ABSENCE OF SECRETION OF ACETYLCHOLINE ON STIMULATION OF NERVES IN AN UNSTRIATED MUSCLE

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The views about chemical transmission at neuromuscular junctions are well known. It is generally believed that such transmission takes place by secretion of acetylcholine, adrenaline or noradrenaline. This idea of neurohumoral transmission has appeared unsatisfactory to some and acetylcholine has been assigned other roles besides its possible action at neuromuscular junctions. According to Nachmansohn (1955), the mechanisms of conduction and transmission are essentially similar. The rhythmic activity of the heart is in some way linked with the enzymatic synthesis of acetylcholine (Burn, 1950). Jullien, Ripplinger and Cardot (1954) believe that acetylcholine is just a product of metabolism which must be removed. They believe that acetylcholine has no motor function.

During estimations of acetylcholine in the stomach muscle of the frog, *Rana tigrina*, it was noticed that though the muscle contained considerable quantities of acetylcholine, none was detected in the eserinised saline in which the muscles were immersed and oxygenated at room temperature. It was therefore decided to investigate the liberation of acetylcholine in this muscle.

Methods

These experiments were performed on the stomach muscle of the frog *Rana tigrina*. The animal was stunned with a blow on the head and rapidly pithed. The stomach was removed along with its nerves and its mucous membrane was gently removed avoiding any injury as far as possible. It was then aerated for 15 mintues in ordinary Ringer's solution and then in eserinised Ringer's solution for two hours. The muscles were removed and the acetylcholine extracted. The Ringer's solution in which the muscles were immersed was tested for acetylcholine.

For testing the effect of stimulation, the muscle along with its nerves was laid in a petri dish on a pair of electrodes and stimulated with strong induction shocks. It was then immersed in 12 c.c. of eserinised Ringer's solution in a beaker and aerated for one hour. Any fluid sticking to the petri dish was washed into the beaker. The aeration was done to allow any acetylcholine liberated to diffuse into the Ringer's solution.

To test the effect of potassium, the muscle was aerated as above and then immersed in a strong solution of potassium chloride, 0·15 M, for 10 minutes and then quickly washed to remove the excess of potassium chloride sticking to the muscle, so that it may not affect the frog's rectus or leech muscle, and then aerated for one hour in eserinised Ringer's solution. Such a strong solution of potassium chloride is a powerful stimulant of the muscle. The Ringer's solution was then tested for acetylcholine.

The effect of calcium was similarly tested, the muscle being immersed in 0·1 M calcium chloride for 15 minutes.

Acetylcholine in the muscle was estimated, using the frog's rectus by the method similar to that used by Richter and Crossland (1945) for brain tissue. After experimentation, the muscle was immediately transferred to a stoppered bottle kept in a freezing mixture, and was allowed to freeze. Eserinised Ringer's solution was prepared and brought to pH 4 by adding N/10 HCl. Twenty c.c. of this was heated to a temperature of 95–100° C. in a water-bath and the weighed muscle transferred to it. It was cut into very small pieces while it was kept at this temperature for 5 minutes. The mixture was decanted, allowed to cool and then centrifuged for 10 minutes. The deposit was stirred in a few c.c. of pH 4 eserinised Ringer's solution and centrifuged again. The supernatant fluid was collected, brought to pH 4, and kept in a refrigerator.

Before testing, the extract was brought to pH 7 to 7·4 with N/10 NaOH. The extract was further diluted with eserinised Ringer's solution, so that 1 c.c. of the extract was equivalent to 100 mg. of the tissue. The assay of acetylcholine was carried out on eserinised frog rectus and sensitive leech preparation against solutions of known acetylcholine concentrations, avoiding errors of sensitisation as suggested by Feldberg (1948).

RESULTS

The acetylcholine content of 10 muscles respectively was 5·9, 5·2, 5·1, 5·3, 4·7, 4·6, 6·7, 5·2 and 4·2 μg./g. of tissue. This is less than that found in mammalian smooth muscle. All of this was intracellular, as shown by immersion in Ringer's solution,

Effect of immersion in Ringer's solution.—In 20 experiments, muscles weighing 2·5–3 g. were immersed and aerated in eserinised Ringer's solution for 2 hours. No acetylcholine could be detected in the solution. This is in agreement with results of Chujyo (1952), who found that no acetylcholine diffused out of guinea pig's intestines under similar conditions. If the muscles were stretched, still there was no liberation of acetylcholine. The frog's stomach muscle thus differs in this respect from guinea pig's intestines, which liberate acetylcholine when stretched (Chujyo, 1952).

