ROLES OF EXTERNAL IONS IN THE EXCITATION-CONTRACTION COUPLING OF FROG SKELETAL MUSCLE

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Since Kahn & Sandow (1950) reported the potentiating effect of NO₃, substituted for Cl in Ringer's solution, on the twitch tension of frog striated muscle without changing the spike potential contour, studies on the coupling process between excitation and contraction have been developed. Lubin (1957) reported that SCN, substituted for Cl, increased the negative after-potential as well as the peak tension, and suggested that this prolonged action potential might play a significant role in the increase in twitch tension. According to Hutter & Padsha (1959), this prolongation was based upon the higher resistance of the muscle membrane due to anion substitution. And it was suggested by Hutter & Noble (1960) that chloride removal might result in increases of both the membrane resistance and twitch tension, although the time scales of them were quite different.

On the other hand, some cations, for example tetraethylammonium (TEA), substituted for Na, showed a similar effect as the above described anion substitution. However, Hagiwara & Watanabe (1955) stated that no significant change in resting membrane resistance was observed under 75% TEA substitution. Furthermore, the important role of Ca or Ca-entry in the excitation-contraction coupling has also been emphasized by Frank (1960), Bianchi & Shanes (1959), Lorkovic (1962) and many other workers.

In the present study, the effects of replacement or concentration change of a constituent ion of Ringer's solution on the tension and action potential were investigated, on the purpose of examining the properties of excitation-contraction link and how each ion in Ringer's solution contributes to the link.

METHODS

The material used was the sartorius muscle of the frog (Rana nigromaculata). The monophasic action potential and twitch tension were recorded simultaneously by the

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partition method. The main set-up of vertical type partition method was the same as described in the previous paper (MASHIMA et al., 1962). The partition method in horizontal type shown in Fig. 1 was also employed in this study, the results obtained did not differ from each other in both types. Tension curve and action potential were recorded simultaneously with a two-gun oscilloscope, the sweep speed of each beam was adjustable independently. Stimulus was supramaximal square pulse with 0.5-1 msec duration, and the anode was always applied to the tibial end of the muscle.

FIG. 1. Arrangement of the partition method in horizontal type.

A, earth plate; Amp₁, DC-amplifier; Amp₂, strain-gauge amplifier; B, stimulating electrode; C, copper-phosphor plate; E, Ag-AgCl lead-off electrode; P, 0.06% procaine-Ringer's solution; R, Ringer's solution; SG, strain-gauge; ST, stimulus isolator. The cross section at the region of the partition is shown in right.

The Ringer's solution contained NaCl, 110 mM; NaHCO₃, 12 mM; KCl, 2 mM; CaCl₂, 1.8 mM; and pH was 7.0. The neuromuscular transmission was always blocked by 6×10⁻⁶ g/dl D-tubocurarine (Amerizol) added to Ringer's solution. The ion substitution was made, keeping the osmotic pressure as close to Ringer's solution as possible.

RESULTS

I. Effects of Na removal

a) Substitution of choline for Na. The monophasic action potential and twitch tension of sartorius muscle were recorded simultaneously by the partition method. Fig. 2, A is the control record obtained in Ringer's solution. When 1/2 of Na was replaced by choline, the spike height of action potential decreased and the rate of rising phase as well as the falling phase became slightly slower, while the increase in twitch tension was obvious (Fig. 2, B). At 1/4 Na concentration, however, not only the spike height but also the twitch tension were diminished, but the latter was still as high as the control (Fig. 2, C). Below 1/6 Na, the spike height decreased gradually, but the twitch tension did more rapidly. At 1/8 Na, no tension was developed at all. The recovery was almost complete when the muscle was returned into Ringer's solution (Fig. 2, D). In Fig. 3, the relative size of the spike height to twitch tension were plotted against Na concentration in logarithmic scale.
Fig. 2. The effect of choline substitution for Na on the action potential (upper curve) and twitch tension (lower curve). A, control; relative concentration of Na was 1 in A, 1/2 in B, 1/4 in C; D, recovery.

