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On the Role of Calcium Ion in the Contractility of the Frog's Stomach Muscle

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That the calcium ion is an indispensable factor for the contraction of skeletal muscle was clearly shown by Heilbrunn & Wiercinski (15) in their experiment injecting the calcium- or magnesium ion into the cell. And, it it a well known fact that the heart is arrested in systolic state when the perfusing fluid contains sodium- and calcium-, and not potassium chloride (Ringer, 1883).

Recently, the relationship between the phenomena in the protoplasmic membrane and the contractile mechanism inside the cell (excitation-contraction coupling: abbr. E-C coupling) is one of the important researches in the field of muscular physiology; and the essential role of calcium ion in the E-C coupling has been recognized by many investigators. In the present paper, the contractility of stomach muscle was mainly examined in the physiological solution, in which the calcium or the potassium content was either decreased or increased.

METHODS

Almost all experiments were carried out on muscle-strip cut out from the pyloric portion of the stomach of bullfrog (*Rana catesbiana*). Toad (*Bufo vulgaris*) was used in some cases. There was no difference between the two in the results reported in this paper. The mucous layer of the excised strip was removed and the preparation mainly consisted of circular muscle. It was hung vertically in Ringer's fluid and the mechanical responses were registered on a kymograph by an isotonic lever with a minimum tension.

The Ringer's solution contained (in millimolar) 110 NaCl, 2.0 KCl, 3.4 $CaCl_2$ and 0.3 $NaHCO_3$ (pH $=$ 7). Composition of the other solution was described in an article of respective experiment. A response to acetylcholine was examined by changing the bath to that containing the drug. Afterwards, the preparation was washed 3~4 times with the previous solution in a beaker and the bathing was continued. The concentration of acetylcholine was 10^{-5} g/ml unless otherwise stated. It was dissolved in Ringer's solution, or in isosmotic NaCl solution in the experiment to deprive the calcium ion. Electric current was led to both

ends of the preparation by chlorided silver electrodes, when stimulation was needed. Outer solution was withdrawn previous to the current flow, so that the muscle was exposed to air during stimulation.

RESULTS

1. *Contractility in Ca-free solution.*

The well-known Ringer's experiment for determining the ionic composition of perfusing fluid (Ringer's solution) for an excised frog's heart was as follows: When the heart was perfused with isosmotic NaCl solution, the heart-beat became weaker and weaker and stopped finally. If the solution contained small amount of $CaCl_2$, the heart did not continue to beat but was arrested in systolic state. And, if the solution contained KCl it also stopped to beat and was ar-
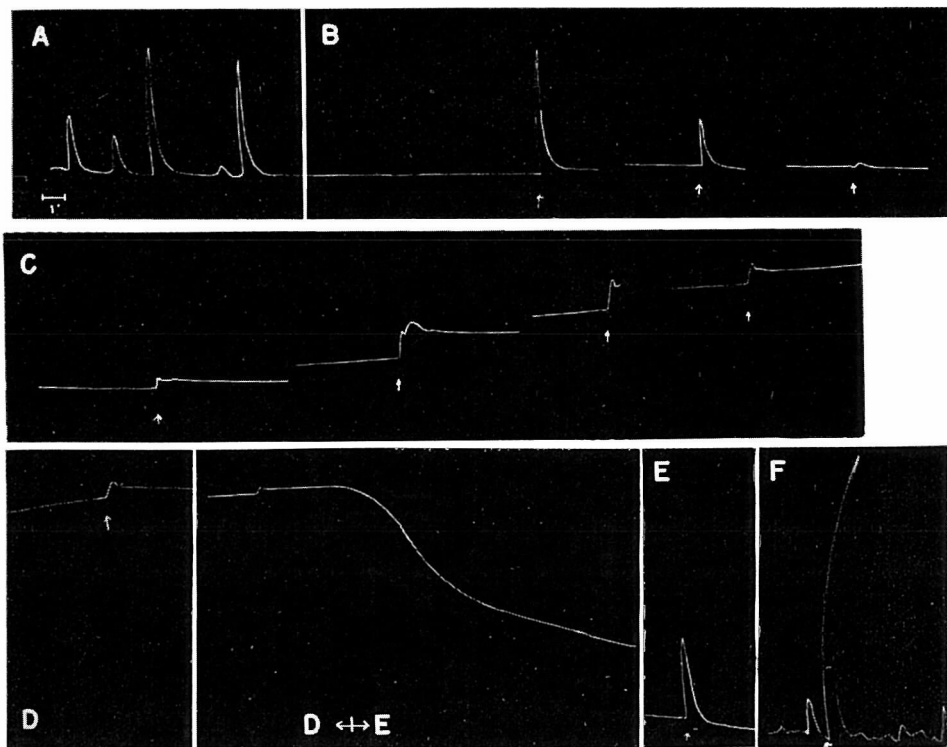

Fig. 1   Continuous record of the length and responses to acetylcholine with one preparation (muscle-strip of frog's stomach). The bath was successively changed as follows.

