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# MEMBRANE RESPONSES OF FROG'S SPINAL GANGLION CELLS IN CALCIUM-FREE SOLUTIONS

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In general, the reduction of the external calcium concentration increases membrane excitability and often leads to spontaneous repetitive activity (Brink, 1954; Shanes, 1958). When external calcium ions are completely removed, however, some excitable tissues, such as squid giant axon (Frankenhaeuser & Hodgkin, 1957), frog myelinated axon (Frankenhaeuser, 1957) or heart muscle fibres (Brooks, Hoffman, Suckling & Orias, 1955; Weidmann, 1955) are found to lose excitability without any significant drop of the membrane potential. Such disappearance of membrane excitability in calcium-free media has been explained on the basis of the concept that the membrane in zero-calcium media is in a state in which the sodium-carrying system is largely inactivated (Frankenhaeuser & Hodgkin, 1957). Action potentials of frog skeletal muscle fibres, however, could be easily produced provided conditioning anodal currents were applied, even after the membrane was strongly depolarized by prolonged immersion in calcium-free Ringer's solution containing ethylenediaminetetraacetic acid (EDTA) (Koketsu & Noda, 1960).

The present experiment was designed primarily for the purpose of studying whether or not frog spinal ganglion cells were capable of maintaining excitability in the complete absence of calcium ions. It will be shown that the cell membrane, strongly depolarized in calcium-free Ringer's solution containing EDTA, is capable of producing action potentials provided conditioning anodal currents are applied, and also that the membrane responses of such depolarized cells resemble those of cells depolarized in potassium-rich media. The present paper will describe the membrane responses of cells immersed in calcium-free media as well as in potassium-rich media.

## METHODS

The spinal ganglion of the frog (*Rana pipiens*), isolated together with the sciatic and dorsal root nerves, was used throughout the present experiments. Intracellular recordings of the membrane potential of individual cells, located in the superficial layer of the ganglion, were taken by using glass capillary micro-electrodes filled with 3M-KCl (Ling & Gerard, 1949). The device used for recording and stimulating simultaneously through a single

intracellular electrode has been described elsewhere (Koketsu, Cerf & Nishi, 1959*a*). When the resistance of the micro-electrode was high, it tended to change during application of strong electrical currents. The time course of the current passing through the micro-electrode was observed by means of a DC amplifier connected to a Wheatstone Bridge circuit (Koketsu *et al.* 1959*a*). All results described in the present experiments were obtained under a condition during which there were no unexpected changes in the strength or the time course of the stimulus currents.

The normal Ringer's solution consisted of (mM): NaCl 112, KCl 2, $CaCl_2$ 1·8 and $NaHCO_3$ 2·4 (pH, 7·0). $CaCl_2$ was simply omitted from the Ringer's solution (Ca-free Ringer) and 4 mM ethylenediaminetetraacetic acid (EDTA) was added to the Ca-free Ringer (Ca-free EDTA Ringer). The potassium-rich medium (K-rich Ringer) was prepared by increasing the concentration of KCl in ordinary Ringer's solution, so that the tonicity of the solution was increased accordingly.

## RESULTS

### *In Ringer's solution*

The value of the resting potential showed a variation among individual cells, the average value being about 70 mV (Ito, 1957; Koketsu *et al.* 1959*a*). When a cell was stimulated directly by an outward current passing through the intracellular micro-electrode, the action potential was initiated at a critical potential level (about 40 mV), as is shown in record 2 of Fig. 1 (Ito, 1957; Koketsu *et al.* 1959*a*). When the membrane was hyperpolarized or depolarized moderately by an anodal or cathodal current (either short pulses of 20–50 msec or continuous current), no marked changes could be observed in the configuration of the action potential (Fig. 1, 1–3).

### *In Ca-free Ringer*

In general, the resting potential showed no appreciable drop when preparations were soaked in Ca-free Ringer up to 3 hr. Longer immersions sometimes resulted in a slight reduction of the resting potential (the external solution was changed frequently during this period). No appreciable changes in the configuration of the action potential were observed as long as cells maintained a normal resting potential value. When the resting potential dropped slightly, the spike potential showed a prolongation by forming a marked hump on the falling phase, at which point it tended to be sustained (Fig. 1, 4).

