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EFFECT OF HYDRAZINE ON THE EXCITABLE MEMBRANE IN SODIUM-FREE MEDIA

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Among the nitrogenous compounds which can replace sodium ions in maintaining electrical excitability, hydrazine seems to be the most favourable (Koketsu, Cerf & Nishi, 1959*b*; Koketsu & Nishi, 1959). Since the action potential of frog spinal ganglion cells could be elicited in sodium-free sucrose solution by injecting hydrazine into the cell-body (Koketsu *et al.* 1959*b*), it would appear that the intracellular hydrazine was responsible for the maintenance of membrane excitability in sodium-free media. Consequently, the possibility that hydrazinium ions may be acting as charge carriers during active membrane depolarization was dismissed (Koketsu *et al.* 1959*b*). The question was then raised as to whether the intracellular hydrazine was also responsible for the generation of spike potentials in the case of frog skeletal muscle fibres.

The present study was aimed primarily at investigating the mechanism of the action potentials of muscle fibres in a sodium-free hydrazine solution. The results indicate that the external rather than the intracellular hydrazine is important for the generation of action potentials of skeletal muscle fibres. The discrepancy between the results obtained with spinal ganglion cells and muscle fibres led the authors to make confirmatory experiments on the effect of the intracellular hydrazine in ganglion cells.

METHODS

Experiments were performed mainly on sartorius muscles and spinal ganglion cells of frogs (*Rana pipiens*) at room temperature (24–26° C). The sodium-free sucrose solution used throughout consisted of (mM) sucrose 224, $KHCO_3$ 2, and $CaCl_2$ 1·8 (pH 6·8–7·0). The sodium-free hydrazine solution was prepared in exactly the same way as has been described in a previous paper (Koketsu *et al.* 1959*b*). Sulphuric acid or nitric acid was sometimes used instead of hydrochloric acid for adjusting the pH of the hydrazine solution. In some experiments the hydrazinium or calcium ions were replaced by equimolar amounts of sucrose. Experimental arrangements for recording intracellular action potentials of muscle fibres and spinal ganglion cells were similar to those described in previous papers (Koketsu, Cerf & Nishi, 1959*a*; Koketsu & Nishi, 1959). The action potential of isolated single muscle fibres was recorded extracellularly by using a pair of large platinum electrodes. The technique of intracellular injection of various nitrogenous compounds into the cell bodies was

similar to that described in previous papers (Koketsu *et al.* 1959*a*, *b*). In the case of muscle fibres, KCl-filled and hydrazine-filled micro-electrodes were used as recording and stimulating electrodes, respectively. After being tested for normal activity in Ringer's solution, the muscles were soaked in sodium-free sucrose solution for 30 min and the spinal ganglia for 60 min in order to eliminate electrical activity, whereupon they were immersed in a sodium-free hydrazine solution.

RESULTS

Isolated single muscle fibre

In this experiment single muscle fibres were isolated from M. iliofibularis. The electrical activity of isolated single muscle fibres was easily eliminated when they were soaked in sucrose solution for 5–10 min. It was promptly restored when the preparations were immersed in 80–112 mM hydrazine solutions (Fig. 1).

Effects of external anions

No appreciable difference in the value of the resting potentials of muscle fibres was observed between the various hydrazine solutions (80–112 mM) containing sulphate, nitrate or chloride ions. The time constant of the resting membrane increased when the chloride ions were replaced by either sulphate or nitrate ions. The electrical activity of muscle fibres was well maintained in hydrazine-nitrate and hydrazine-sulphate solutions, and no appreciable change in the configuration of the spike potentials was observed in these solutions in comparison to that in hydrazine-chloride solution (Fig. 2).

Effects of different concentrations of hydrazinium ions

The resting and action potentials of muscle fibres soaked in 80–90 mM hydrazine solutions were described in a previous paper (Koketsu & Nishi, 1959). In this study the concentration of hydrazine was changed and the relationship between the action potential and the external hydrazine concentration was studied.