A: Ringer's solution. B: isosmotic sucrose. C: sucrose and 7 mM $CaCl_2$. D: isosmotic NaCl and 7 mM $CaCl_2$. E: NaCl and 5 mM KCl. F: Ringer's.

At the discontinuous parts of the curve, the recording was not carried out and the preparation was left in the respective bath for 10~20 minutes.

rested in diastolic state. In the case where the NaCl solution contained both CaCl₂ and KCl in adequate amounts, it continued to beat for a long time.

A similar experiment was carried out with the stomach muscle and represented in Fig. 1, which was a continuous record (A→F) with one preparation. The bath was changed, successively, from Ringer's (A) to isosmotic (230 mM) sucrose (B), isosmotic sucrose containing 7 mM CaCl₂ (C), 110 mM NaCl + 5 mM CaCl₂ (D), 110 mM NaCl + 5 mM KCl (E) and further to Ringer's solution (F). The length of the preparation and the response to acetylcholine were recorded in each solution.

The spontaneous movement could be recorded in Ringer's solution (Fig. 1-A). When the bath was changed to isosmotic (230 mM) sucrose solution, the movement disappeared and the responsibility to acetylcholine was gradually diminished (1-B). Calcium chloride was added to the sucrose solution) final concentration: 7 mM), and a shortening of the preparation and a reappearance of the responsibility could be observed (1-C). When the sucrose solution containing calcium was changed to 110 mM NaCl + 5 mM CaCl₂ solution, maximum shortening and the retained responsibility could be observed (1-D). After this shortening, the bath was changed to 110 mM NaCl solution containing 5 mM KCl. It relaxed gradually and attained the original length after 30 minutes. At this time, the responsibility to acetylcholine could be also recorded but it was not so remarkable as in Ringer's solution at the recovery (1-E & F).

In the experiment shown in Fig. 1, it was noted that the contractile response to acetylcholine was not retained in the Ca-free solution but it did, in the solution containing calcium ion. When the NaCl solution was used instead of sucrose, in another experiment, the results were the same. The recorded response in Fig. 1-E was due to the short time of soaking. It could not be recorded if the preparation was soaked for more than 1 hour. Concerning the spontaneous movement Bozler (5) reported that, although the movement of frog's muscle-strip disappeared in the isosmotic sucrose solution, it could be seen again when the calcium (0.5～2 mM; final concentration) was added or when the bath was changed to the NaCl solution. Those results, however, was rarely recognized in the present experiment.

Coutinho and Csapo (8) reported that the contractile tension produced by an application of oxytocin was decreased by washing the uterine strip of rabbit with Ca-free Ringer's solution. That the tension of twitch or contracture decreases in the Ca-free physiological solution have been reported by many investigators (1, 10, 13, 14, 25). On the other hand, since the publication of Huxley's experiments (17), it has been generally believed that the change of membrane potential is an essential step in setting off the contractile process. Indeed, Bülbring (7), Kuriyama & Csapo (23) and other authors reported a decrease of membrane potential (depolarization) in the Ca-free or -deficient me-

dium.

In the previous reports (26), however, the depolarization in Ca-free Ringer's solution (in mM, 110 NaCl, 2.8 KCl and 0.3 NaHCO$_3$) could not be observed in the stomach muscle. It was also observed that the membrane potential had increased in isosmotic sucrose solution and that the spontaneous movement or the contraction induced by acetylcholine had not accompanied the potential change of the membrane. Seemingly, there may exist another contractile mechanism which is alternative to membrane depolarization.