### *In K-rich Ringer (less than 10 mM)*

If the resting potential maintained a value higher than the threshold potential in these solutions, the action potential could be initiated by cathodal pulses (Fig. 1, 5). In this case the duration of the spike tended to be prolonged.

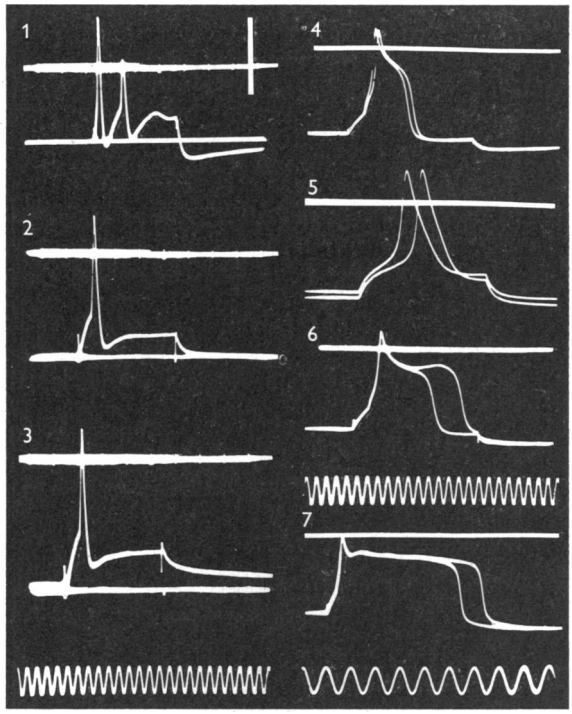


**Fig. 1.** Action potentials of spinal ganglion cells produced by intracellular direct stimulation in Ringer's solution (1–3), Ca-free Ringer (4), K-rich (6 mM) Ringer and Ca-free Ringer containing 4 mM EDTA (6–7). Resting potential was depolarized (1) and hyperpolarized (3) by continuous cathodal and anodal currents. Time marker, 500 cycles except for record 7 (100 cycles). Calibration, 50 mV: this calibration can be applied to records shown in Figs. 2–7.

### *In Ca-free EDTA Ringer*

In general, the resting potential of preparations soaked in this solution dropped to about 15–20 mV within 15–30 min; no further drop was observed even with longer immersions. The effective resistance of the depolarized membranes showed a marked reduction under this condition. In some cases the preparations remained relatively unaffected in this solution, and the membranes of some cells were capable of maintaining a relatively large resting potential. Nevertheless, sufficiently prolonged soaking eventually resulted in a uniform drop of the resting potential to 15–20 mV. When the resting potential was maintained above the threshold potential, a cathodal pulse produced a very prolonged action potential exhibiting a sustained plateau phase (see Fig. 1, 6, 7). This type of action potential was also observed when various kinds of onium ions were added to the external solution (Koketsu, Cerf & Nishi, 1959*a*, *b*).

When an anodal pulse was applied to the membrane that was depolarized to 15–20 mV, a characteristic response was found to be produced at the critical level (about 40 mV) of the membrane potential (Fig. 2, 1–3). Such a response was similar to the hyperpolarizing response in K-rich media observed with frog spinal ganglion cells (see p. 7) or with other tissues (Stämpfli, 1958; Segal, 1958; Moore, 1959; Tomita, Saimi & Toida, 1961; Hagiwara, Kusano & Saito, 1961). Typical examples of such responses are shown in records 1–3 of Fig. 2. As is seen in these records, the

