons, a transient inward current (excitatory response) or slow outward current (inhibitory response). The Glu-activated outward current ($n = 5/47$) developed slowly (3–6 s) and there was no evidence of desensitization. This current could be blocked by tetraethylammonium (0.1 mM) and its E_r was -85 ± 4 mV. These features fit to the characteristics of Glu-activated potassium current described in literature (Bolshakov *et al.*, 1991).

Second group of neurons ($n = 4/47$) responded to Glu-application with picrotoxin-resistant transient inward current which E_r was near 0 mV. This current was similar to Glu-elicited cationic current observed by others (Dale and Kandel, 1993; Bravarenko *et al.*, 2003).

In the majority of cells ($n = 38/47$), Glu application induced transient inward current which reversed at potentials between −35 and −55 mV in different cells ($E_r = -45 \pm 6$ mV). The value of E_r allowed to suggest that the main component of this response was Cl^--current (Bolshakov *et al.*, 1991; Kehoe and Vulfius 2000). In further experiments, we used only the neurons displaying this type of response.

The Effects of Picrotoxin and Furosemide on Glu-Activated Cl^--Current

In presence of 0.3 mM picrotoxin, a blocker of Cl^--channels, the amplitude of Glu-induced inward current recorded at −60 mV decreased by 74 ± 12% ($n = 6$).

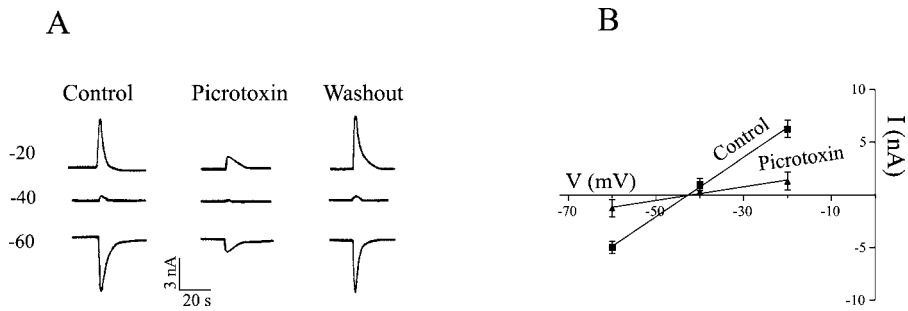

Fig. 1. The inhibition by picrotoxin of the Glu-activated currents of snail neurons. (*A*) Current traces recorded from single cell at different potentials in control solution, in the presence of 0.3 mM picrotoxin and after the substance was washed off. The values of potentials are shown in mV on the left of current traces. (*B*) The average I–V curves of Glu-activated current in the control solution (■) and in the presence of picrotoxin (▲) ($n = 6$).

The picrotoxin effect was the same on both sides of E_r, and was completely reversible after 12–18 min washing of cells with control solution (Figs. 1(A) and (B)). The absence of the shift of the E_r in the presence of picrotoxin indicates that the contribution of non-chloride-conducting glutamate receptors to the response was negligible.

To get an additional confirmation of Cl^- nature of Glu-response we used furosemide. This drug is known to block Cl^- permeability in different membranes (Nicoll, 1978; Bolshakov *et al.*, 1991). Besides, furosemide was described to be a potent inhibitor of the $Na^+/K^+/Cl^-$-cotransporter accumulating Cl^- (Russel, 2000). In our experiments, furosemide was added to the extracellular solution at the concentration of 0.5 mM during 4 min. In presence of furosemide, the amplitude of Glu-activated inward current recorded at −60 mV decreased by 81 ± 9% ($n = 6$). The furosemide effect depended on the potential: at −100 mV, the response to Glu decreased by 62 ± 8%, whereas at −20 mV, the response decreased only by 25 ± 5%. The furosemide effect was completely or partially reversible during 15–30-min washing of the cell with control solution. Figures 2(A) and (B) shows the current traces and the average current–voltage (I–V) relations of Glu-responses in the control solution or in the solution containing furosemide. One can see that furosemide both decreased the I–V curve slope relative to the potential axis and shifted the I–V curve to the left along the potential axis. The slope factor decreased from 0.29 ± 0.02 (control) to 0.14 ± 0.02 (furosemide), whereas the intersection of the curve and the potential axis shifted from −43.9 mV (control) to −55.8 mV (furosemide) ($P < 0.05$). These results indicate that furosemide has a dual effect on Glu-activated channels: conductance decrease and shift of E_r towards more negative potentials.