2. Contractility in K-rich solution

The responses to acetylcholine or electric stimulus were examined in the solution containing potassium in high concentration, and shown in Fig. 2. The used electric stimulus was D. C. having the strength of two times of rheobase and the duration of 3 seconds.
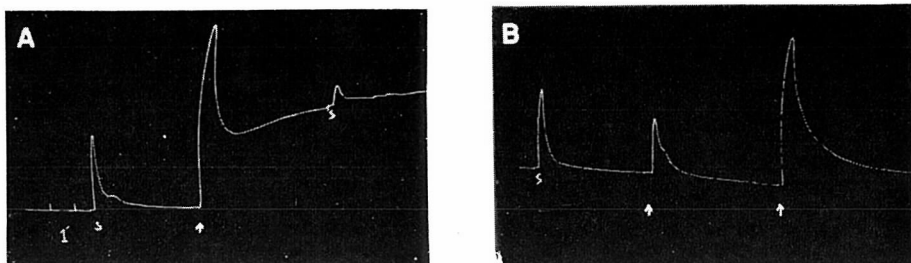

Fig. 2   Responses to electric stimulus (s), high-potassium or acetylcholine (arrow) in the presence (A) and absence (B) of calcium. The response to acetylcholine in B was obtained with a depolarized muscle. In detail, see in the text.

In Fig. 2–A, the response to the electric stimulus was firstly recorded in Ringer's solution and the bath was changed to the solution prepared by mixing the same amount of isosmotic (95 mM) K$_2$SO$_4$ and Ringer's solution. A remarkable contracture induced by high concentration of potassium could be observed. After relaxing to some extent, however, it slowly shortened again and maintained the shortening for more than 10 minutes. The responsibility to the electric stimulus was retained in this shortened state. Figure 2–A represented a similar record when the calcium was not contained in the bath. If the soaking time in Ca-free Ringer's solution was less than 1 hour, the preparation retained its responsibility to acetylcholine, as noted above. It also responded to the electric stimulus. At the first arrow in Fig. 2–B, the bath was changed to the isosmotic K$_2$SO$_4$ solution. Contrary to the finding in Fig. 2–A, the extent of relaxation after the contracture was enough and the shortening was not maintained. Acetylcholine was administered and washed off with the previous K$_2$SO$_4$ solution at the second arrow. The responsibility was also retained in this case.

A vast number of experiments have been reported showing that the proto-plasmic membrane of the nerve, skeletal-, cardiac- or smooth muscle is depola-rized in the K-rich solution.   This phenomenon was also recognized in the sto-mach muscle and reported previously (26).   It is noteworthy, in Fig. 2, that the muscle depolarized in K-rich solution retains its responsibility to acetylcholine or to electric stimulus and that the preparation maintains its shortening of con-tracture in the presence of calcium ion in outer solution but not, in the absence of it.

As a working hypothesis, Sandow (27) suggested in the skeletal muscle that an action potential or depolarization of muscle fibre permits or promotes the entrance of calcium ions into the fibre and that these ions then initiate the mechanical events.   The reports of Hodgkin & Keynes (16), Ishiko & Sato (18) and Bianchi & Shanes (2), showing the increased influx of calcium during elect-ric stimulation or potassium contracture, have supported the hypothesis.   The increased Ca-influx during the contraction induced by the administration of adrenaline was reported by Briggs & Melvin (6) in the aortic muscle of rabbit. The non-specific increase of the membrane permeability of the smooth muscle in the medium containing acetylcholine has been generally recognized (22).   It may be assumed that the influx of calcium is also increased by the administra-tion of acetylcholine, although the report clearly showing this phenomenon could not be seen in the literature (cf. 28).

On the other hand, Evans et al. (12) reported that the mammalian smooth muscle depolarized with potassium retains its ability to contract in response to acetylcholine or oxytocin.   Similar experiments were carried out by Edman & Schild (11), Csapo (9) and Sperelakis (29) with the same results.   From the facts that the contractile tension of the depolarized muscle had a linear rela-tionship to the logarithm of Ca-concentration in the bath and that the influx of calcium was also increased accompanying the response, they concluded that there exists also a contractile mechanism not mediated by the membrane depo-larization but by the calcium entering into the muscle-cell.

In short, it is likely that there is two kinds of contractile mechanism, one is mediated by the depolarization of the protoplasmic membrane and the other, is not.   The experiment in Fig. 2 would have a relation to the latter mecha-nism.