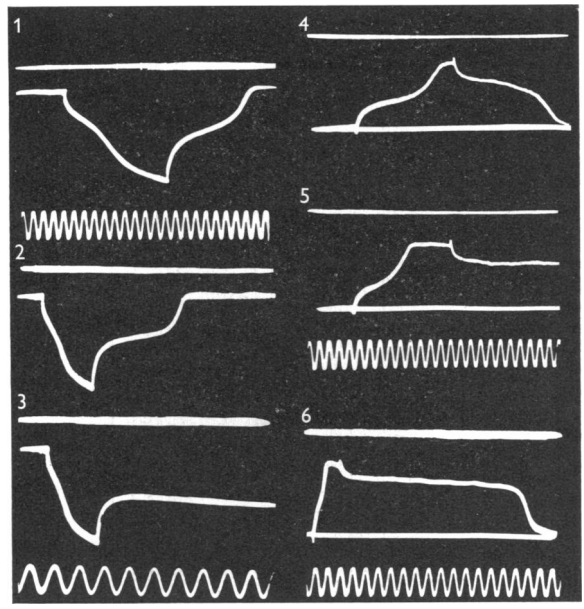


Fig. 2. Membrane responses induced by anodal pulses (1–3) and by cathodal pulses during conditioning hyperpolarizing currents (4–6). Preparations were soaked in Ca-free Ringer containing 4 mM EDTA. Duration of anodal or cathodal pulses were about 20 msec. Time marker, 500 (records 1, 4 and 5) and 100 cycles (records 2, 3 and 6).

membrane potential returned to its original level by forming a step of about 40–50 mV when an anodal pulse was discontinued, the potential, in some cases, being sustained at this level for as long as 1 sec. Membrane responses, such as those shown in Fig. 2, were observed in a number of cells, particularly when relatively weak anodal pulses were applied. On the other hand, with sufficiently strong anodal pulses most cells were found to produce a break response upon cessation of pulses, as is seen in record 1 of Fig. 3. The break responses obtained from three different cells depolarized to 37–40 mV in Ca-free EDTA Ringer are also demonstrated in the other three records. The break response consists of an initial spike

which is followed by a prolonged plateau phase; and the potential level of the plateau phase appears to be constant (about 15–20 mV), notwithstanding the value of the original resting potential level.

If the membrane was hyperpolarized by an anodal current to approximately the normal resting potential level (about 70–80 mV), a cathodal pulse could produce a characteristic response (Fig. 2, 4–6). This response,

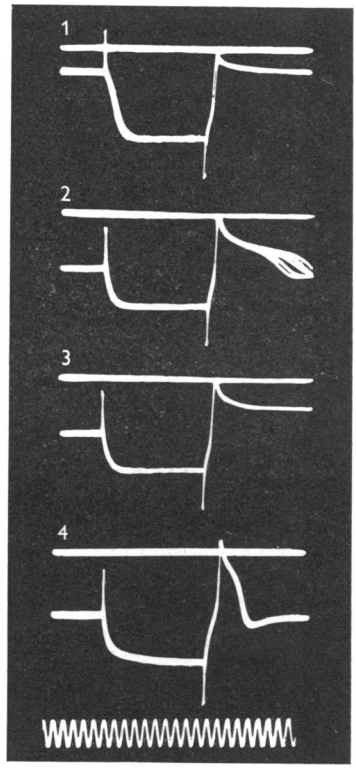


Fig. 3. Break responses of cells which were depolarized to varying degrees in Ca-free EDTA Ringer. Note the value of the resting potential and the level of the plateau phase of break responses. Time marker, 500 cycles.

which appeared to be a reverse reaction of the hyperpolarizing response, sometimes remained for as long as 1 sec after the cessation of the cathodal pulses.

When the strength of both the conditioning anodal current and cathodal pulse was large enough, the rate of rise of the response increased, and the initial spike component appeared. The records shown in the left-hand column of Fig. 4 were obtained by gradually increasing the strength of both the conditioning anodal and stimulus cathodal currents. The amplitude

and rate of rise of the response is increased according to the increase of the currents, and the peak of the response may either approach or exceed the zero resting-potential level. This type of response was observed in Ca-free EDTA Ringer when there was no marked drop of the resting potential (see Fig. 1, 6). Records 3 and 4 of Fig. 4 show that the potential reaches the peak by forming a step (Fig. 4, 3, 4). This phenomenon was comparable

