INTRODUCTION

It is now generally accepted that an increase in the intracellular concentration of ionized calcium, closely associated with membrane excitation, is the essential factor for initiation of contraction in various muscles. In fast skeletal muscles such as the frog sartorius, the sarcoplasmic reticulum has been considered as a chief source of supply of the ionized calcium. However in smooth muscles the intracellular membranous structures are poorly developed, therefore the possibility is considered by many authors that the calcium necessary for contraction may be derived from an extracellular space or surface membrane.

The anterior byssal retractor muscle (ABRM) of Mytilus, a kind of lamellibranch smooth muscle, is capable of two types of contraction; a tonic contraction, so-called 'catch' in response to a direct current stimulation or acetylcholine, and a phasic one in response to an alternating current or repetitive stimulation. Potassium contracture is also the phasic type. It has been reported that the external calcium concentration has no selective influence on catch, but the tension development is dependent on the external calcium concentration.

TWAROG (1967) has studied the electrophysiological nature of Mytilus ABRM by intracellular microelectrodes and observed that the action potential is not affected by tetrodotoxin (10^{-4} g/ml). We have observed also that potassium contracture of ABRM is reduced by Mn (10 mM). These facts suggest that the excitation of ABRM may be accompanied by a Ca-spike and that the tension development of ABRM may be based on the same mechanism as other smooth muscles.

It is therefore of interest to examine the efflux and influx of Ca at rest and during potassium contracture of Mytilus ABRM and to compare the

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Eiichi HAGIWARA AND Torao NAGAI

Department of Physiology, Sapporo Medical College, S. 1, W. 17, Japan

45CA MOVEMENTS AT REST AND DURING POTASSIUM CONTRACTURE IN MYTILUS ABRM
results with those obtained in various other muscles.

METHODS

*Mytilus coruscus* Gould was obtained from Oshoro, Hokkaido and kept in an open tank at 5°C with filtered-aerated sea water. The intact whole muscle of ABRM with shell was prepared according to Jou et al (1967) and mounted on ⁴⁵Ca experimental chamber.

⁴⁵Ca experimental chamber

The diagram of the chamber is shown in Fig. 1-a. This was made from a water repellant plastic to minimize the adsorption of a quantity of the tracer. The chamber was set on a magnetic stirrer and the bathing solution was stirred constantly. (Fig. 1-b). The exchange of solutions was performed by sucking out the medium with a stream pump and putting a new solution into the chamber with a pipette.

⁴⁵Ca efflux

The whole muscle mounted on the chamber was equilibrated with normal artificial sea water for an hour, then was loaded for 4 hours in 4 ml of radioactive solution containing ⁴⁵Ca at a concentration of about 500,000 CPM/ml. The ⁴⁵Ca solution was changed every 30 min. After loading the muscle was rinsed with non-radioactive solution for 15 sec (to strip off loosely adherent radioactive solution from the muscle surface) and then washed successively with 3 ml of the non-radioactive solution at first for 5 min, then at 10 min intervals for a total of 120 min. After the washout for 120 min, the washout solution was changed to 320 mM K artificial sea water and the muscle was soaked at frequent intervals (2, 8, 10, 10, 10, 10, 10, 10 min) for a total of 60 min. The washout solutions were collected at the end of each period and dried on planchets.

After washout the muscle was removed from the chamber and the portions, without being soaked in radioactive solution, were cut off. The muscle piece soaked in radioactive solution was blotted on a filter paper and weighed on a balance. The tissue weighed was ashed in an electric furnace for 16 hours at 600°C. The residue was taken up in 2 ml of 0.1 N HCl and dried on planchets. The radioactivity of ⁴⁵Ca of wash samples and aliquots of the residue was obtained by counting in a Nuclear Chicago gas flow counter with window.

⁴⁵Ca desaturation curves were obtained from a summation of the ⁴⁵Ca contents of the collection and ⁴⁵Ca remaining in the muscles at the end of 180 min.