Fig. 3. The effect of Na removal on the relative size of spike height and twitch tension. A, twitch tension in choline substitution; B, theoretical line by Nastuk & Hodgkin; C, twitch tension in TEA substitution; D, spike height in TEA substitution. S, spike height; T, twitch tension.

Except below 1/4 Na concentration, the course of decrease in the spike height showed a good coincidence with the line B in Fig. 3, which was the result of Nastuk & Hodgkin (1950) with the intracellular electrode. On the other hand, the twitch tension increased and showed the maximum at 1/2 Na concentration.
After all, none of parallel relation between the spike height and twitch tension was observed.

According to Mashima et al. (1962), the coupling efficiency (CE) was defined as follows:

\[
CE = \frac{\text{relative size of twitch tension } (P/P_0)}{\text{relative size of spike height } (A/A_0)}
\]

Where \( P \) or \( P_0 \) is the peak tension in test or Ringer's solution, and \( A \) or \( A_0 \) is the spike height in test or Ringer's solution respectively. The CE values in substitution of choline for Na are shown in Table 1. In all cases, CE is larger than 1, that means an improvement of coupling efficiency. However, where the relative concentration of Na is less than 1/4, \( P/P_0 \) is less than 1. In these concentrations, it is difficult to distinguish whether this is really or looks like an improvement.

### Table 1.

The coupling efficiency in choline solution, substituted for Na.

<table>
<thead>
<tr>
<th>relative concentration of Na</th>
<th>( P/P_0 )</th>
<th>( A/A_0 )</th>
<th>CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3/4</td>
<td>1.17</td>
<td>0.93</td>
<td>1.24</td>
</tr>
<tr>
<td>1/2</td>
<td>1.29</td>
<td>0.83</td>
<td>1.52</td>
</tr>
<tr>
<td>1/4</td>
<td>0.94</td>
<td>0.67</td>
<td>1.43</td>
</tr>
<tr>
<td>1/6</td>
<td>0.38</td>
<td>0.33</td>
<td>1.15</td>
</tr>
</tbody>
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b) Substitution of sucrose for NaCl. When NaCl was replaced by osmotically equivalent amount of sucrose, the similar effects on the relative size of the spike height and twitch tension were observed as in the case of choline substitution (Fig. 3). From this fact, the specific effect of choline should not be essential for the twitch augmentation and the Na removal is considered to be the main cause for it.

c) Substitution of Li for Na. When Li was substituted for Na, the spike height was almost unchanged until Na concentration was reduced to 1/8 (Fig. 3). The twitch tension also showed only little change, but it was assured that at 3/4 Na concentration the tension clearly increased by about 10%. Then, it was concluded that Li could fill the place of Na very well but not completely, as far as the effect on the excitation-contraction coupling is concerned.

d) Substitution of TEA for Na. Sodium in Ringer's solution was replaced by TEA. Sometimes repetitive firing to a single stimulus was observed at more than 25 mEq (milliequivalent per litre) of TEA, but in many cases single twitch response was obtained. Fig. 4 shows simultaneously recorded action potential and twitch tension by the partition method at various concentrations of TEA. With concentration of TEA, the spike height decreased but the spike
duration and the negative after-potential increased (Fig. 4, B to E). These changes were observed apparently at 7 mEq of TEA, but at over 84 mEq they increased no more. The recovery from TEA solution was fairly good (Fig. 4, F).

The relations between the spike height or twitch tension and the concentration of TEA, which is represented by the relative concentration of Na, are shown in Fig. 3. The spike height decreased with TEA concentration in a straight line (Fig. 3, D), which was slightly over the line B. However, the twitch tension was potentiated very much (Fig. 3, C), and it was far larger than that of choline substitution. It is unreasonable to assume that such a slight difference in spike height can lead the great difference in twitch tension.