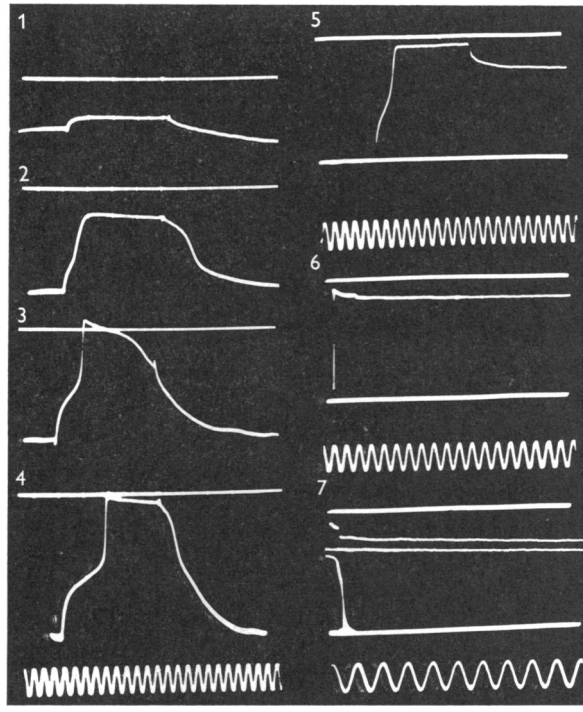


Fig. 4. Responses to cathodal pulses during conditioning hyperpolarization. Recordings of left column (1–4) were taken from cells depolarized in Ca-free EDTA Ringer. The strength of anodal current and cathodal pulse was gradually increased from 1 to 4. Records 5–7 were taken from other cells which showed extremely prolonged plateau phases. In record 8 the entire time course of the plateau (approximately 4 sec) was recorded by superimposing three traces. Time marker, 500 (1–5), 100 (6) and 10 (7) cycles.

to that observed in K-rich Ringer (see p. 7). The potential sometimes was sustained at the plateau level for several seconds, as is seen in records 5–8 of Fig. 4.

The changes of the membrane responses observed in Ca-free EDTA Ringer seem to be due to the chelating action of EDTA rather than some other unknown effect of EDTA. Indeed, the use of citrate or oxalate showed qualitatively similar effects.

*In K-rich Ringer (more than 10 mM)*

If the external potassium concentration is increased to more than 10 mM, the membrane is generally depolarized below the threshold potential (40 mV) (Koketsu *et al.* 1959*b*). The hyperpolarizing response of the membrane, depolarized to 15–20 mV in K-rich Ringer, appeared to be induced at almost the same level (about 40 mV) as in Ca-free EDTA Ringer (Figs. 5 and 6). In general, when anodal pulses were discontinued, the potential returned to the original resting potential level at which point

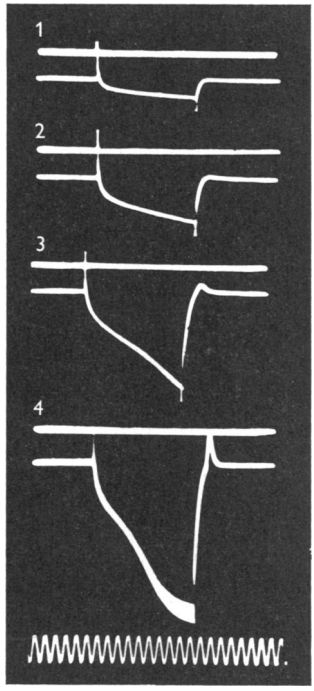


Fig. 5. Hyperpolarizing and break response of a cell which was depolarized in K-rich (40 mM) Ringer. The strength of anodal pulses was increased gradually from 1 to 4. Time marker, 500 cycles.

a spike potential was induced (Fig. 5). In some cases no break responses were produced even though strong anodal pulses were given, and the potential returned to the original resting potential level by forming a step, as is seen in records 1 and 2 of Fig. 6.