Effect of electrical stimulation.—In 20 experiments stimulation of the muscle and its nerves, with induction shocks, did not liberate any acetylcholine. The frog's stomach muscle becomes less excitable if its initial length decreases, and if it is aerated in saline, it gradually shortens to a constant length, and may become inexcitable to electric current. Under such conditions only nerves will be stimulated. Such differential stimulation did not result in the liberation of any acetylcholine, so that neither the stimulation of muscle nor its nerves liberate any acetylcholine.

Effect of depolarisation.—Excess of potassium is known to produce depolarisation of muscle or nerve membranes. In 16 experiments, strong solutions of potassium chloride, such as 0·15 M, failed to liberate any acetylcholine. Similarly in 16 muscles, strong solution of calcium chloride, such as 0·1 M, failed to liberate any acetylcholine. Thus the membrane of the frog's stomach muscle appears to be very resistant, or acetylcholine is very strongly bound.

Effect of heating.—If the muscle, is heated to 50° C. then acetylcholine is liberated in traces in 1 hour. If heated to 60° C., then there is marked liberation of acetylcholine. This shows that acetylcholine comes out of the muscle cells only when the membrane is damaged. The contractile mechanism also undergoes change when heated to 50–60° C., as it relaxes actively (Singh and Singh, 1954). The contractile proteins are denatured and possibly they release the bound acetylcholine.

The extract of the muscle produced by heating to 60° C., causes frog's stomach muscle to contract and then relax (Fig. 1). The contraction is presumably due to acetylcholine and relaxation to adrenaline or noradrenaline. Frog's stomach muscle is quite sensitive to adrenaline; in sensitive muscles, 1 in 20 million produces appreciable relaxation. No relaxing substance was found in the solution surrounding the muscle after immersion for 2 hours on stimulation with induction shocks.

Acetylcholine was also liberated from the muscle if treated with eserinised Ringer to which some ether had been added. Change in osmotic pressure of the saline did not produce any liberation.

