J. Exp. Biol. (1973), **59**, 767–780
With 10 *text-figures*
Printed in Great Britain

REPETITIVE FIRING IN MOLLUSCAN GIANT NEURONES

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(*Received* 30 *May* 1973)

INTRODUCTION

The somata of molluscan neurones respond with long trains of spikes to steady injected outward current. During such repetitive firing the maximum rate of rise of action potentials usually decreases as a result of inactivation of the transport mechanism for inward current. Significant inactivation of this transport system does not often change the peak level of the action potential (Magura, 1967). According to the investigations of Connor & Stevens (1971 *a*, *c*) the neurone's behaviour in the interval between spikes depends upon a potassium-conductance mechanism which is different from that in the squid giant axons. This mechanism represents the fast transient potassium channels which are on several criteria operationally distinct from channels of delayed rectification (Connor & Stevens, 1971 *b*; Neher, 1971; Gola, 1972).

The purpose of this work was to perform an analysis of neuronal behaviour during the train of spikes. We shall conclude that during repetitive firing one may observe the inactivation of transport systems for inward current and for potassium current. The potassium inactivation is an important factor in stabilization of the peak level of action potentials during repetitive firing.

It is also concluded that Ca^{2+} ions in the external solution play an important role in the mechanisms which stabilize the peak level of spikes.

In some neurones of *Limnea stagnalis* the fast transient potassium channels participate in carrying potassium current during the falling phase of the action potential at the beginning of firing.

METHODS

The perioesophageal ring of ganglia of *Limnea stagnalis* or *Planorbis corneus* was isolated and fixed in a chamber under Ringer solution suitable for these molluscs. In the experiments the giant neurones of right and left parietal ganglia were used. The sheath of connective tissue of these ganglia was usually so thin that the somata could be readily impaled by microelectrodes. The diameter of the somata in the neurones used reached 120–150 μ. The experiments using the voltage-clamp method were performed on giant neurones of *Limnea stagnalis* (Magura, Kiss & Kryshtal, 1971).

The data obtained from the experiments on neurones of different species of molluscs are fully comparable.

To study the action potential generation with minimal damage to the cell only one microelectrode was inserted into the soma. A bridge circuit made it possible to use it both for recording and for stimulation of a neurone by electric current. Microelectrodes were filled with 3 M-KCl solution. Their resistance varied from 4–7 MΩ.

Table 1. *Composition of Ringer solutions for* Planorbis corneus *and* Limnea stagnalis (*mM/l*)

	NaCl	KCl	$CaCl_2$	$MgCl_3$	Sucrose
Normal Ringer	60	2	10	—	—
High-calcium Ringer	15	2	40	—	—
Low-calcium Ringer	60	2	1	—	25
Magnesium Ringer	60	2	—	10	—

In experiments using the voltage-clamp method two microelectrodes filled with 3 M-KCl solution were inserted into the soma. The device for voltage-clamp was similar to that used by Chamberlain & Kerkut (1969).

To analyse the changes in mechanism of action potential generation during repetitive firing we recorded not only the spikes but also their first derivatives. The time constant of the condenser and resistance used for differentiation was 30 μsec. The curves of first derivative could be used for quantitative evaluation of ionic currents through the membrane during the action potential (Hodgkin & Katz, 1949; Narahashi, 1964; Magura, 1968). The maximum rate of rise of the action potential $(dV/dt)_{max}$ is proportional to the maximum intensity of inward current $(I_{In})_{max}$; $(I_{In})_{max} = -C(dV/dt)_{max}$, where C is the capacity of the soma membrane.

The pH of the solution was adjusted to 7·5 by use of Tris.

By adding of TEA to external solution the equivalent amount of NaCl was removed.

RESULTS

The rhythmic discharge in normal Ringer solution

In molluscan neuronal somata when several action potentials appear following one another at intervals not exceeding 200 msec, one can easily observe marked differences in their course. These differences are especially clearly seen in the curves of first derivatives of action potentials. In Fig. 1 is represented the 'spontaneous' firing recorded from the soma of a giant neurone of *Planorbis corneus*. Comparing the curves of first derivatives of action potentials one can clearly see that the maximum rate of rise of the action potential varies markedly. It depends on both the character of membrane potential changes during the interval between spikes and on the duration of this interval. The increase in the duration and the more significant shift of the membrane potential in the negative direction cause the increase of the maximum rate of rise of the action potential.

There was a tendency during the discharge of the duration of the falling phase of the spikes to increase and of the maximum rate of its decay to decrease. Attention should be paid to the fact that the changes in the rising and falling phases occurred independently of each other.

It is convenient to study the behaviour of the excitable membrane of molluscan neuronal somata during the repetitive firing in response to a steady applied outward current. The change of polarizing current intensity made it possible to alter within certain limits the frequency of firing and to choose the optimal conditions for experiment.