cAMP Analogue Produces Long-Lasting Augmentation of Glu-Activated Cl^--Current Recorded at Resting Potential

cAMP cell-permeable analogue, 8-Br-cAMP, was bath applied for 2 min in the concentrations of 100 μM. In the presence of this substance, Glu was administered once. It was found that in the presence of 8-Br-cAMP, the amplitude of Glu-response

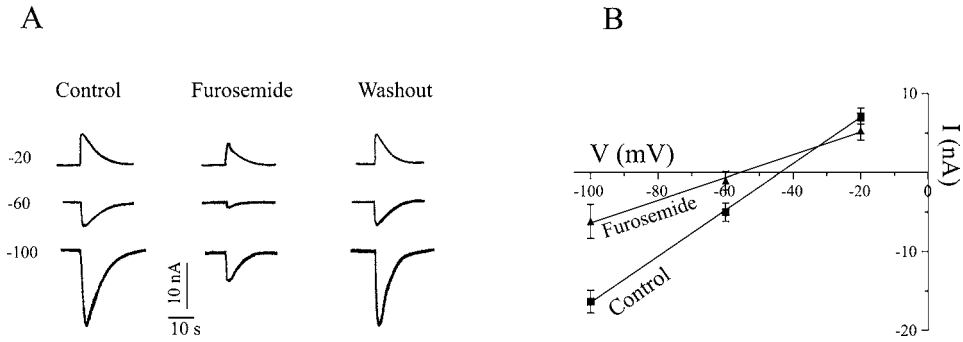

Fig. 2. The effect of furosemide on Glu-activated currents. (*A*) Current traces recorded from the same cell at different potentials in control solution, in the presence of 0.5 mM furosemide and after the substance was washed off. The values of potentials are shown in mV on the left of current traces. (*B*) The average I–V curves of Glu-activated current in the control solution (■) and in the presence of furosemide (▲) ($n = 6$).

was not changed significantly. The effect of the drug became remarkable 6 min after the beginning of the cell washing and lasted at least 30 min. After the cell treatment with 8-Br-cAMP, a pronounced increase in the amplitude of the Glu-activated inward current registered at −60 mV was observed ($n = 7/8$).

Figure 3 shows the time course and magnitude of the effect of 8-Br-cAMP on Glu-response recorded at −60 mV. Figure 3(A) illustrates 8-Br-cAMP-induced long-term Glu-response augmentation in seven neurons, and Fig. 3(B) depicts representative experiment. The enhancement of Glu-activated inward current was observed 6–30 min following 8-Br-cAMP withdrawal and reached 166 ± 26% of

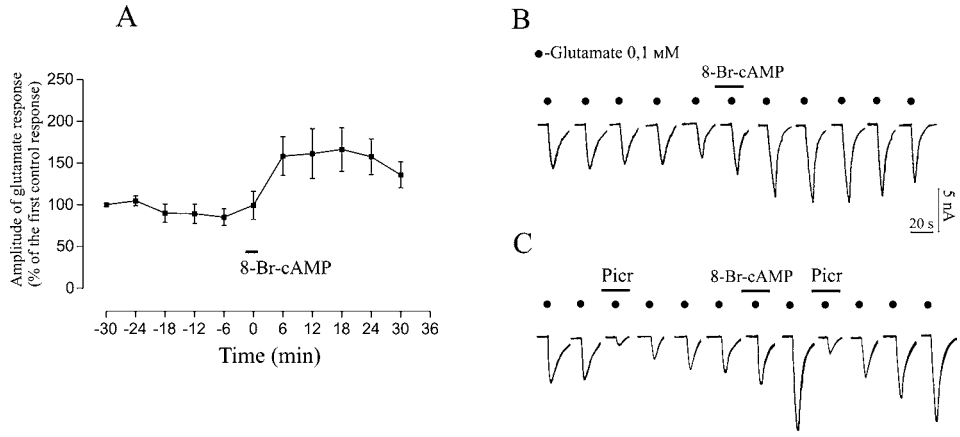

Fig. 3. Long-term potentiation of Glu-activated inward current by 8-Br-cAMP. (*A*) The time course of the 8-Br-cAMP-induced effect on the average Glu-responses recorded at −60 mV ($n = 6$ for Glu-responses obtained at −30, −12, −6, 0, 6, and 30 min; $n = 4$ for Glu responses obtained at −24, −18, 12, 18, and 24 min). (*B*) Current traces recorded in one of the cells at −60 mV before the 8-Br-cAMP application, in the presence of 0.1 mM 8-Br-cAMP, and after the substance was washed off. Intervals between Glu applications were 6 min. (*C*) The maintenance of picrotoxin effect after 8-Br-cAMP treatment. Another cell than in *B*.