EDWARDS et al. (1956) and HUTTER & NOBLE (1960) have suggested that the duration of action potential could be related to the contraction height. In many cases it was difficult to measure the duration of action potential precisely, because the falling phase of spike potential transfered smoothly into the after-potential. Therefore, the duration at the 40% of spike height (D40) was measured. Assuming from the data with microelectrode that the membrane potential and overshoot potential were $-90\,\text{mV}$ and $+35\,\text{mV}$ respectively, 40% of spike height represents the level of $-40\,\text{mV}$. According to HODGKIN & HOROWICZ (1960a), the tension of single muscle fibre reached the maximum when the membrane potential was depolarized to the level of $-40\,\text{mV}$ by potassium. Then, D40 may represent the effective duration of necessary and sufficient depolarization for the maximum contraction. The D40 was 2.8-
3.2 msec in control and 9.9-11.7 msec in 84 mEq of TEA. These values were slightly different from those of single fibre with microelectrode, but the relative prolongation of D40 by TEA was of the same order of single fibre. In Fig. 10, the relative twitch tension was plotted against the relative D40 at various concentrations of TEA. The relation was not linear. Within 200% prolongation of D40, the tension increased with D40, but over 200% it was saturated.

These results suggest that not only the spike height but also the duration of action potential is not responsible directly for the potentiation in twitch tension. Rather, TEA substituted for Na has two different effects besides the effect of Na removal; one is a prolongation of action potential at the membrane level and the other is a potentiation of tension probably at the level of excitation-contraction coupling.

II. Effects of K concentration. According to JENERICK & GERARD (1953), the muscle lost its excitability when excess KCl more than 10 mM was added and the resting potential was depolarized beyond the critical level of −57 mV. In the present study, the twitch became undetectable between 9 mEq and 10 mEq of external K concentration. And it was observed that at less than 9 mEq of K the twitch increased slightly (Fig. 5, A), despite the fall in spike height (Fig. 5, B), due to the reduced membrane potential.

![Fig. 5. The effect of excess K on the spike height and twitch tension. A, twitch tension; B, spike height; C, twitch tension in the medium containing 9 mEq of Ca.](image)

III. Effects of Ca concentration. To prevent the Ca precipitation, NaHCO₃ was removed from Ringer's solution. Therefore, the control solution in this experi-
ment contained NaCl, 120 mM; CaCl₂, 1.8 mM; KCl, 2 mM. Many investigators have shown the potentiating effect of Ca on the tension in potassium contracture, but it was rather peculiar that, in the non-depolarized muscle, the twitch tension produced by a single electrical shock was hardly affected by external Ca concentration between 0 and 5.4 mEq. When excess CaCl₂ was added to Ringer's solution, the spike potential was potentiated between 1.8 mM and 50 mM, but it was depressed and slowed down at 72 mM, while the twitch tension was depressed with the increase in Ca concentration (Fig. 6). The depression in the twitch was not based upon the hypertonicity by excess CaCl₂, because none of such depression was observed when 54 mM of NaCl was added, instead of 36 mM of CaCl₂. But when 108 mM of NaCl was added, instead of 72 mM of CaCl₂, the depressions of the same order were observed both in the spike height and tension. Therefore, the differential action of hypertonic solution on the twitch and action potential (HODGKIN & HOROWICZ, 1957) could not be neglected at that high concentration. Furthermore, when 54 mM of NaCl was reduced and 36 mM of CaCl₂ was added, keeping osmotic constancy, the spike height was depressed, despite a larger twitch than before the reduction (Fig. 6). When 108 mM of NaCl was reduced and 72 mM of CaCl₂ was added, the spike height decreased down to about 35% of the control and almost no tension

![Graph](image-url)
was developed. These facts are quite understandable, considering the summed effect of Ca addition and Na removal.