In Fig. 2(*a*) are shown the superimposed action potentials of the repetitive response,

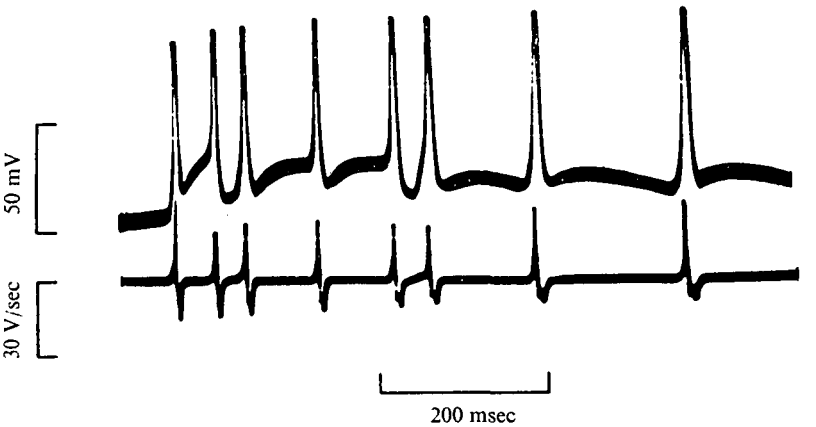

Fig. 1. Changes in course of the action potential during the 'spontaneous' firing in a neurone of *Planorbis corneus*. Upper trace, membrane potential; lower trace, rate of change of potential.

One can see the considerable lowering of the maximum rate of rise of the action potentials during the response. Simultaneously the duration of the falling phase of the action potentials is significantly increased. The lowering of the maximum rate of rise of action potential results from the decrease in the intensity of the inward current and could be regarded as the sign of inactivation of the appropriate mechanism of ion transfer through the membrane. According to the changes in maximum rate of rise during the response represented in Fig. 2(*a*) the intensity of the inward current during the action potential has fallen to $\frac{1}{3}$ of the initial value. Such significant inactivation of the transport mechanism for inward current resulted neither in marked increase of the threshold nor in the lowering of the peak level of the action potential. One could suggest that stability of these parameters of the action potential during the response was due to the simultaneously developing potassium inactivation. Its manifestation is the considerable increase in the duration of the falling phase of the action potential.

The relatively high rate of decay of the falling phase of the action potentials at the onset of the firing is characteristic of some neurones of *Limnea stagnalis*. It markedly exceeds the maximum rate of their rise. During firing one can observe the signs of potassium inactivation only in some of these neurones. The maximum rate of rise of the action potential was not markedly changed. In Fig. 2(*b*) one can see that in this case during the repetitive firing some increase in the peak level of the action potential takes place (Fig. 2*b*).

In experiments on molluscan neuronal somata it has been shown that during long-duration depolarization under voltage-clamp conditions the delayed outward current declined after reaching the maximum value (Alving, 1969; Leicht, Meves & Wellhöner, 1970; Neher & Lux, 1971). This decline is caused both by a potassium inactivation and by a shift in potassium equilibrium potential in the positive direction (Magura, Krishtal & Valeyev, 1971). Potassium inactivation under voltage-clamp conditions may be also observed during relatively short repetitive depolarization. Fig. 3 presents three current traces corresponding to identical shifts of membrane potential at intervals of 2 sec. A decrease of the maximum level of outward current was observed.