⁴⁵Ca uptake

Resting uptake was measured by exposing the muscle to a radioactive solution for 30 min (see the results), then washing out the muscle in 3 ml of non-radioactive solution at frequent intervals (5, 10, 25, and 50 min), for a total of 90 min. By this washout, the ⁴⁵Ca in the extracellular space and on the superficial binding sites of the fiber surface and in the connective tissue could be removed routinely. After washout, the muscle was weighed, ashed and the residue was taken in 0.1 N HCl. The radioactivity of ⁴⁵Ca of the aliquot was measured.

During the last 2 min in the loading of 30 min, the experimental condition of 320 mM K solution was introduced for one of a pair of muscles. After loading the muscle was washed for 90 min, ashed and ⁴⁵Ca content was measured as mentioned above.

All experiments were performed at room temperature of 18 to 25°C.

Composition of solutions

The normal artificial sea water was of the following composition; NaCl 450 mM, KCl 10 mM, CaCl₂ 10 mM, MgSO₄ 51 mM, buffered to pH 7.2 at 20°C by Tris HCl 50 mM. 320 mM K solution was made by replacing 320 mM NaCl by corresponding KCl.
Fig. 1. Diagrams of $^{45}\text{Ca}$ experimental chamber (a) and set of $^{45}\text{Ca}$ experiment (b).

Fig. 1-a.

Exp. solution

Shell

Cotton soaked in vaseline

Bar

Suction

Magnetic stirrer

Fig. 1-b.
RESULTS

1. $^{45}$Ca efflux

The desaturation curve for ABRM is plotted on a semilogarithmic scale (Fig. 2). Each point is the average of 6 experiments (Table 1). The curves in normal artificial sea water are composed of at least two exponentials; after an initial period of rapid loss the rate of emergence of the isotope becomes much slower and the slope of the curve becomes essentially constant. The fast component has exponential time constant ($\tau_f$) of about 6 min and that of the slow component ($\tau_s$) is 108 min (S.E. 10 min). The former may be regarded as extracellular origin and the latter as derived from within the fibers.

When the washout solution is changed to 320 mM K artificial sea water after 120 min, the increase in $^{45}$Ca loss from the fibers is evident. In this case the rate of the loss during first 10 min is apparently larger than that

![Fig. 2. $^{45}$Ca desaturation curve of Mytilus ABRM. ○: in normal artificial sea water. ●: in 320 mM K artificial sea water](image-url)
of following periods as shown in Fig. 2. The approximate exponential time constant of the former (\( \tau_{k1} \)) is 19 min (S. E 2 min), whereas that of the latter (\( \tau_{k2} \)) is 46 min (S. E. 3 min).

TWAROG (1954) has observed that the isotonic K induced contracture of ABRM is distinctly phasic and that relaxation is completed within 10 to 20 min. We have confirmed also that 320 mM K induced contracture of muscle bundle (200 to 500 \( \mu \) in diameter) of ABRM is phasic and the relaxation is completed within 2 to 5 min. Therefore it may be regarded that \( \tau_{k1} \) is the exponential time constant during potassium contracture and that \( \tau_{k2} \) is that of depolarized and relaxed ABRM. Within the limits of these results the Ca efflux during potassium contracture increases about 6 times that of the resting muscle.

The amount of \(^{45}\text{Ca} \) taken up during exposure to radioactive solution is calculated by adding the CPM lost during 180 min washout period and the CPM remaining in the muscle at the end of the washout. \(^{45}\text{Ca} \) space is calculated by dividing the total CPM/g wet wt. muscle by the CPM/ml of radioactive solution. In 6 experiments the total \(^{45}\text{Ca} \) space averages 0.421 (S. E 0.04) ml/g. Since the zerotime intercept of slow component averages 19.2\%, fast and slow compartment sizes are 0.340 ml/g, and 0.081 ml/g, respectively.

In this paper the fast compartment size of 0.34 ml/g is regarded as the extracellular space of Mytilus ABRM and used to estimate an intracellular calcium concentration. This value of the space is almost similar those of the mammalian smooth muscle\(^{18}\).