The resting potential of muscle fibres in a solution containing 80–90 mM hydrazine was 90 mV after immersion for 10–30 min, and gradually dropped to about 80 mV after 60–120 min (Koketsu & Nishi, 1959). These values were not appreciably altered by changing the concentration of hydrazine from 50 to 220 mM. When the hydrazine concentration was reduced to less than 50 mM the resting potential tended to fall. The value of the resting potential shown in Fig. 3 is an average of twenty fibres, which were first immersed in a 112 mM hydrazine solution for 30 min and then in a solution containing a given amount of hydrazine for another 30–60 min. The values of the electrical constant of the resting membrane in solutions containing 50–220 mM hydrazine did not show appreciable deviations from those reported in a previous paper (Koketsu & Nishi, 1959).

When the muscles were immersed in a 112 mM hydrazine solution the electrical activity was restored within 1 min. If the amount of the intracellular hydrazine which diffused into the muscle plasma were an important factor, dominating the electrical activity, the action potential could be affected by further immersion. No appreciable differences, however, were found by comparing action potentials recorded immediately after, and between 90 and 120 min after, immsersion in a 112 mM hydrazine

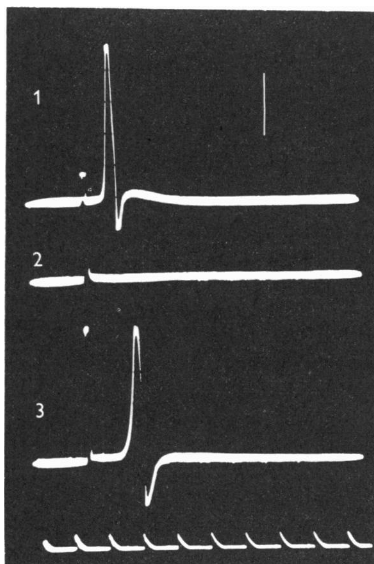

Fig. 1

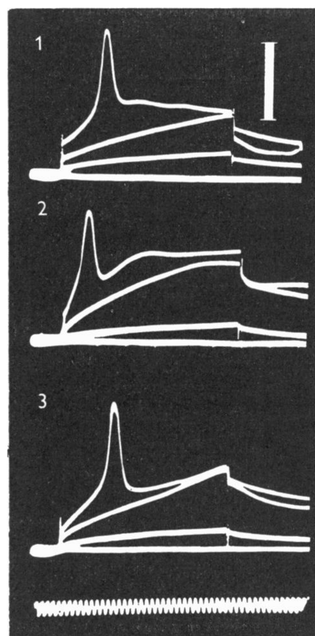

Fig. 2

Fig. 1. Action potentials recorded extracellularly from isolated single muscle fibres in Ringer's solution (1), 10 min after soaking in sucrose solution (2), and (3) 5 min after immersion in 112 mM hydrazine solution. Calibration, 5 mV; time marker, 1 msec.

Fig. 2. Intracellular action potentials of muscle fibres recorded in 112 mM hydrazine solution of which pH was adjusted with HNO_3 (1), H_2SO_4 (2), and HCl (3). Calibration, 40 mV, time marker, 1 msec.

solution. Figure 3 shows the relationship between the peak potential of spikes (active membrane potential) and the external concentration of hydrazine. This result was obtained from a muscle preparation which was first immersed in a 112 mM hydrazine solution for 30 min and then successively immersed in solutions containing 112, 70, 50 and 30 mM hydrazine for 30–40 min, respectively. The active membrane potential decreased according to the decrease of the external hydrazine concentration; the

slope being practically identical with that obtained by the same experimental procedure with Ringer's solution, where the external sodium concentration was altered. In both the sodium and hydrazine solutions the reduction of the concentration to one-tenth resulted in a decrease of the spike height of approximately 58 mV (the value of RT/F at room temperature). A similar relation was observed in muscle preparations which