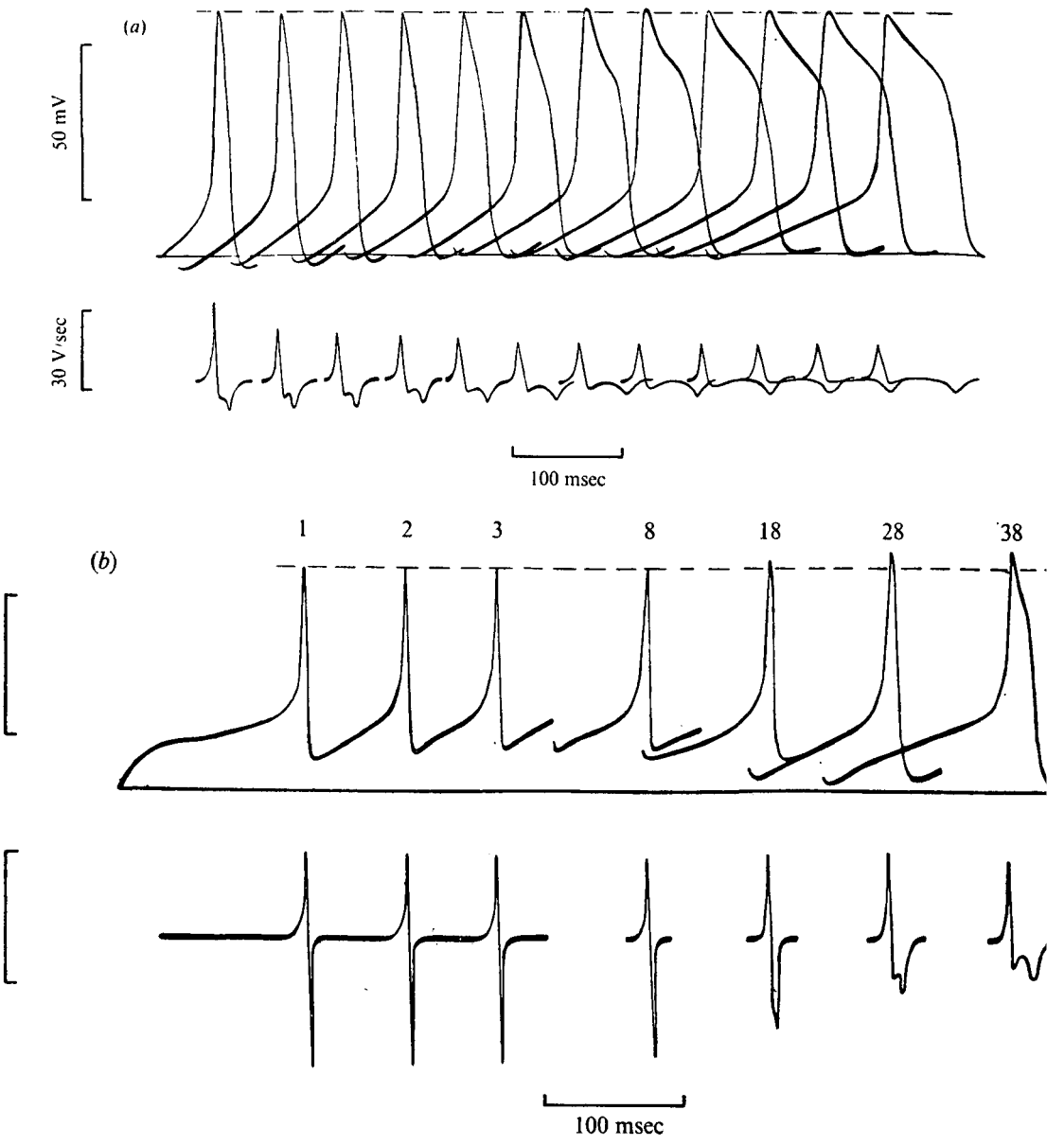

Fig. 2. Action potential during repetitive firing. Upper trace, membrane potential; lower traces, rate of change of potential. (*a*) Action potentials and their first derivatives superimposed during repetitive firing (*Planorbis corneus* neurone). (*b*) Action potentials and their first derivatives during repetitive firing (*Limnea stagnalis* neurone). Figure indicated the number of the spike in the train.

Influence of the change in external concentration of calcium ions on the action potentials during firing

For the effective stabilization of the peak level of the action potential during firing the calcium concentration in the external solution should not be lower than 4–5 mM.

In Fig. 4 the repetitive responses from the soma of the giant neurone of *Planorbis*

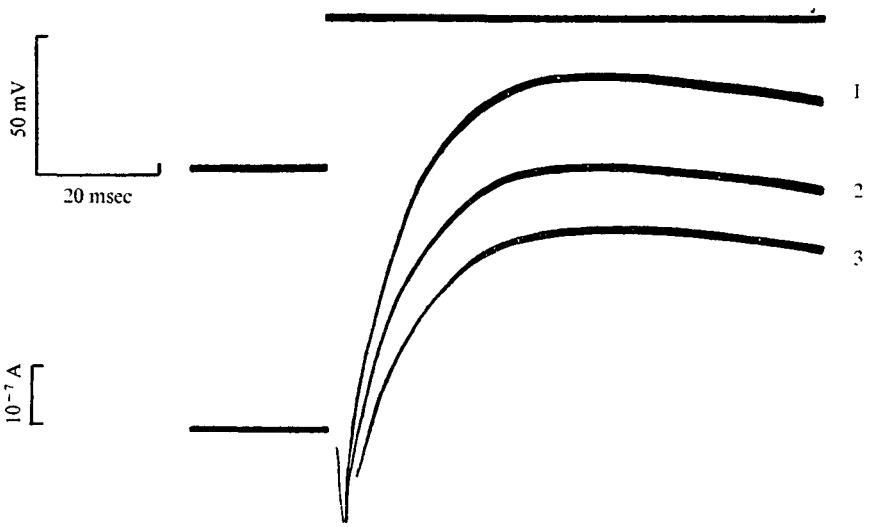

Fig. 3. Membrane currents (lower traces 1, 2, 3) superimposed during repetitive depolarization under voltage-clamp condition. Upper trace, voltage step.