Since the calcium concentration of the radioactive solution is 10\( \mu \) mol/ml,

\[
\begin{array}{lcccccc}
\text{TABLE 1.} \\
\text{Desaturation curve characteristics of each efflux experiments.} \\
\hline
 & \text{Total }^{45}\text{Ca} & \text{Zero time} & \text{ }^{45}\text{Ca} \text{ uptake} & \tau_f & \tau_{k1} & \tau_{k2} \\
 & \text{Space (ml/g)} & \text{intercept} & \text{ (}^{45}\text{Ca} \text{ mol/g)} & \text{ (min)} & \text{ (min)} & \text{ (min)} \\
A & 0.487 & 20 & 0.974 & 91 & 14 & 49 \\
B & 0.316 & 21 & 0.664 & 127 & 24 & 56 \\
C & 0.358 & 18 & 0.644 & 111 & 23 & 38 \\
D & 0.400 & 20.5 & 0.820 & 75 & 20 & 40 \\
E & 0.564 & 17 & 0.958 & 134 & 13 & 49 \\
F & 0.402 & 19 & 0.764 & 108 & 22 & 42 \\
\text{Mean} & 0.421 & 19.2 & 0.806 & 108 & 19 & 46 \\
\text{(S. E.)} & \pm 0.040 & \pm 0.6 & \pm 0.063 & \pm 10 & \pm 2 & \pm 3 \\
\end{array}
\]
the amount of the calcium taken up by the fibers is $0.808\mu\text{mol/g}$. If the exponential time constant for $^{45}\text{Ca}$ uptake by the fibers equals that for the washout (108 min) the intracellular calcium exchange is incomplete in the 4 hours. Assuming a simple exponential uptake, the exchange and uptake in infinite time would have been theoretically $0.906\mu\text{mol/g}$ instead of $0.808\mu\text{mol/g}$. This may be regarded as the approximate amount of exchangeable calcium inside the fibers of ABRM. This figure is two to three times those of frog ventricle$^{19}$ and frog rectus abdominis$^{20}$.

2. $^{45}\text{Ca}$ uptake

The muscles loaded in radioactive solution for various periods were ashed without washing. The radioactivity of the residue was counted and the total $^{45}\text{Ca}$ space was calculated as described in the previous section. As illustrated in Fig. 3, $^{45}\text{Ca}$ is at first rapidly absorbed by the tissue but subsequent uptake is small and slow. It is difficult to analyze the curve quantitatively because of a large deviation of values, but the $^{45}\text{Ca}$ space after 25 to 30 min of exposure is about 80% of that found after 240 min. This suggests that the initial rapid phase originate in extracellular space and tissue surface and that a relatively small fraction moves into the fibers in the following slow phase.

A rough estimate of extracellular space of $0.35\text{ml/g}$ is obtained by ex-

![Fig. 3. $^{45}\text{Ca}$ uptake curve in Mytilus ABRM. Vertical bar=1×S.E. about the mean.](image-url)
trapolating the slow phase back to zerotime. This value is about the same as that obtained in efflux experiments. If this extrapolation is valuable, the extracellular space of ABRM may be equilibrated effectively within the first 30 min of exposure. Consequently in the following study, 45Ca uptake of resting ABRM exposed to radioactive solution for 30 min was measured and compared with that of the contracting one. After loading, the residual 45Ca space of the tissue, washed out for 90 min in non-radioactive solution, was estimated and the value was corrected to 45Ca space at the beginning of the washout by \(\tau_r\) and \(\tau_{kl}\).

In 7 resting muscles (Table 2) the 45Ca space after 90 min washout and the 45Ca space corrected to zerotime average 0.0076 (S. E. 0.0008) ml/g and 0.0175 (S. E. 0.0019) ml/g, respectively. Whereas in the experimental with value averages 0.0139 (S. E. 0.0018) ml/g and 0.0320 (S. E. 0.0240) ml/g, respectively. There is a difference of 0.0145 ml/g between the corrected values of the control and experimental, therefore the increase of 45Ca uptake during potassium contracture in 2 min is 0.145\(\mu\)mol/g wet wt. muscle. This figure roughly equals that of barnacle muscle fibers.21)

3. 45Ca kinetics

The feature of the results mentioned above is in the large exchangeability of Ca during potassium contracture of ABRM and the time course of the exchange is approximately exponential.