the initial value ($n = 4$, $P < 0.01$ comparing the mean amplitude of Glu-response 18 min after 8-Br-cAMP withdrawal to the average for 6 min before 8-Br-cAMP application).

The potentiation of Glu-evoked current after 8-Br-cAMP treatment was not accompanied by changes in the sensitivity of the response to picrotoxin. Enhanced Glu-responses were rapidly and reversibly suppressed by picrotoxin by $69 \pm 13\%$ on average ($n = 3/3$). The little difference between the effects of picrotoxin before ($74 \pm 12\%$) and after ($69 \pm 13\%$) cell treatment with 8-Br-cAMP is statistically insignificant ($P > 0.05$). Figure 3(C) illustrates the maintenance of picrotoxin effect after 8-Br-cAMP treatment in one of the cells.

The Shift of E_r of Glu-Activated Cl^--Current After 8-Br-cAMP Treatment

To understand the mechanism(s) of 8-Br-cAMP-induced long-term facilitation we studied Glu-activated current at different potentials (−100, −60, and −20 mV) before and 6–24 min after 8-Br-cAMP treatment. It was found that the 8-Br-cAMP effect depended on the potential. The increase in Glu-response was observed at −100 mV ($126 \pm 10\%$ of the initial value, $n = 5$). In contrast, at −20 mV, we observed a significant decrease in Glu-induced outward current ($43 \pm 12\%$ of the initial value, $n = 5$). Figures 4(A) and (B) depicts the current traces and the average I–V curves of Glu-activated current in the control solution and 6–24 min after treatment with 8-Br-cAMP. One can see that treatment with 8-Br-cAMP shifted the I–V curve of Glu-activated current to the right along the potential axis, whereas the slope of the curve remained unaltered: 0.2263 ± 0.01823 (8-Br-cAMP) versus 0.2191 ± 0.01609 (control) ($P > 0.05$). The control curve crossed the potential axis at −41.8 mV, whereas after the 8-Br-cAMP treatment, the intersection point shifted to −29.3 mV ($P < 0.05$).

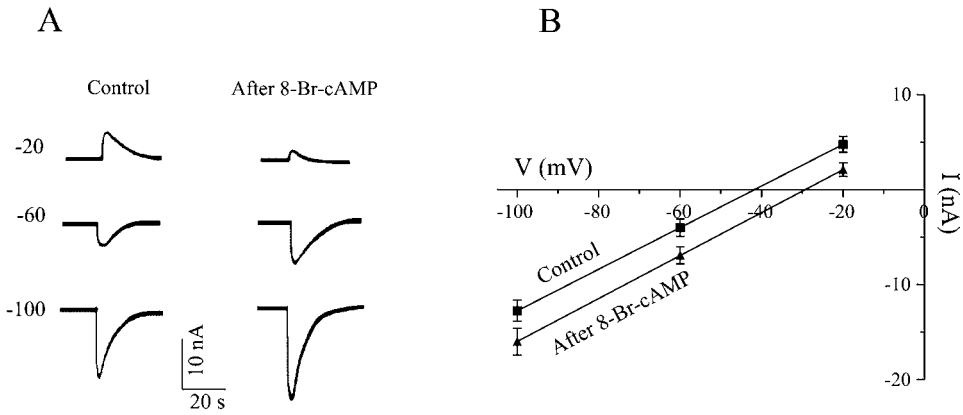

Fig. 4. The shift of the reversal potentaial of Glu-response after the treatment with 8-Br-cAMP. (*A*) The recordings of Glu-activated currents made from a single cell at different potentials in the control solution and 12–24 min after 2 min treatment with 0.1 mM 8-Br-cAMP. The values of potential are shown in mV on the left of the current traces. (*B*) The average I–V curves of Glu-activated current in the control solution (■) and 12–24 min after the cell treatment with 8-Br-cAMP (▲) ($n = 5$).