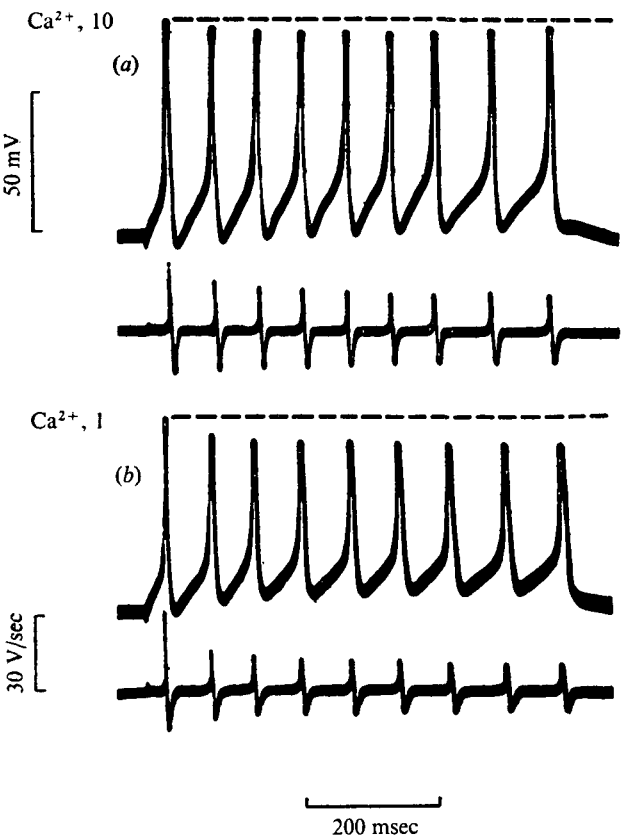

Fig. 4. Repetitive firing pattern in Ringer solution with 10 mM Ca^{2+} (*a*) and Ringer solution with 1 mM Ca^{2+} (*b*). Upper traces, membrane potential; lower traces, rate of change of potential. Peak level of the first action potential is indicated by dashed line.

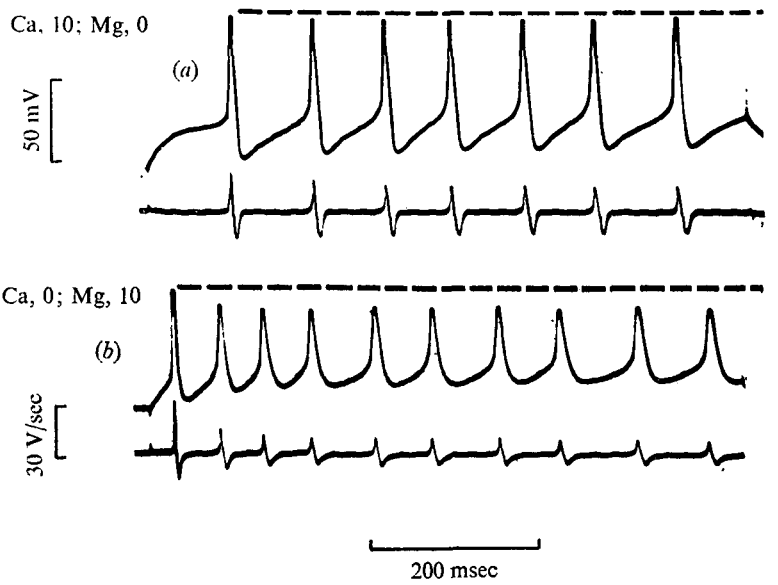

Fig. 5. Effect of Mg^{2+} ions on action potentials during repetitive firing. Upper trace, membrane potential; lower trace, rate of change of potential. Peak level of the first action potential is indicated by dashed line. (*a*), Normal solution; (*b*) solution in which Ca^{2+} ions were replaced by Mg^{2+} ions.

corneus are compared in normal Ringer solution (with 10 mM Ca^{2+}) and in Ringer solution with Ca^{2+} concentration reduced to 1 mM. In the solution with low Ca^{2+} concentration the peak level of the action potential markedly decreased during repetitive firing. Besides this there was a more significant tendency to reduce the maximum rate of rise of action potential. One could suggest that the lowering of the external calcium concentration caused more distinct manifestation of inactivation of the transport mechanism for inward current during repetitive firing. When the external calcium ions were replaced by magnesium ions the signs of inactivation of the transport mechanism for inward current were significantly more pronounced (Fig. 5). By adding to the external solution Co^{2+} ions in concentration exceeding 10 mM the somata of some giant neurones were unable to produce the repetitive firing.

In solutions with high calcium concentration the sodium ion concentration was lowered. It was noted earlier that in some cases considerable reduction of the external sodium concentration occurring simultaneously with the rise of the calcium concentration did not markedly affect the peak level of the action potential, though the maximum rate of rise decreased appreciably (Krishtal & Magura, 1970). In other cases the peak level of the action potential markedly decreased with simultaneous increase of the maximum rate of its decay. In this case one could suggest that the increase in the external calcium concentration resulted in earlier and more significant increase in the potassium membrane conductance during the action potential. This may be a reason for the decrease of the peak level of the action potentials.

Preliminary depolarization of the membrane in this case gave rise to the increase both in the maximum rate of the action potential rise and in the peak level (Krishtal & Magura, 1970).