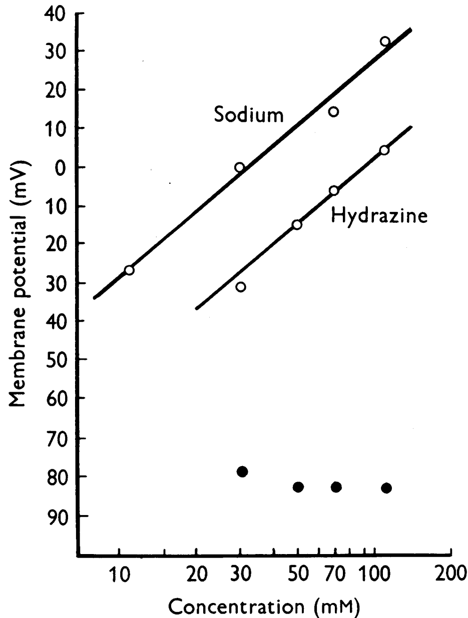

Fig. 3. Relation between active membrane potential and external concentration of sodium and hydrazinium ions obtained from two different preparations according to experimental procedure described in text. ○, average value of active membrane potential obtained from six different muscle fibres of a preparation. ●, mean value of resting potential obtained from six different preparations soaked in hydrazine solutions (for details see text). Semi-log. scale.

were directly immersed in various concentrations of hydrazine for 60–90 min without previous soaking in 112 mM hydrazine. These experimental results were in contrast with those obtained from spinal ganglion cells, where no appreciable change occurred in the potential height with variation of the external hydrazine concentration (Koketsu *et al.* 1959*b*).

Figure 4 shows the action potentials obtained in different concentrations of hydrazine. These records demonstrate that (1) the rate of rise of the action potential is apparently increased according to the increase of concentration and (2) the threshold for the initiation of the action potential and the negative after-potential increases as the external concentration decreases.

Effects of different concentrations of calcium ions

The resting potential of muscle fibres fell slightly with a decrease of the external calcium concentration and increased slightly in raised concentrations. The variation of the values observed in 80 mM hydrazine solutions containing 0·3–32 mM calcium ions was within ± 10 mV. When

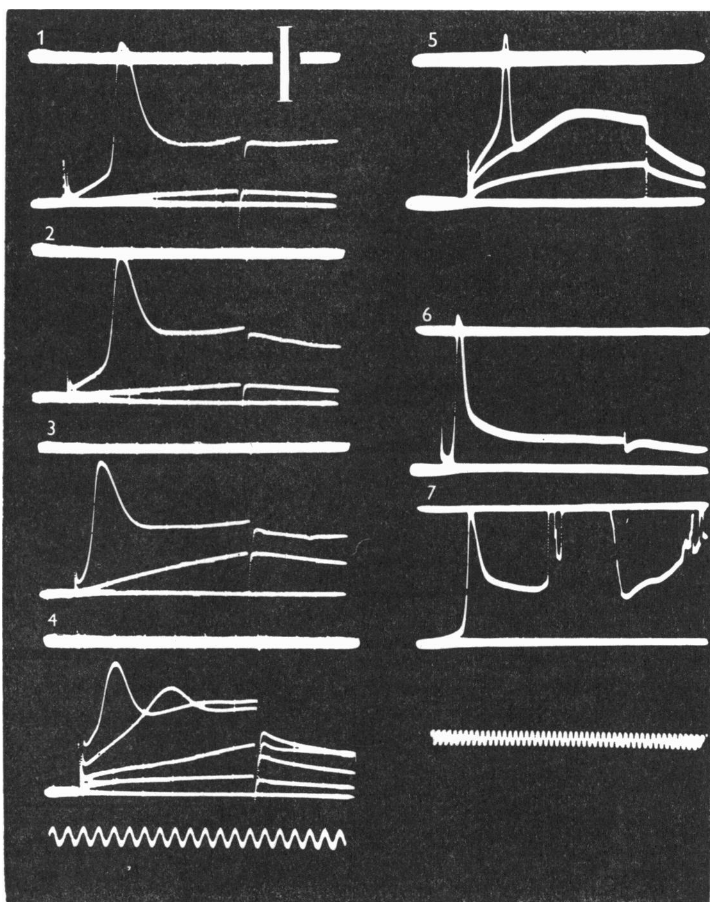

Fig. 4. Intracellular action potentials of muscle fibres soaked in solutions containing various concentrations of hydrazine. 1–4 are records contributing to Fig. 3, obtained in 112, 70, 50, and 30 mM hydrazine, respectively. Record 5 is obtained in hypertonic hydrazine solution (224 mM). Records 6 and 7 are conducted action potentials obtained in 224 and 90 mM hydrazine solutions, respectively (note rising phase, spike duration, and negative after-potential). The distance of recording and stimulating micro-electrodes inserted into a single fibre was approximately 0·5 (record 5), 1·0 (records 1–4), and 5 mm (records 6 and 7). Calibration, 40 mV, time marker, 1 msec.