According to NIEDERGERKE22), the equation to be used in the calculation

| Table 2. |
| Comparison of Ca uptake as measured by 45Ca in paired Mytilus ABRM subjected to normal and 320 mM K artificial sea water. |

<table>
<thead>
<tr>
<th></th>
<th>Residual 45Ca space (ml/g)</th>
<th>Corrected 45Ca space (ml/g)</th>
<th>Ca uptake ((\mu)mol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.0055</td>
<td>0.0157</td>
<td>0.0127</td>
</tr>
<tr>
<td>b</td>
<td>0.0083</td>
<td>0.0098</td>
<td>0.0191</td>
</tr>
<tr>
<td>c</td>
<td>0.0104</td>
<td>0.0150</td>
<td>0.0239</td>
</tr>
<tr>
<td>d</td>
<td>0.0080</td>
<td>0.0170</td>
<td>0.0184</td>
</tr>
<tr>
<td>e</td>
<td>0.0043</td>
<td>0.0111</td>
<td>0.0098</td>
</tr>
<tr>
<td>f</td>
<td>0.0082</td>
<td>0.0142</td>
<td>0.0188</td>
</tr>
<tr>
<td>g</td>
<td>0.0088</td>
<td>0.0146</td>
<td>0.0202</td>
</tr>
<tr>
<td>Mean (\pm) (S. E)</td>
<td>0.0076 (\pm) 0.0139</td>
<td>0.0175 (\pm) 0.0320</td>
<td>0.175 (\pm) 0.320</td>
</tr>
</tbody>
</table>
of influx during activity is:

\[ m_{in} = \frac{[^{45}Ca]_i}{\tau_s(1 - \exp(-t/\tau_s))} \]  

(1)

where \( m_{in} \) is influx/fiber volume, \([^{45}Ca]_i\), the \(^{45}\)Ca gain during activity, \( \tau_s \), the exponential time constant of release during activity. Applying the equation (1) to calculate the influx during potassium contracture of Mytilus ABRM and regarding \([^{45}Ca]_i\) as 0.145 \(\mu\)mol/g, the increased amount of \(^{45}\)Ca taken up during 2 min of potassium contracture and as 19 min, \( \tau_{kt} \) obtained in efflux experiments, the influx during activity should be 0.0759 \(\mu\)mol/g min.

Considering the magnitude of efflux during activity, this can be calculated by the following equation:

\[ m_{out} = \frac{[Ca]_i}{\tau_s} \]  

(2)

where \( m_{out} \) is efflux/fiber volume, \([Ca]_i\), the concentration of exchangeable Ca inside the fiber. Approximate estimate of \([Ca]_i\) is 0.906 \(\mu\)mol/g, so that the efflux during activity is 0.0477 \(\mu\)mol/g min.

In the resting state the fibers are close to a steady state in radioactive solution, hence the approximate resting influx and efflux are given by

\[ m_{in} = m_{out} = \frac{[Ca]_i}{\tau_r} \]

where \( \tau_r \) is the exponential time constant at rest given in TABLE 1. The resting fluxes are estimated to be 0.0083 \(\mu\)mol/g min.

To express these figures as concentration/1 ABRM fibers, the results were divided by the factor 0.612, which was given by \((1/d-e)\), where \( d \), the density of the ABRM has been assumed to be 1.05 (HILL, 1931), \( e \), the extracellular space taken equal to be 0.34 ml/g obtained in efflux experiments.

### TABLE 3.

Ca fluxes of Mytilus ABRM at rest and during activity. \( M_{in} \) and \( M_{out} \) values were calculated from the conversion factor of 0.612 ml/g which was given by \((1/d-e)\), where \( d \), the density of the ABRM has been assumed to be 1.05 (HILL, 1931), \( e \), the extracellular space taken equal to be 0.34 ml/g (see the text), and of surface/volume ratio of 8000 cm\(^{-1}\) calculated by assuming the cell diameter as 5 (HEUMANN and ZEBE, 1968).

<table>
<thead>
<tr>
<th></th>
<th>Influx</th>
<th>Efﬂux</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( m_{in} ) ( (\mu)mol/g. min)</td>
<td>( M_{in} ) ( (p)-mol/cm(^2) ) sec</td>
</tr>
<tr>
<td>At rest</td>
<td>0.0083</td>
<td>0.0282</td>
</tr>
<tr>
<td>During activity</td>
<td>0.0759</td>
<td>0.2584</td>
</tr>
</tbody>
</table>
Furthermore to obtain fluxes/fiber surface, the values thus obtained were divided by surface/volume ratio of 8000 cm$^{-1}$ calculated by assuming the cell diameter as 5$\mu$ (HEUMANN and ZEBE, 1968). All these results are summarized in Table 3.