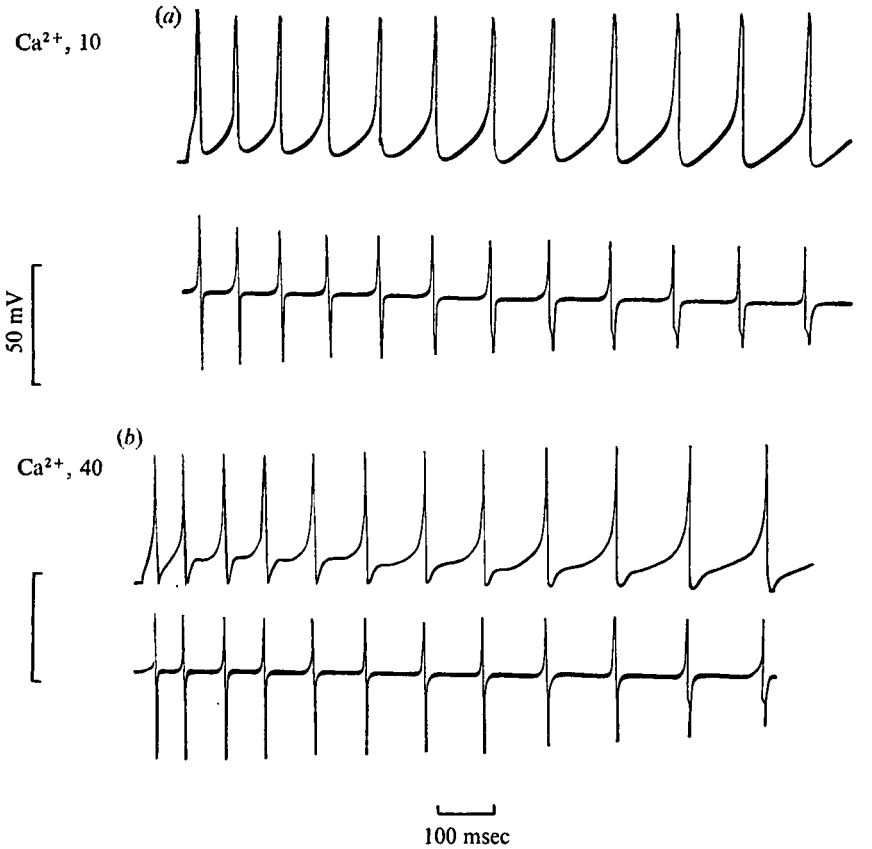

Fig. 6. Repetitive firing pattern for solution with 10 mM Ca^{2+} (*a*), and solution with 40 mM Ca^{2+} (*b*). Upper traces, membrane potential; lower traces, rate of change of potential.

In Fig. 6 is represented the repetitive firing of the giant neuronal soma of *Limnea stagnalis* in normal Ringer solution (10 mM of Ca^{2+}) and in Ringer solution with 40 mM of Ca^{2+}.

In the latter case the maximum rate of rise of the action potential during firing was more stable than in normal Ringer solution. The maximum rate of decay of the action potential increased. In the beginning of the repetitive firing, changes in the course of the inter-impulse intervals took place which could be due to acceleration of the rate at which the high potassium conductance shuts off just after the action potential was over.

According to our observations a rise of the external calcium concentration caused in some neurones the appearance of the fast transient potassium current during depolarization of the membrane under voltage-clamp conditions. The fast transient potassium current could overlap to a considerable extent the transient inward current. It usually led both to a decrease of the peak level of the action potential and to a decrease of the maximum rate of its rise.

Appearance of the fast transient potassium current under voltage-clamp conditions was earlier noticed in those cases when depolarizing shift of the membrane potential