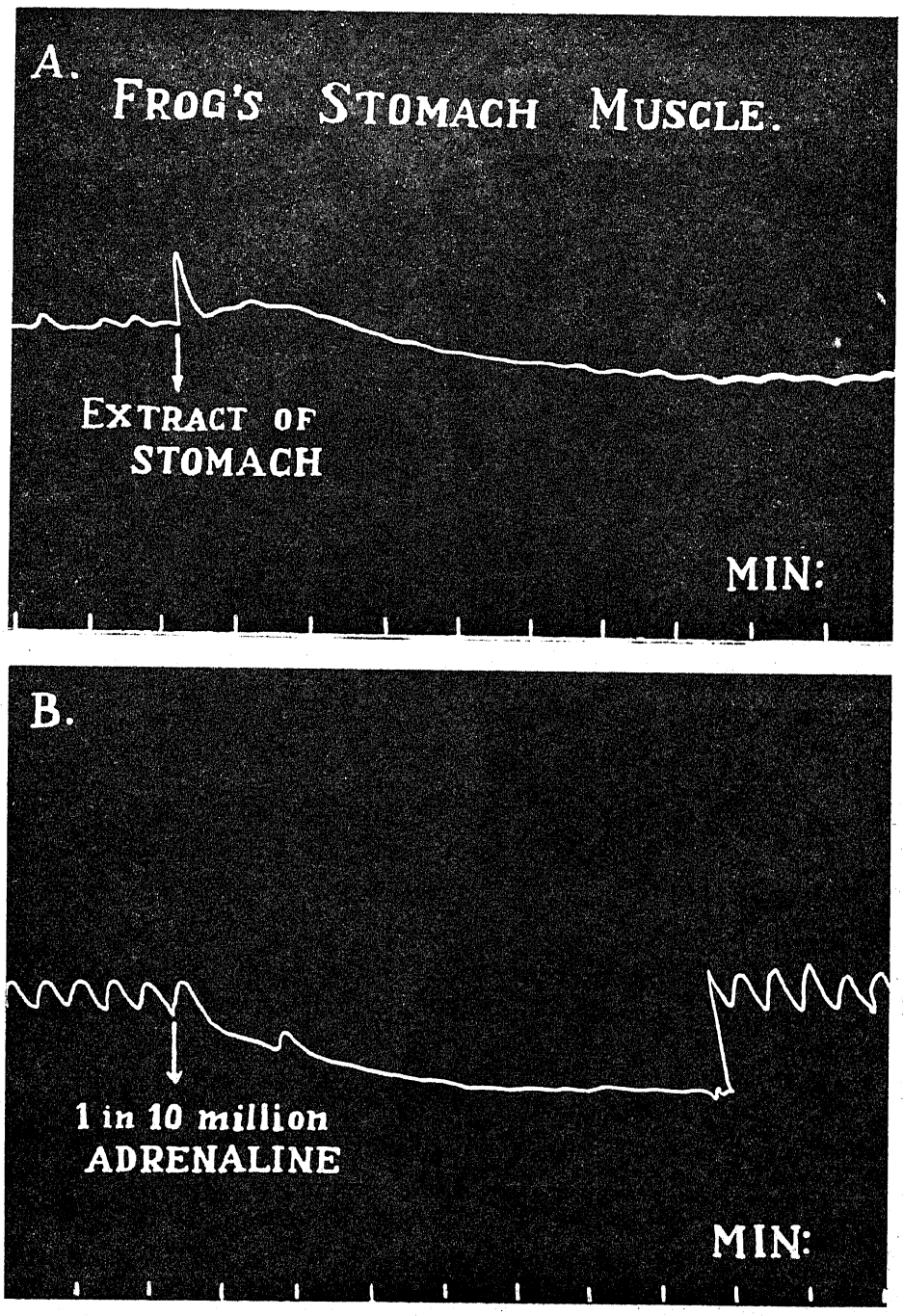

FIG. 1. Frog's stomach muscle. A. Effect of extract of stomach heated to 60° C. for one hour. B. Effect of 1 in 10 million adrenaline.

Just freezing the muscle for one hour and then thawing does not release any acetylcholine. This suggests that acetylcholine is not contained in the muscle fibres by the membrane, which would be destroyed on freezing and thawing. It must be then chemically bound and keeping temperature low, there is a chance of extracting the bound acetylecholine as such and knowing whether it is bound to the contractile mechanism.

The above experiments were performed on autumn frogs, room temperature 20–25° C. In winter the results were quite different (room temperature, 13–15° C.). At this temperature no acetylecholine diffused out of the muscle, but raising the temperature to even 20° C. caused liberation. This shows that if the muscle had acclimatised to low temperature, then acetylecholine is more easily liberated by a rise of temperature. Besides seasonal variations, individual variations may occur.

Discussion

The stomach muscle from *Rana tigrina* is remarkable in several ways. It reacts like a normal muscle and shows action potentials in the complete absence of any electrolyte in the external medium (Singh, and Bhatt, 1957; Singh and Acharya, 1957 *a*). It reacts in saline in which all the sodium had been replaced with potassium and can be excited in the absence of any ionic gradient of sodium or potassium across the membrane (Singh and Acharya, 1957 *b*). If glycerinated, it fails to react to adenosine triphosphate (Singh, 1958).

The acetylcholine in this muscle is intracellular as in nervous tissue and it is most likely that it has some important intracellular function to perform which may or may not have any relation with neuromuscular transmission. This intracellular function may be its chief function, and its leakage from the muscle cells into the surrounding fluid may be the " smoke coming out of the chimney ", and its role in neuromuscular transmission may be " the utilisation of a by-product".

If in muscle, these neurohormones are attached to the contractile mechanism, they may be playing an important role in contraction and relaxation. Acetylcholine appears to cause contraction and adrenaline, relaxation of the contractile mechanism (Singh and Singh, 1950). It is interesting to note that if the muscle is heated to 50–60° C., it relaxes actively, and acetylcholine leaks out. It might be that the dissociation of acetylcholine from the contractile mechanism leads to active relaxation, so that acetylcholine would be concerned with the contractility of muscle. This might explain the findings of Burn, that acetylcholine revives a heart which has stopped

beating, and of Chujyo (1952), who correlates the acetylcholine of intestines with peristalsis.

As this muscle contains two contractile systems, one of which relaxes passively, and the other actively (Singh and Acharya, 1958 *a*), it is possible that acetylcholine acts on the first system and adrenaline or noradrenaline on the second. One should therefore look for some complex between the actomyosin system and the neurohormones.

SUMMARY

1. Frog's stomach muscle when immersed in eserinised Ringer's solution for 2 hours shows no leakage of acetylcholine, whether extended or unextended.

2. Electrical stimulation of the muscle or its nerves does not release any acetylcholine.

3. Depolarisation with strong solutions of potassium chloride does not liberate acetylcholine.

4. Immersion in strong solutions of calcium chloride does not liberate acetylcholine.

5. Heating to 50–60° C., or treatment with ether liberates acetylcholine; the muscle also relaxes actively if heated. Adrenaline or noradrenaline is also liberated by heating.

6. As heating to 50–60° C. causes active relaxation as well as liberation of acetylcholine, it is suggested that acetylcholine is in some way connected with contractility by direct action on the contractile mechanism.

7. The above results were obtained in autumn, but in winter acetylecholine was liberated.

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