the calcium concentration was reduced to 0·3 mM or increased to 9 mM, there was only a slight decrease of the threshold in lower concentrations and a slight increase in higher concentrations. Action potentials of the all-or-none type were no longer detectable when the calcium concentration was increased to 19 mM and most of the fibres then produced only small graded responses.

Effect of withdrawal of calcium ions

In frog spinal ganglion cells the excitability of cells was found to be eliminated when the calcium was completely withdrawn from the sodium-free chlorine, tetra-ethylammonium (TEA), or hydrazine solution. In the case of frog sartorius muscles the results were not as clear as those on spinal ganglion cells. When the muscle preparations, which were previously soaked in a sucrose solution for 30–120 min, were immersed in a Ca-free hydrazine solution, the value of the resting potentials of the individual fibres was found to vary. Some fibres had relatively high resting potential (70–80 mV), while other cells showed lower potentials (50–60 mV); the average value obtained from twenty-two fibres of a preparation was 71 mV. None of these fibres twitched spontaneously, which was generally observed in Ca-free Ringer's solution. The fibre having relatively high resting potentials (70–80 mV) produced action potentials, but many of them were of low or graded amplitude. The fibres with relatively low resting potentials (50–60 mV) did not produce action potentials, although some of these gave graded responses.

If the preparation was soaked previously in a calcium-free sucrose solution for 30–60 min, all fibres had low resting potentials (40–60 mV) and completely lost their electrical activity in the Ca-free hydrazine solution. The excitability of these preparations, however, could be restored upon reimmersion in the hydrazine solution containing Ca.

Intracellular injection

Muscle. Attempts to produce action potentials of muscle fibres by means of intracellular injections of hydrazinium ions were unsuccessful. Repeated injections of hydrazinium, as well as of TEA, did not induce any sign of restoration of the electrical activity in the sucrose, choline, or TEA solutions.

Spinal ganglion cell. The situation in spinal ganglion cells was quite different. Action potentials of cells soaked in sucrose solution were easily produced by intracellular injections of TEA (Koketsu *et al.* 1959*a*), hydrazine (Koketsu *et al.* 1959*b*), choline and acetylcholine. In every case local responses appeared initially and were followed by full spike potentials. The duration of these action potentials was gradually

prolonged by continuing the injections. Spontaneous activity, however, could not be observed even after repeated injections, indicating that these nitrogenous substances cannot activate the membrane without electrical stimuli.

It might be argued that the intracellularly injected substances might have diffused out across the membrane and in essence were acting extracellularly. In order to test this possibility, a KCl-filled electrode was inserted into a cell and hydrazinium ions were applied electrophoretically on the immediate outside of this particular cell by positioning a hydrazine-filled electrode close to the membrane surface. This procedure yielded negative results. Repeated intracellular injections of sodium ions did not restore the excitability of cells immersed in sucrose solution. It is also worth while to note that intracellular injections of hydrazine did not restore the excitability of cells which were immersed in a Ca-free sucrose solution.

After-effects of prolonged immersion

Muscle. Crustacean muscle fibres maintained their electrical activity in sodium-free solutions after previous immersion in a solution of quaternary ammonium ions (Fatt & Katz, 1953; Fatt & Ginsborg, 1958). This finding suggests that the ions which diffuse into the fibres during immersion are responsible for maintaining activity in sodium-free solutions. The same kind of experimental procedure was applied to the frog muscle to find whether it could maintain electrical activity in sodium-free sucrose solution after prolonged immersion in hydrazine solution. The electrical activity of the muscle was abolished within a minute when the preparations were placed in sucrose solution after prolonged previous immersion (up to 20 hr at 2–3° C) in a 112 mM hydrazine solution. The activity of these preparations was again restored within a minute by reimmersing in hydrazine solution, which suggests that the external hydrazine, rather than the internal, is important for the maintenance of electrical activity.