On the other hand, in the depolarized muscle, the potentiation of twitch by excess Ca was obviously demonstrated. When the muscle was depolarized by 10 mEq of K, no tension was detectable, but under the existence of 9 mEq of Ca, a larger twitch than the control was observed (Fig. 5). Consequently, the curve A in Fig. 5 shifted to the right (Fig. 5, C). This fact coincides with the result reported by CSAPO (1960), which was obtained about the maximum tetanic tension produced by a.c. stimulation. From these observations it was concluded that external Ca could potentiate the tension under some amount of depolarization. However, when the muscle was immersed into Ca-free Ringer's solution, a fibrillary movement began to occur and lasted for about 20 min. But the muscle could still show a twitch response of more than 90% of the

![Figure 7](image)

**Fig. 7.** The effect of Ca-free medium on the maintenance of tetanus tension. A, control; B, in Ca-free solution; C, recovery. Stimulating frequency was 50 c/sec.

![Figure 8](image)

**Fig. 8.** The effect of Cl removal on the action potential (upper curve) and twitch tension (lower curve). From A to E, NO₃ was substituted for Cl. NO₃ concentration was 0 in A, 28 mEq in B, 56 mEq in C, 84 mEq in D, 112 mEq in E. From F to J, SCN was substituted for Cl. SCN concentration was 0 in F, 7 mEq in G, 14 mEq in H, 28 mEq in I, 56 mEq in J.
control, and no significant change was observed in the course of twitch height decrease during repetitive stimulation at 2 c/sec. Only the difference was the maintenance of tetanic tension, namely the tension curve during 50 c/sec stimulation fell down far quickly in Ca-free solution (Fig. 7). Unless a long lasting depolarization existed, low Ca in the medium could hardly affect the contraction. The muscle seems to reserve a sufficient amount of Ca on or within the fibre, and a very brief depolarization during single action potential may not be sufficient to let the muscle lose the Ca, although the depolarization might facilitate the in- and/or outflux of Ca (WINEGRAD & SHANES, 1962; LORKOVIĆ, 1962). Eventually, according to FRANK (1960), Ca was bound so tightly on the membrane that the muscle lost its contractility after a long pre-soaking in Ca-free medium containing EDTA.

**IV. Effects of Cl removal.** Chloride in Ringer's solution was replaced by the anomalous anion, such as NO₃, I or SCN. The negative after-potential was

![Graph](image-url)

**Fig. 9.** The effect of Cl removal on the relative size of twitch tension. A, NO₃; B, I; C, SCN; D, SO₄ was substituted for Cl respectively.
prolonged and the twitch tension was greatly potentiated (Fig. 8), coincided with the observation by LUBIN (1957), using the intracellular electrode. The coupling efficiency was remarkably improved at 28 mEq of SCN and reached three times larger than the control at 28 mEq of SCN or at 84 mEq of NO₃. In Fig. 9, the relative size of twitch tension was plotted against the Cl concentration. The potentiation of tension occurred in the order of lyotropic series, namely SCN > I > NO₃.

Besides these anomalous anions, SO₄ was also substituted for Cl. In this case, the standard SO₄-solution contained Na₂SO₄, 95 mM; NaHCO₃, 12 mM; KCl, 2 mM; CaCl₂, 1.8 mM. Substitution was made by mixing Ringer’s solution with the standard SO₄-solution. The osmotic pressure of the standard SO₄-solution was supposed to be isotonic (ADRIAN, 1956). In some cases, especially in winter, the concentration of CaCl₂ was raised up to 9 mM to stop the fibrillary movement. The influences of the hypertonicity, if any, or excess Ca are not so serious, because of the above results. Sulphate is known as an impermeable ion for membrane, but it potentiated the peak tension in similar way as anomalous anions. This fact suggests that not only the specific action of these anions on the membrane or inside of it but also the Cl removal is an essential factor for the twitch potentiation. Of course, the quantitative difference of SCN-curve (Fig. 9, C) from SO₄-curve (Fig. 9, D) should depend upon the specific action of SCN.