A reverse reaction of these hyperpolarizing responses, such as those seen in Ca-free EDTA Ringer, was also observed when a weak cathodal pulse was applied during a conditioning anodal polarization (Fig. 6, 3). The rate of rise of the responses was increased and the spike appeared

when the strength of the conditioning anodal and stimulus cathodal pulses was increased (Fig. 6, 4). In these cases the spikes were initiated after the potential increased in a stepwise fashion (Fig. 6, 4, 5), as observed in Ca-free EDTA Ringer (Fig. 4, 3, 4), and overshot the zero resting-potential level (Fig. 6, 5, 6). In contrast to the responses observed in Ca-free EDTA Ringer, the following plateau potential level varied according to the

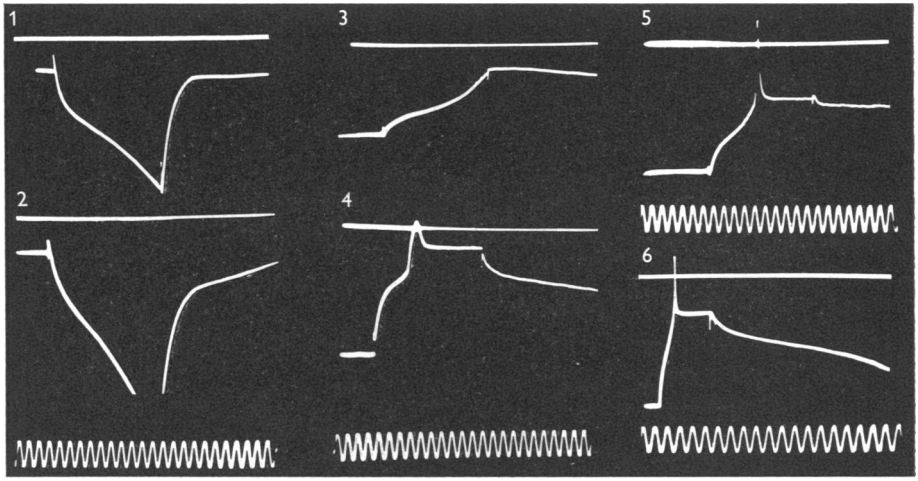


Fig. 6. Hyperpolarizing and depolarizing responses of cells which were depolarized in K-rich media. Records 1–4 were obtained from cells which were depolarized to about 15 mV in 40 mM potassium, and records 5 and 6 were obtained from cells which were depolarized to about 40 mV in 15 mM potassium. Records 3–6 were obtained by applying cathodal pulses during conditioning anodal polarization. Time marker, 500 (1–5) and 100 (6) cycles.

original resting-potential level (Lüttgau, 1959). The relationship between the resting potential and the potential level of the plateau phase is demonstrated in the records of Figs. 6 and 7, which were obtained from cells depolarized to various degrees in K-rich media. The plateau phase was sometimes sustained for an extended period (Fig. 7, 4, 5), and returned to its original level by occasionally forming a large undershoot (Fig. 7, 6).

## DISCUSSION

When direct intracellular stimulation is applied to spinal ganglion cells, action potentials may be produced initially in the membrane of the initial axonal segment (or axon hillock) inasmuch as its threshold is lower than that of the cell body (Ito, 1957). The responses recorded in the present experiment seem to be produced mainly in the membrane of the cell body, judging from the fact that they are too large to have been originated

elsewhere. The responses characteristic of the cell body have been demonstrated in sodium-free media (Koketsu *et al.* 1959*a*, *b*). The typical responses observed in calcium-deficient media are considered actually due to the characteristics of the membrane property of the cell body. Indeed, with small myelinated nerve bundles calcium removal caused only a small depolarization (Stämpfli & Nishie, 1956; Straub, 1956), and with isolated single nodes of Ranvier no appreciable drop of the resting potential nor any marked changes in the configuration of the action potential could be observed (Hashimura & Wright, 1958).