Spinal ganglion cells. The excitability of spinal ganglion cells could not be maintained for an extended time in 80–112 mM hydrazine solutions; the resting potential tended to drop gradually and many of the cells lost their excitability when they were soaked in these solutions for more than approximately 2 hr. This made it difficult to test the after-effect of an extended soaking. The excitability of cells was usually eliminated within 30–60 min in sucrose solution, but apparently maintained for a longer time than if they were previously soaked in a hydrazine solution for 1–2 hr (see Fig. 5).

Such an after-effect was more clearly demonstrated by using TEA solution. If the spinal ganglia were previously immersed in TEA

solution for a certain period (4–20 hr.), the excitability of cells was well maintained and produced potentials for several hours in sucrose solution (see Fig. 5).

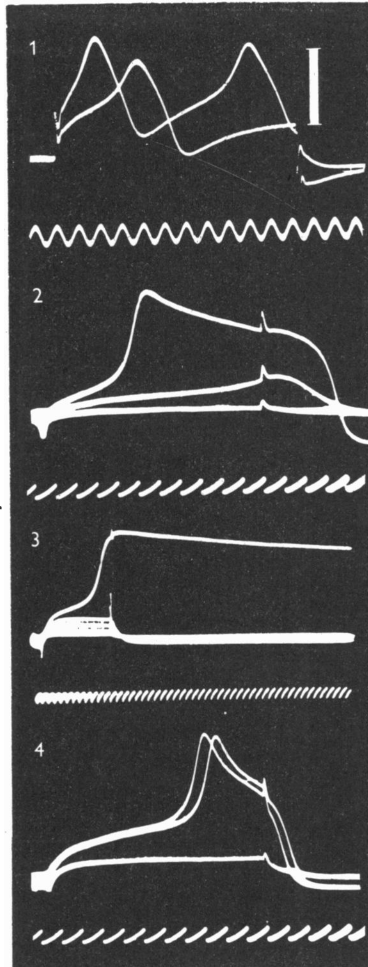

Fig. 5. Intracellular action potentials of spinal ganglion cells soaked in sucrose solution after previous immersion in hydrazine (1) and TEA (2–4) solutions. Record 1 obtained 90 min after soaking in sucrose (2 hr previous immersion in 112 mM hydrazine at room temperature). Record 2 obtained 2·5 hr after soaking in sucrose (4 hr previous immersion in 112 mM TEA at room temperature). Records 3 and 4 obtained 30 min and 5 hr after soaking in sucrose (20 hr previous immersion in 112 mM TEA at 2° C). Calibration, 40 mV, time marker, 1 msec.

DISCUSSION

According to the ionic hypothesis (Hodgkin, 1951), the action potential of nerve impulses is generated by the influx of the sodium ions which shifts the resting membrane potential toward the sodium equilibrium potential. It must be that trace amounts of sodium ions are retained in the intracellular space or immediately outside the membrane, even when the preparations are soaked in sodium-free solutions for an extended time. Thus, it might be argued that these sodium ions are still responsible for the production of action potentials in sodium-free media. This possibility, however, seems to be inconceivable, since, first, the action potentials produced in hydrazine solutions are too large to be explained in this way, secondly, the electrical activity of isolated single muscle fibres is maintained indefinitely in hydrazine solution, and finally, the electrical activity of the muscle is easily eliminated in sodium-free sucrose or choline solutions.

If the excitatory process in hydrazine solution is essentially identical with that in normal Ringer's solution, it would be expected that the hydrazinium ions are acting exactly in the same manner as sodium ions. A significant relationship between the concentration of the external sodium ions and the spike height of the action potential has been found in various excitable tissues, which, supports the sodium hypothesis (Hodgkin & Katz, 1949; Nastuk & Hodgkin, 1950; Huxley & Stampfli, 1951). A similar result was observed in muscle fibres immersed in hydrazine solution, although the spike height in hydrazine was smaller than that in sodium. Comparable results were obtained from frog nerve axons soaked in a guanidinium solution (Lüttgau, 1958). It may be possible to introduce a hypothesis involving such factors as the equilibrium potential, the permeability constant or even a pumping mechanism of hydrazinium ions to explain the role of hydrazine in the maintenance of membrane excitability. It also seems feasible, however, to assume that hydrazinium ions are acting in a manner which cannot be explained by the sodium hypothesis.