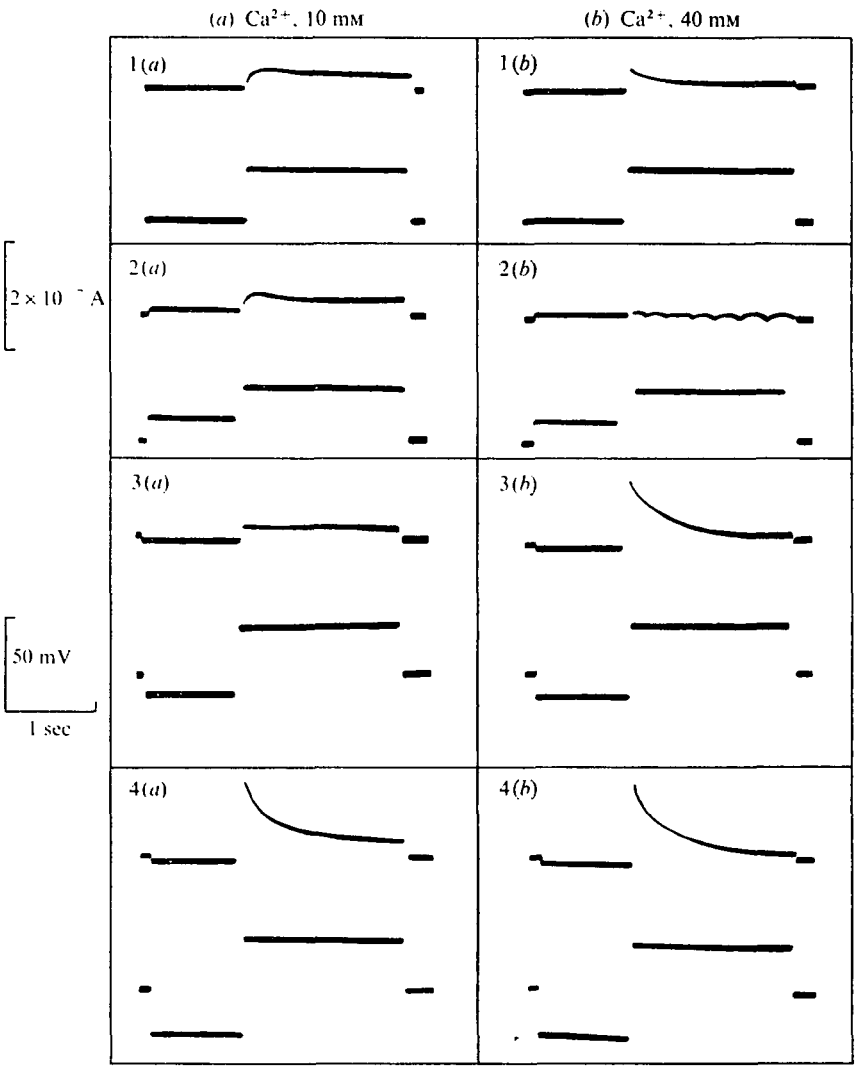

Fig. 7. Effect of conditioning pulse upon the outward current traces under voltage-clamp condition. (*a*) Ringer solution with 10 mM Ca^{2+}; (*b*) Ringer solution with 40 mM Ca^{2+}. Holding potential in both solution, −45 mV. Upper traces, membrane current; lower traces, membrane voltage steps.

was preceded by hyperpolarization of the membrane (Hagiwara, Kusano & Saito, 1961; Connor & Stevens, 1971*b*; Neher, 1971; Gola, 1972).

In our experiments increase in the external calcium concentration in the range from 10 to 40 mM was roughly equivalent to a hyperpolarization of 7–8 mV.

In Fig. 7 are shown the effects of increasing the external calcium concentration and of a preliminary shift of membrane potential upon the outward current under voltage-clamp conditions.

The holding potential in solutions with 10 mM and 40 mM of Ca^{2+} was −45 mV. The shifts of the membrane potential in both solutions were identical.

In the solution with 10 mM Ca^{2-} oscillograms 1(*a*) and 2(*a*) no clear signs of the

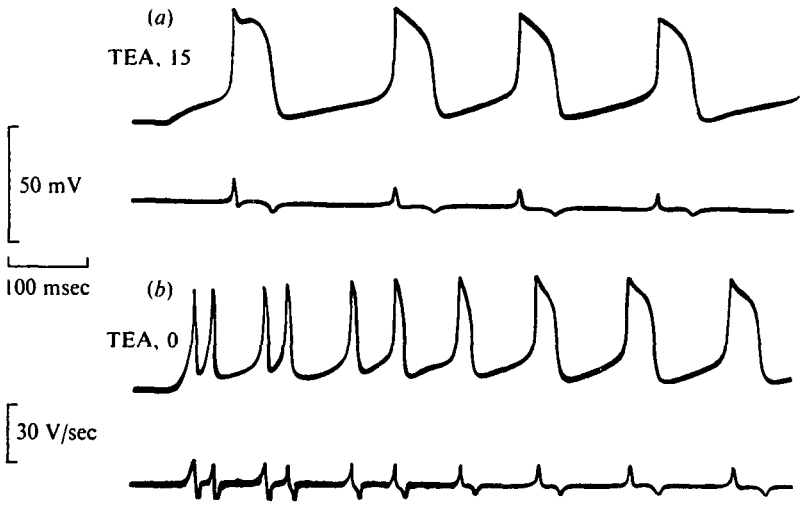

Fig. 8. Repetitive firing pattern in Ringer solution with 15 mM TEA (*a*), and after complete removal of TEA from the external solution (*b*). Upper traces, membrane potential; lower traces, rate of change of potential. Ganglia were kept for 1 h in the Ringer solution containing TEA.

potassium inactivation show. The conditioning depolarization of the membrane in this solution did not evoke appreciable changes of the outward current. The conditioning hyperpolarization of the membrane led to the increase in the rate of rise of the outward current, and simultaneously there appeared the clear signs of the potassium inactivation (Figs. 3*a* and 4*a*). These data could be regarded as a result of the removal of inactivation of the fast transient potassium channels.

In the solution with 40 mM of Ca^{2+} the fast potassium current appeared without preliminary hyperpolarization of the membrane. At the same time the threshold depolarization required for the appearance of the delayed outward current shifted towards a significantly greater depolarization. The depolarization of the membrane to -15 mV caused only the fast potassium current, which was inactivated with a time constant 300 msec. The preliminary depolarization of the membrane fully suppressed the appearance of the fast transient potassium current. The conditioning hyperpolarization of the membrane markedly increased it.