8-Br-cGMP Shifts E_r of Glu-Evoked Cl^--Current

The effect of 8-Br-cGMP on Glu-response was found to be similar, in general, to that of 8-Br-cAMP in the majority of the cells examined ($n = 8/12$). The treatment of the cell with 100 μM 8-Br-cGMP during 2 min resulted in the enhancement of Glu-activated inward current that lasted for at least 30 min and reached $153.8 \pm 19.7\%$ of the initial value ($n = 5$, $P < 0.01$ comparing the mean amplitude of Glu-response 18 min after 8-Br-cGMP withdrawal to the average for 6 min before 8-Br-cGMP application). The measuring of Glu-response at different potentials showed that Glu-activated inward current (at -100 and -60 mV) increased while Glu-activated outward current (at -20 mV) decreased after the treatment with 8-Br-cGMP. Slope factor of the I–V curve was unaffected (0.2775 ± 0.01443 before and 0.2762 ± 0.02165 after treatment with 8-Br-cGMP) ($P > 0.05$). The intersection point shifted to the right by 13.1 ± 4.6 mV ($P < 0.05$) (data not shown).

DISCUSSION

In our experiments, three types of Glu-responses were recorded in neurons isolated from parietal and visceral ganglions of snail *Helix*. They differed in both E_r values and pharmacological properties, and, according to literature data (Bolshakov *et al.*, 1991; Dale and Kandel, 1993; Kehoe and Vulfius, 2000; Bravarenko *et al.*, 2003), were believed to be controlled by cationic-, K^+- or Cl^--conductance.

In the majority of the cells examined ($n = 38/47$), Glu-responses were believed to be associated with the change in Cl^--conductance. In this group of neurons, the values of E_r of Glu-effects varied between -35 and -55 mV in different cells. These values are in line with those described by other authors. In molluscan neurons, the E_r value of Glu-activated Cl^--current was estimated to be: -41 mV in *Planorbarius* neurons (Bolshakov *et al.*, 1991), $(-46) \div (-51)$ mV in *Aplysia* neurons (Kehoe and Vulfius, 2000), -57 mV in *Heobania* neurons (Eusebi *et al.*, 1978). Further evidence of the Cl^--nature of our Glu-responses was obtained in the experiments with picrotoxin and furosemide. Picrotoxin, a well-known blocker of Cl^--channels (Etter *et al.*, 1999), strongly suppressed Glu-induced response in our experiments. Furosemide is a well-known antagonist of both Cl^--channels (Nicoll, 1978; Bolshakov *et al.*, 1991) and $Na^+/K^+/Cl^-$-cotransporter pumping Cl^- into the cell (Russel, 2000). We observed a dual effect of furosemide on Glu-response which included both a decrease of conductance and a negative shift of E_r. Our results with furosemide do not quite agree with the results obtained by Bolshakov *et al.* (1991) in neurons of mollusc *Planorbarius*. In their study, no change in E_r of Glu-response during the blocking effect of furosemide was observed. The authors have concluded that in their cases furosemide had a direct effect on Glu-controlled Cl^- channels rather than on the intracellular Cl^- concentration. However, our results with furosemide indicate the participation of both mechanisms: the blockade of Glu-activated Cl^- channels and the decrease in intracellular Cl^- concentration induced by the inhibition of $Na^+/K^+/Cl^-$-cotransporter.

Thus, the value of E_r and strong sensitivity of Glu-response to picrotoxin and furosemide allow us to conclude that the main component of these response was Cl^- current.

The neurons displaying Glu-activated Cl^- current were used in the experiments with cyclic nucleotides. We found that a short-term (2 min) cell treatment with permeable analogues of cyclic nucleotides, 8-Br-cAMP or 8-Br-cGMP, led to a long-lasting (at least 30 min) increase in the amplitude of Glu-activated and picrotoxin sensitive inward current. Our work is the first one describing a long-term augmentation of Glu-response of molluscan neurons as affected with cyclic nucleotides. In neurons of mollusc *Helix*, EPSP potentiation after intracellular cAMP injection was described (Borisova and Skrebitsky, 1991). In mammals' hippocampus, perfusion of slices with membrane permeable analogues of cAMP and cGMP such as Sp-cAMPS (50, 100 μM) and 8-Br-cGMP (1, 100 μM) produced long-lasted potentiation of EPSP (Son *et al.*, 1998; Lu *et al.*, 1999; Yu *et al.*, 2001).