Entrance of hydrazinium ions could not be held responsible for active depolarization if intracellular injections of hydrazinium ions are necessary to produce action potentials in sucrose solution. In ganglion cells intracellular hydrazine appears to be essential for the restoration of electrical activity. The possibility that hydrazinium ions injected into the cell body diffuse out and act extracellularly may be eliminated for the following three reasons: (1) an external hydrazine concentration less than 80 mM could not produce full-spike potentials (Koketsu *et al.* 1959*b*); (2) external application of hydrazinium ions immediately outside the membrane did not produce action potentials; and (3) action potentials were not produced

by intracellular sodium injections in sucrose solution. No explanation is available at this moment why no action potentials were produced by intracellular injections of hydrazinium ions into the muscle fibres. The results of the present experiments rather suggest that the external hydrazinium ions are essential for the production of action potentials. Two possibilities may be considered: (1) hydrazinium ions are acting as charge carriers, probably like sodium ions, in the muscle but not in spinal ganglion cells; and (2) hydrazinium ions can exert their specific effect externally on the muscle membrane although internally on the cell membrane.

There seems to be a reduction of the membrane resistance during the action potential in sodium-free solutions (Koketsu *et al.* 1959*a*). If the increase of conductance is due to an increased permeability of the membrane to certain kinds of ions, the active depolarization may be explained either by an increased influx of external cations or an efflux of internal anions. Calcium ions are among the external cations which must be considered in this case. Fatt & Ginsborg (1958) attempted to explain the electrical activity of muscle fibres of crayfish in sodium-free media on the basis that the substituted ammonium compounds increased the permeability of the active membrane to calcium ions. In the present experiment, however, no appreciable relationship was found between the amplitude, duration or maximum rate of rise of the action potentials and the calcium concentration in the hydrazine solution, suggesting that calcium is not acting as charge carrier for the generation of action potentials. A similar result was also observed in the nerve axon in sodium-free guanidinium solution (Lüttgau, 1958). The presence of calcium ions, however, seems to be indispensable for the restoration of electrical activity in muscle fibres as well as in spinal ganglion cells (Koketsu *et al.* 1959*b*) in sodium-free solutions. The importance of external calcium for the electrical activity in sodium-containing solutions has been reported for the squid giant axon (Frankenhauser & Hodgkin, 1957) and frog motor axon (Frankenhauser, 1957), but the mechanism of calcium action in the excitatory process is still obscure.

An important question which remains is whether the excitatory process in sodium-free solutions is essentially identical with that in normal Ringer's solution. It is conceivable that the various nitrogenous compounds substituted for sodium exert some specific action on the tissues and produce action potentials whose mechanism is fundamentally different from the normal excitatory process.

SUMMARY

1. The electrical activity of isolated single muscle fibres can be maintained in a sodium-free hydrazine solution.

2. External chloride ions can be replaced by nitrate or sulphate ions for maintaining excitability of muscle fibres in a sodium-free hydrazine solution.

3. Calcium ions are essential for maintaining excitability in a sodium-free hydrazine solution. They did not seem, however, to be acting as charge carriers during active membrane depolarization.

4. The relationship between the active membrane potential and the external hydrazine concentration indicates that a reduction to one-tenth of the external hydrazine concentration results in approximately 58 mV decrease of the spike height.

5. Intracellular injections of hydrazine into the muscle fibres did not restore excitability. There was, however, strong experimental evidence indicating that the intracellularly injected hydrazine was responsible for maintaining the excitability of spinal ganglion cells.

6. No experimental evidence for an essential role of intracellular hydrazine was found in muscles. Results rather suggest that external application of hydrazine is important for the maintenance of muscle excitability.

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