**Fig. 10.** The relation between the effective duration of depolarization during action potential (D₄₀) and the twitch tension. A, NO₃, I or SO₄ was substituted for Cl, almost linear relation was obtained. B, substitution of SCN for Cl; substitution of TEA for Na.
Another possibility for the twitch potentiation is in the prolonged negative after-potential produced by anomalous anions. In Fig. 10, the peak tension was plotted against D40. As for several ions, such as NO₃, I and SO₄, the longer is the D40, the larger becomes the peak tension and there is a linear relation between them. But this line (A, in Fig. 10) did not coincided with the curve of SCN or TEA substitution (B and C in Fig. 10). Consequently, as a whole, it was impossible to deduce a quantitative relation between the duration of depolarization and the peak tension of twitch. The prolonged negative after-potential may play a part for the potentiation of twitch tension, but the amount of it depends upon the specific action of each ion, which is superposed on the general effect of Cl removal.

DISCUSSION

In the previous paper (MASHIMA et al., 1962), the action potential obtained by the partition method was compared with that of the intracellular recording, and it was assured that the former showed a good coincidence with the latter, as far as the relative size of spike height or duration was concerned.

When Na in Ringer's solution was replaced by choline, the excitation-contraction coupling in muscle was improved. Although SANDOW (1952) stated that the twitch tension did not alter until 75% deficiency of Na, it was increased clearly under 50% deficiency of Na. In heart muscle, the effect of Na deficiency on peak tension was more obvious (LÜTTGAU & NIEDERGERKE, 1958), but in the skeletal muscle, only little effect has been pointed out by HODGKIN & HOROWICZ (1960a), as to the potassium contracture.

EDWARDS et al. (1956) suggested that the mechanism of the twitch potentiation in choline solution would be based upon the specific action of choline. But the similar increase in peak tension was observed by substituting sucrose for NaCl. SCHAECHTELIN (1961) described that Na depletion directly produced a contracture, and the potassium induced contracture was augmented by 24% even when Li was substituted for Na. Then it is more likely, the absence of Na in the external solution is favorable for the excitation-contraction coupling of skeletal muscle.

When Na was replaced by TEA, a prolonged spike potential followed by a large negative after-potential, as well as a far larger tension than in choline-Ringer's solution, were observed. As far as TEA is concerned, the specific actions on both the membrane and the excitation-contraction coupling can not be neglected, and it might be superposed upon the general effect of Na deficiency.

When the external K concentration was raised up to 9 mEq, the twitch tension increased slightly, despite the decrease in spike height. In these concentrations the inactivation of Na-carrier caused by depolarization (OOGAMA &
WRIGHT, 1962) could be neglected. Can excess potassium activate the excitation-contraction coupling or can it activate the contractility of skeletal muscle? According to HODGKIN & HOROWICZ (1960a), the maximum tension in potassium contracture was 10% higher than that of electrical stimulation, because the mean depolarization in the former was about twice deeper than in the latter. Of course, this fact does not mean that the depolarization by excess potassium can potentiate the contractility. The maximum tetanic tension elicited by electrical stimulation in excess potassium never exceeded over the value in normal Ringer's solution (CSAPO, 1959; SUZUKI, 1959). Therefore, it is more likely that the slight increase of twitch tension in excess potassium is based upon an improved excitation-contraction coupling. As suggested by HODGKIN & HOROWICZ (1960a), the depolarization may be favorable to produce some substance, which is necessary for contraction and would be destructed quickly.

LORKović (1962) stated that the height and duration of potassium contracture or the Ca-entry during the contracture was a function of external Ca concentration. And it was emphasized by BIANCHI & SHANES (1959) and WINEGRAD & SHANES (1962) that Ca influx was an essential factor for the excitation-contraction coupling in the heart muscle, and that additional Ca would be derived from readily accessible Ca-bounding sites on the muscle fibre. All these observations indicate that a higher concentration of Ca in the medium or some amount of depolarization accelerate the Ca-entry, and the Ca-entry is favorable for the excitation-contraction coupling. At higher than 5.4 mEq of Ca, however, the twitch was depressed, coincided with the report by PAUL (1960), while the spike height was potentiated. According to ISHIKO & SATO (1957), excess Ca led to a hyperpolarization of muscle membrane. Then it is possible to consider that both low Ca in the medium and a hyperpolarization by excess Ca would result in a reduced Ca-entry.