The effect of TEA on the action potential during firing

In Ringer solution containing TEA ions the somata of molluscan neurones generates prolonged action potentials (Hagiwara & Saito, 1959). If ganglia were placed in TEA solution for 15–20 min the subsequent removal of TEA ions led to recovery of the initial shape of the action potential in several minutes. These data could evidence that TEA ions blocked the potassium channels of the soma membrane from the external surface. During more prolonged exposure to TEA ions their effect becomes irreversible. Complete removal of TEA from the external solution in such case does not eliminate the effect of TEA upon the action potential.

In connexion with these observations one could believe that TEA ions slowly passed through the membrane and exerted their blocking action from its inner surface also.

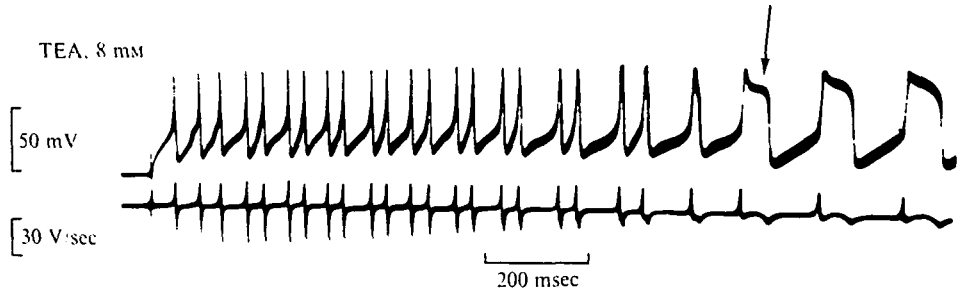

Fig. 9. Repetitive firing pattern in Ringer solution with 8 mM TEA. Upper trace, membrane potential; lower trace, rate of change of potential. Arrow indicates the moment when the TEA effect was clearly manifested.

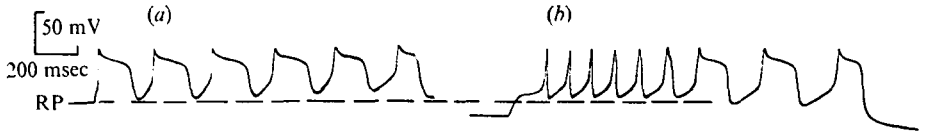

Fig. 10. Effect of preliminary hyperpolarization on repetitive firing in Ringer solution with 15 mM TEA. (*a*) Repetitive response without preliminary hyperpolarization; (*b*) effect of hyperpolarization. Resting potential is indicated by dashed line.

Earlier it was suggested that TEA applied in the outside medium actually penetrated the membranes of spinal ganglion cells in frogs and exerted its action inside the membrane (Koketsu, Cerf & Nishi, 1959). A similar suggestion was discussed by Pichon (1971) as to the action of TEA on the cockroach giant axon.

In Fig. 8 is shown the repetitive firing in Ringer solution containing 15 mM of TEA. Ganglia were kept in this solution for 1 h.

Repetitive firing was again recorded half an hour after the removal of TEA from the external solution. The removal of TEA decreased the duration of the action potential only at the beginning of the response. The somata produced the prolonged action potentials as in the presence of TEA.

The change in efficacy of the TEA effect on the action potential during firing was especially clearly manifested when solutions with rather small TEA concentrations were used. The repetitive firing in the soma of the giant neuron of *Limnea stagnalis* in the solution with 8 mM of TEA is represented in Fig. 9. In this figure one should pay attention to the fact that the TEA effect was clearly manifested just when the rate of decay of the falling phase of the action potential considerably decreased, i.e. when a significant portion of the potassium channels had been inactivated. In connexion with these observations one could suggest that those potassium channels which were inactivated at the onset of repetitive firing are less sensitive to the blocking effect of TEA ions than those whose activity was maintained during the whole repetitive response.

The preliminary hyperpolarization of the membrane increased the portion of the potassium channels which were little affected by TEA. After the preliminary hyperpolarization the duration of the prolonged action potential in Ringer solution containing TEA markedly shortened at the beginning of the firing (Fig. 10).

DISCUSSION

It is concluded that in the most cases during repetitive firing in the somata of the molluscan giant neurones the signs of inactivation of the transport mechanism for the inward current were manifested in a decrease of the maximum rate of rise of the action potentials. Simultaneously, potassium inactivation was observed. It was manifested in prolongation of the duration of the falling phase of the action potential. Sometimes one might observe the appearance of a plateau on the falling phase. The changes in the transport mechanism for the inward current and for the potassium current took place independently of each other.

The inactivation of the transport mechanism for the inward current during firing was often accompanied by only slight increase of threshold potential. The peak level of the action potentials usually remained unchanged. Such stability of these parameters of the action potentials during firing was due to a considerable extent to the potassium inactivation.

The main feature of the potassium inactivation in molluscan neurones is that it is triggered by a depolarizing shift of the membrane potential and is then maintained for several seconds after repolarization of the membrane (Neher & Lux, 1971). It could be suggested that during repetitive responses the potassium inactivation developed not only during the action potential but also during the interval between spikes.