3.   *Two kinds of shortening in response to acetylcholine*

It is already known that there are at least two kinds of shortening in smooth muscle, which were designated by previous authors rapid or phasic contraction and slow or tonic contraction (4, 20, 29).   Recently, Bohr (3) observed these contractions in an aortic muscle of rabbit responding to adrenaline, and desig-nated fast and slow components.   Figure 3 represents the similar phenomena

obtained in the present material responding to acetylcholine.   They can also be seen in Fig. 1-D.

In Bohr's experiment, the extent of shortening of the fast component was smaller, and that of the slow one was greater in the bath containing higher concentration of calcium (range; 0.2~3.2 mM).   In our experiment, however, the results were somewhat different, as shown in Fig. 4.
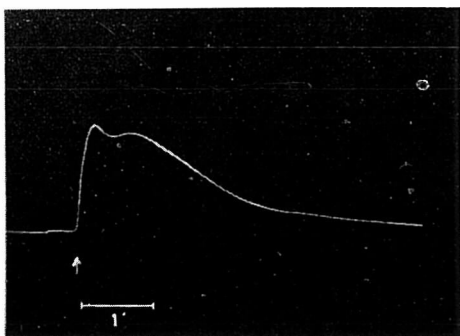

Fig. 3   Fast and slow components can be seen in a response to acetylcholine in Ringer's solution. (Stomach muscle-strip of bullfrog)
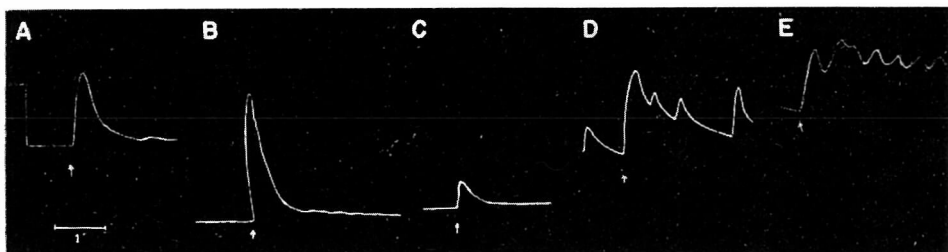

Fig. 4   Responses to acetylcholine.

Continuous record with one preparation.   The bath was successively changed as follows.   A: Ca-free Ringer's solution, after 30 min soaking.   B & C: 110 (mM) NaCl, for 50 and 80 min after A, respectively.   D: 110 NaCl and 0.5 $CaCl_2$, 10 min after C.   E: 110 NaCl and 6 $CaCl_2$.   Between D and E, the preparation was soaked in 110 NaCl solution containing 1.0, 2.5, 4.0 and 6.0 $CaCl_2$ for each 15~20 minutes.

Figure 4-A represented the response to acetylcholine after the preparation was soaked in Ca-free Ringer's solution for 30 min; 4-B and 4-C, in isosmotic NaCl solution for 50 and 80 min after recording 4-A, respectively.   The relaxation seen before the application of acetylcholine in 4-A and 4-B was caused, perhaps, by the small tension of isotonic lever.   The fast component in Fig. 4-B was greater than in 4-A.   It was thought that this phenomenon was not caused by the deprivation of calcium, but by the passively relaxed state of the preparation.   Because, the response-shortening in 4-B was greater than in 4-C and it was reasonable that the calcium deprivation was more complete in the latter.

The slow component could be seen a little, in Fig. 4–A. Perhaps, the calcium was not enough washed out at this time.

After the recording of Fig. 1–C, the bath was changed to isosmotic NaCl solution containing 0.5 mM $CaCl_2$ and the preparation was left for 10 minutes. It was shortened during this time to the first level of Fig. 1–D. Acetylcholine was administered and washed out at the arrow. The concentration of calcium was increased to 1.0, 2.5, 4.0 and 6.0 (mM) and the similar procedures were repeated with varying time-interval. Figure 4–E represented the final record, in which the preparation was soaked in 6 mM $CaCl_2$ and 110 mM NaCl solution for 20 min after the preceding record. It was noteworthy in Fig. 4–D that the distinct response to acetylcholine reappeared and that the shortening was maintained for a short time. Although each record was not illustrated in the figure, the preparation was more shortened and maintained the response-shortening for longer time in the bath containing higher concentration of calcium. Contrary to the Bohr's finding, the fast component was not decreased in high Ca-concentration. As to the similarity of the slow component with the maintained shortening, some considerations seemed to be necessary.