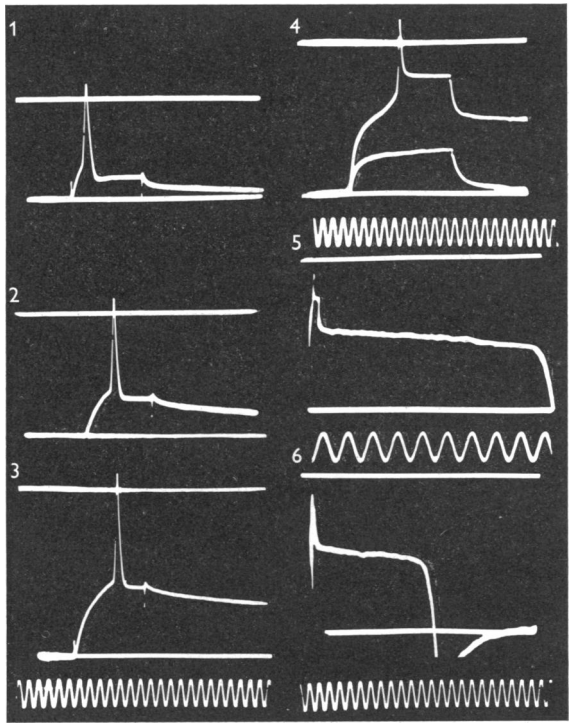


Fig. 7. Responses of cells which were depolarized to about 45 mV in a solution containing 10 mM potassium (records 1–3) and to about 30 mV in a solution containing 20 mM potassium (records 4–6). Note level of plateau potential. All records were obtained during anodal polarization. The strength of anodal currents was gradually increased in records 1–3. Time marker, 500 (1–4) and 10 (5–6) cycles.

According to the present experiments, neither the existence nor the entry of external calcium ions seems to be essential for the production of action potentials. It has been reported that the membrane of excitable tissue in Ca-free solution is in a state in which the sodium-carrying system is largely inactivated (Frankenhaeuser & Hodgkin, 1957). In the case of

frog spinal ganglion cells anodal polarization appears to be sufficient to remove such inactivation of the sodium-carrying system.

Two possible explanations for the hyperpolarizing responses observed with the depolarized nerve membrane in K-rich media have been proposed, based on the concept of the ionic theory (Stämpfli, 1958, 1959; Moore, 1959; Grundfest, 1961) and of the two-stable-state hypothesis (Tasaki, 1959). Hyperpolarizing responses without prior depolarization were observed in lobster muscle fibres and also in several kinds of electroplaques (Grundfest, 1961). It was shown in the present experiment that the membrane, depolarized in Ca-free Ringer, was also capable of producing hyperpolarizing responses. According to the ionic theory (Stämpfli, 1958, 1959; Moore, 1959; Grundfest, 1961), the hyperpolarizing responses observed under these experimental conditions would be explained as being caused by the changes in the membrane permeability to K, Na and Cl ions.

The present experimental results indicated that the cell membrane, whether depolarized in Ca-free or K-rich media, was capable of undergoing a reversible change between two states when electric currents were passed through the membrane (Tasaki, 1959). The resemblance between the membrane responses of cells in Ca-free and K-rich solutions may be an indication that the membrane property is undergoing a similar alteration in the two different solutions. In connexion with this, it is interesting that tissue calcium, presumably located in the membrane, appears to be removed in Ca-free or K-rich media (Koketsu & Miyamoto, 1961; Noda, Koketsu & Miyamoto, 1961). Further investigation on the physicochemical property of the membrane of excitable tissues in Ca-free or K-rich media may provide the key for understanding the fundamental mechanism of membrane responses, such as those observed in the present experiment.

## SUMMARY

1. The membrane property of frog spinal ganglion cells immersed in Ca-free or K-rich media was studied.

2. When the resting potential of cells dropped slightly in Ca-free Ringer, the action potential tended to form a plateau phase.

3. Cells depolarized to 15–20 mV and rendered inexcitable in Ca-free EDTA Ringer produced hyperpolarizing responses upon application of anodal pulses.

4. These depolarized cells were capable of responding to cathodal pulses applied during conditioning anodal polarization. Responses which showed no initial spike component were produced when the cathodal pulses were not strong enough, whereas the spike and the following plateau components could be produced by a strong cathodal pulse.

5. The cell membrane, depolarized in K-rich (40–50 mM) media, produced hyperpolarizing responses upon application of anodal pulses, and also produced prolonged action potentials when cathodal pulses were applied during conditioning anodal polarization.

6. The potential level of the plateau phase was about 15–20 mV in Ca-free EDTA Ringer, but that in K-rich Ringer changed according to the external potassium concentration.

7. It was suggested that the membrane property might be altered similarly when the external calcium ions were omitted or the external potassium concentration was increased.

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