Besides the channels of the delayed rectification in the membrane of molluscan neuronal somata the fast transient channels have been revealed (Hagiwara *et al.* 1961; Connor & Stevens, 1971*b*; Neher, 1971, Gola, 1972). During the depolarization of the membrane these channels provide the transient increase in the potassium conductance of the membrane. Fast potassium channels are activated much more rapidly than channels of delayed rectification, and are also inactivated more rapidly.

In neurones of *Helix pomatia* and also in those of *Anisodoris* and *Archidoris* the fast transient potassium channels were fully inactivated at a membrane potential of -50 mV. At a membrane potential of -100 mV their inactivation was completely abolished (Connor & Stevens, 1971*b*; Neher, 1971). According to our observations in some neurones of *Limnea stagnalis* and *Helix pomatia* the removal of inactivation of the fast potassium channels could take place at a membrane potential of -45–50 mV if the external calcium concentration was increased; a change in calcium concentration in the range from 10 to 40 mM was equivalent to a membrane potential change of 7–8 mV. Fast transient potassium channels are only moderately affected by external concentrations of TEA which completely block the channels of delayed rectification (Connor & Stevens, 1971*b*; Neher & Lux, 1972). Similar potassium channels may be present in the supramedullary cells of the puffer fish *Spheroides maculatus* (Nakajima & Kusano, 1966; Nakajima, 1966).

When using TEA ions one should pay attention to the fact that in some neurones of *Limnea stagnalis* at the beginning of repetitive firing an important role in the potassium current transfer across the membrane is played by those potassium channels which are little affected by TEA.

In this connexion one could suggest that in *Limnea stagnalis* neurones the fast transient potassium channels were not fully inactivated at the resting potential (of -45–50 mV).

It has been established in *Helix pomatia* and *Aplysia* neurones that potassium channels of the soma membrane could be blocked by TEA ions both from the external surface and from the internal one (Kehoe, 1972; Neher & Lux, 1972). Similar results have been obtained in Ranvier nodes (Koppenhöfer & Vogel, 1969; Armstrong & Hille, 1972). According to our observations, during a prolonged stay of ganglia in TEA-containing solution, TEA ions penetrated the cells and exerted their action inside the membrane.

An important role in the mechanism of stabilization of the peak level of the action potentials during repetitive firing is played by the calcium ions. The decrease in their external concentration or their replacement by magnesium ions causes considerable manifestation of inactivation of the transport mechanism for inward current. In Ringer solution with increased calcium concentration the inactivation of the transport mechanism for the inward current is displayed less clearly than in normal solution. It is difficult to exclude the possibility that the increase in the external calcium concentration contributes to the enhancement of the role of these ions in the inward current transfer, and that during the rhythmic discharge, as the inactivation of the transport mechanism for sodium current progressed, the role of calcium ions as carriers of the inward current increased.

The data concerning the participation of sodium and calcium ions in transfer of the inward current during the action potential have been obtained in neurones of molluscs (Gezasimov, Kostyuk & Mayskii, 1965; Junge, 1967; Kezkut & Gardner, 1967; Geduldig & Junge, 1968; Meves, 1968; Moreton, 1972; Wald, 1972), in neurones of the bullfrog sympathetic ganglion (Koketsu & Nishi, 1969) and in neurosecretory neurones of the crayfish (Iwasaki & Satow, 1971). It has been shown that magnesium is unable to substitute for calcium (Meves, 1968; Wald, 1972).

In our paper we did not discuss the problem of change in ionic concentration in the narrow intercellular cleft during firing. Important data on this problem were recently published by Moreton (1972).

SUMMARY

1. The repetitive responses of molluscan neurones to steady injected outward current have been examined using intracellular microelectrodes. Voltage-clamp experiments were also performed to investigate the participation of transport mechanisms for potassium current in neurones during firing.

2. The maximum rate of rise of action potentials usually decreases during repetitive firing. Simultaneously an increasing duration of the falling phase of spikes is observed. The peak level of spikes in most cases is relatively stable if external Ca^{2+} concentration exceeds 3–4 mM.

3. The peak level of action potentials markedly decreases during firing in solutions with low Ca^{2+} concentration. A similar effect is observed if the Ca^{2+} ions in normal Ringer solution are completely replaced by Mg^{2+} ions.

4. With increasing external Ca^{2+} concentration the maximum rate of rise of action potentials becomes more stable during repetitive firing.

5. With increasing external Ca^{2+} concentration the steady-state inactivation of fast transient potassium channels is removed. Increase in Ca^{2+} concentration (in range from 10–40 mM) is roughly equivalent to a hyperpolarization of 7–8 mV.

6. Experiments with TEA show that in some neurones of *Limnea stagnalis* the fast transient potassium channels participate in carrying potassium current during the falling phase of the action potentials at the beginning of repetitive firing.

7. The role of potassium inactivation as well as of Ca^{2+} ions in mechanisms which stabilize the peak level of action potentials during repetitive firing is discussed.

The authors express their gratitude to Professor P. G. Kostyuk for his kind support and valuable advice.

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