It is a custom to call the sustained state of shortening as "tone", when physiologically produced and maintained for relatively long time. The sustained contraction is often referred to as "contracture", when produced by the action of drugs or ions. Concerning smooth muscles, the two terms are used in an almost synonymous sense. If there is any difference in the nuance, it is highly subjective and we know nothing yet what differentiates two types of contractile mechanisms. Therfore, it seems necessary to take into consideration only two types of contraction; the one, fast or phasic, and the other, slow or tonic component (of shortening in isotonic recording: or of tension-development in isometric one).

The data presented here indicate that the fast and the slow components can also be distinguished in the smooth muscle of frog's stomach. Concerning the mechanisms, Bohr (*loc. cit.*) proposed an opinion that the former is mediated by the changes in the protoplasmic membrane and the latter is related directly to the contractile mechanism inside the cell. However, the fast component can be observed in the muscle depolarized by potassium (Fig. 2–B). The response to acetylcholine can not be obtained if the preparation is soaked in Ca-free medium for a long time (Fig. 1). It seems reasonable to consider that the entrance of calcium, and not the depolarization itself, is an essential factor for the fast component. The possibility, however, that the depolarization of the membrane permits the Ca-entrance can not be denied.

In the practical record, it is usual to observe that the fast and the slow components are involved, in different proportions, in the shortening caused by acetylcholine or high potassium concentration. The spontaneous movement

having a character of the fast component (rf. 21) can be observed in variously shortened state of the preparation. When it is previously relaxed in the Ca-free solution, the extent of maintained shortening is greater in the medium containing higher concetration of calcium ion. It is possible to postulate that the graded state of shortening has some relationship with the concentration of calcium existing within the muscle-cell.

In 1962, Johnson (19) and Lowy & Hanson (23) observed two kinds of filament in anterior byssus retractor muscle of *Mytilus*, with electronmicroscope. The former researcher considered that the one is actomyosin- and the other, paramyosin- filament; and the latters, that they are myosin- and actin-filament. Both agreed in an opinion that there is a mutual sliding of filaments in the case of contraction. Although the authors have no experimental data concerning the two filaments and the sliding phenomenon, it is tempting to speculate the contractile mechanism according to Johnson (*loc. cit.*) who offered the working hypothesis that there is some "catch mechanism" after the sliding. That is, the administration of acetylcholine permits or promotes the entrance of calcium ions which exist in the surrounding medium or in the protoplasmic membrane, into the cell. The calcium-entry causes the sliding of contractile filament, or the phasic shortening. After the sliding, the shortening is maintained (catched) in that state by yet uncleared mechanism, in which calcium ion would also play an important role. In the case where the catch mechanism is not activated, the slow component of shortening would not be resulted.

4. *Addendum*

Ethylenediamine-tetraacetic acid (EDTA) are often used in order to deprive the calcium ion from the bathing solution. This procedure, however, causes the decrease of pH. For example, when it was added, in 0.1~0.5 mM final concentration, to Ca-free Ringer poorly buffered with sodium bicarbonate, the pH was decreased from 7.3 to 3.7~3.9. The response of the present material diminished or disappeared when the pH of Ringer's solution was less than 5.0.

SUMMARY

1. The isotonic contraction (shortening) of the muscle-strip from the pyloric portion of frog's stomach was investigated in the physiological solution, in which the calcium or the potassium content was either decreased or increased.

2. When the calcium ion was deprived from the bathing solution, the contractile response to acetylcholine could not be observed after one hour or more. Generally, the preparation relaxed gradually to a full extent in the calcium-free solution. If the bath was changed to that containing the calcium, it shortened again and the responsibility to acetylcholine reappeared.

3. The contractile response to acetylcholine or to electric stimulus could be observed with the depolarized muscle in a potassium-rich solution. The shortening of contracture induced by high-potassium was maintained in Ca-containing solution but was not, in Ca-free one.

4. In the contractile response to acetylcholine, two kinds of shortening, a fast component and a slow component, could be distinguished in different proportions. The slow component disappeared much earlier than the fast one in a Ca-free solution. There was no finding to differentiate the former and the maintained shortening or contracture.

5. Concerning the role of calcium ion in the contractile mechanism, it was discussed as a working hypothesis that the entrance of calcium ion from the extracellular fluid or the protoplasmic membrane into the cell would be promoted by the administration of acetylcholine, and that the maintained state of shortening would have a relationship with the concentration of calcium existing inside the muscle fibre.

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