The construction of the I–V relations before and after the cell exposure to 8-Br-cAMP or 8-Br-cGMP showed that E_r of Glu-response shifted to depolarizing direction after the treatment. We think that the above shift of E_r is a good explanation for the augmentation of Glu-response observed at -60 mV.

The positive shift of E_r of Glu-activated Cl^- current may have various reasons. First of all, such a shift can result from the forthcoming of Glu-gated cationic current. The insertion of additional AMPA-type Glu receptors activating cationic channels into postsynaptic membrane is a popular explanation for both serotonin-induced facilitation in molluscan neurons (Chitwood *et al.*, 2001) and LTP in hippocampus (Milner *et al.*, 1998). However, the above explanation doesn't seem to work for our results because (i) cyclic nucleotides failed to increase in the slope of the I–V curve of Glu-response, and (ii) the sensitivity to picrotoxin was unchanged after cyclic nucleotides treatment. It seems that potentiations of different types of glutamate-activated channels in molluscan neurons can occur through different mechanisms.

In our opinion, the likely mechanism of the right shift of E_r of Glu-activated Cl^- current is the increase in Cl^- intracellular concentration. Such increase may be due to the regulating action of cyclic nucleotides dependent protein kinases on transport proteins that control Cl^- homeostasis. In neurons, two types of cation-Cl^- cotransporters are described that regulate intracellular Cl^- level in opposite directions: $Na^+/K^+/Cl^-$-cotransporter accumulating Cl^-, and K^+/Cl^--cotransporter extruding Cl^- (Delpire, 2000; Russel, 2000). The activation of $Na^+/K^+/Cl^-$-cotransporter may be regarded as a possible mechanism for the right shift of E_r of Glu-activated Cl^--current observed in our experiments. Large body of evidence supports the activating effect of cAMP (Lytle, 1997; Bachmann *et al.*, 2003; Niisato *et al.*, 2004) and cGMP (O'Donnel and Owen, 1986; Delporte *et al.*, 1993) on $Na^+/K^+/Cl^-$-cotransporter in different tissues. Our results indicating the left shift of E_r of Glu-response in the presence of inhibitor of $Na^+/K^+/Cl^-$-cotransporter, furosemide, is in favor of this suggestion.

The right shift of E_r of Cl^--current may also result from the inhibition of the K^+/Cl^--cotransporter pumping Cl^- out of the cell (Russel, 2000). However, this mechanism looks less suitable for the explanation of the observed effect of cyclic nucleotides. We could not find any literature data supporting the ability of cyclic

nucleotides to inhibit K^+/Cl^--cotransporter. On the contrary, cGMP was shown to activate K^+/Cl^--cotransporter in some tissues (Delporte *et al.*, 1993; Di Fulvio *et al.*, 2001).

Another possible explanation of the right shift of E_r of Glu-activated Cl-current is an increased production of bicarbonate (HCO_3^-), which, like Cl^-, may serve as a current-carrying ion for Cl^--channels but has a more positive E_r (Staley *et al.*, 1995; Sun and Alkon, 2002). The change of a balance in Cl^--current/HCO_3^--current flowing through Glu-activated anionic channels will result in a shift of E_r of Glu-response from Cl^- toward HCO_3^- (more positive). Inasmuch as carbonic anhydrase, an enzyme producing HCO_3^-, was shown to be activated being phosphorylated by cAMP-dependent protein kinase (Narumi and Miyamoto, 1974), the contribution of HCO_3^- current seems to be the possible mechanism of the observed shift of E_r of Glu-response. The involvement of the above mechanism in the described effect of cyclic nucleotides needs further investigation.

The idea that the change in the Cl^--homeostasis of postsynaptic neurons is one of the important mechanisms of synaptic plasticity is now intensively discussed with reference to the neurons of higher animals. It was shown that, during LTP in the hippocampus that accompanies the learning process in animals, the so-called "disinhibition" of GABA response occurs; i.e., GABA inhibition is substituted by GABA excitation (Paulsen and Moser, 1998; Gusev and Alkon, 2001). A depolarizing shift of the E_r of the GABA-activated Cl^--current is supposed to underlie the change of GABA response sign (Collin *et al.*, 1995; Sun and Alkon 2002).

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