LÜTTGAU & NIEDERGERKE (1958) reported the antagonism of Na against Ca for the contraction height of the cardiac muscle. As for the skeletal muscle, the interaction of both ions was not simple. Ca removal did not affect the twitch height but Na removal potentiated it. Excess Ca depressed the twitch but excess Na did not affect it below 72 mEq. It is plausible that Na and Ca enter the membrane through a common path, disturbing each other. Then, Na removal may open the path for Ca-entry and excess Ca may close the path for Na. Eventually, excess Ca caused a hyperpolarization by reducing the Na-permeability of membrane (ISHIKO & SATO, 1957; TRAUTWEIN & KASSEBAUM, 1961). Reversely, Ca removal resulted in a high excitability of membrane by increased Na-permeability. BRECHT et al. (1961) reported the interesting observations that the muscle pre-soaked in 18 mEq of Ca showed a contracture without action potential by isotonic KCl solution, but that the muscle pre-soaked in Ca-free solution showed a tetanic contraction accompanied by action potentials. Strong depolarization overcame the reduced Na-permeability by
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By the substitution of anomalous anion for Cl or the substitution of TEA for Na, the duration of spike potential was prolonged and the negative after-potential was increased. However, the quantitative relation between the twitch tension and the effective duration of action potential, D40, differed from ion to ion, probably the strength of effect partly depends upon the size of ion (ARAKI et al. 1961). After all, the action potential contour is not a crucial factor for the amount of tension. If the mechanical threshold was lowered by these anions (HUTTER & PADSHA, 1959) by about 10%, then the effective duration of depolarization, D30, could be estimated as long as sufficient for the mechanical response. But, more likely, as suggested by HODGKIN & HOROWICZ (1960b) and FRANK (1961), that more tension was produced with the same amount of depolarization after the anion substitution; in other words, the coupling efficiency was improved by anomalous anions. And the prolongation of active state duration may have an intimate relationship with the improvement of coupling efficiency (RITCHIE, 1954; LUBIN, 1957), even in the case of TEA substitution (EDWARDS et al., 1956).

It is noticeable that an impermeable anion, such as SO4, could also increase the peak tension. Chloride removal may primarily improve the coupling efficiency and the specific action of these anions might be superposed on it. According to HUTTER & NOBLE (1960), impermeable anions had the effect to increase both the membrane resistance and the duration of active state. From these observations it is evident that the absence of Cl and/or Na improved the excitation-contraction coupling, and the specific effect of substituted ion was superposed on it, probably, the specific action on the Ca-entry. High membrane resistance may not be a direct cause of this improvement, rather, it would be a result of reduced permeability of membrane for Na and/or Cl.

SUMMARY

1. The effects of replacement or concentration change of a constituent ion of Ringer's solution on the twitch tension and action potential of frog sartorius muscle were investigated.
2. The Na removal within the limit of 75% improved the excitation-contraction coupling.
3. The excess K facilitated the excitation-contraction coupling under the limit of 9 mEq.
4. In the depolarized muscle, the excess Ca facilitated the coupling, but in the non-depolarized muscle, the twitch tension was depressed by the excess Ca, while the spike height increased.
5. In Ca-free Ringer's solution, none of significant change in mechanical response was observed at single or low frequency stimulation. But the
maintenance tension at high frequency stimulation fell down far quickly than the control.

6. The Cl removal improved the excitation-contraction coupling.

7. TEA, substituted for Na, and anomalous anions, substituted for Cl, prolonged the duration of spike potential and increased the negative after-potential as well as the twitch tension. The relation between electrical change and mechanical response, however, was not uniform. Because the specific effect of each ion was superposed on the general effect of Na and/or Cl removal.

8. The significance of Ca-entry to the excitation-contraction coupling as related to the depolarization and Na-permeability, and the dual (general and specific) effects of ion substitution were discussed.

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REFERENCES


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