The resting fluxes are 0.0282 p-mol/cm$^2$. sec. During potassium contracture the influx increases to 0.2584 p-mol/cm$^2$. sec and the efflux to 0.1623 p-mol/cm$^2$. sec. These are about nine- and sixfold increases in comparison with those at rest, respectively.

**DISCUSSION**

The main feature of the present work is that Ca influx and efflux markedly increase during potassium contracture of Mytilus ABRM. As shown in Table 3 $^{45}$Ca influx during contracture is nine times larger than that at rest. The efflux increases to about six-fold that at rest.

Resting flux of 0.0282 p-mol/cm$^2$. sec roughly equals those of the guinea pig atria, cat intestine and frog ventricle but less than that of the frog sartorius. Ca influx during activity is 0.0759 $\mu$mol/g.min or 0.2584 p-mol/cm$^2$. sec. This figure is somewhat similar that of the barnacle muscle fiber (90 $\mu$M/Kg min). NIEDERGERKE has proposed that the initiation of contraction of the frog ventricle is the consequence of an increase of 'activator calcium' within the fiber. According to his model the concentration of activator-Ca, [Ca$_a$], should be related to the influx, $m_{in}$, and the rate constant $\beta$, of the deactivation reaction by the equation:

$$\frac{d[Ca_a]}{dt} = m_{in} - \beta[Ca_a]$$

Applying our results mentioned above to this equation, we obtain the results that the ionized calcium within the ABRM fiber amounts to about 2$\mu$ M/l ABRM fiber within 1 sec after application of high K solution and thereafter linearly increases for about 10 sec. For $\beta$ a value of 0.05 sec$^{-1}$ was chosen from the twitch half decay time of 14 sec. This amount of 'activator Ca' is beyond the intracellular threshold concentration of ionized Ca for contraction in crab muscle fiber.

Potassium contracture of ABRM is phasic and abolished in Ca-free medium. Manganese ions inhibit the contracture. The action potential is not affected by tetrodotoxin. These facts and our results in this paper suggest that the activation of contraction in Mytilus ABRM is achieved at least in part, by the entry of extracellular calcium.

On the other hand the intracellular membranous structures such as sarcoplasmic reticulum observed in skeletal muscle fibers are poorly developed.
in ABRM and similar structures are irregularly distributed in the periphery of the fibers\(^2\). Transverse tubules are scarcely observed in the fibers.

Recently STÖSSEL and ZEBE\(^2\) have isolated microsomes from ABRM and compared them, with respect to their Ca binding activity, with those obtained in various other muscles. The Ca binding activity is one nineth that of the mouse skeletal muscle and one fourth to eleventh those of other invertebrate striated muscles. These morphological and biochemical evidences suggest that the calcium regulating mechanism of the intracellular membranous system is not developed adequately in Mytilus ABRM.

Other possible relations of calcium entry to contraction become apparent from the ratio of numbers of myosin molecules of ABRM and Ca ions which entered during activity. According to RÜEGG\(^2\) 50% of the total muscle protein of ABRM is the fraction of fibrillar protein and 37% of the fraction is actomyosin. Assuming that 1 g wet weight muscle of ABRM contains 20% of protein, the muscle contains about 30 mg of myosin per g wet weight muscle. Thus if the molecular weight of myosin is 500,000\(^3\), each gram of ABRM contains about 0.06\(\mu\) mol of the protein. The increase of Ca uptake during potassium contracture is 0.0759\(\mu\) mol/g. min. If this entered calcium is distributed uniformly within the fibers, the ratio of the number of calcium ions to that of myosin molecules after one minute of application of 320 mM K solution should be approximately 1:1. In fact the tension development of ABRM in isotonic K or 320 mM K solutions reaches the maximum after about one minute\(^9,14\). Since the time course of potassium contracture of ABRM is apparently concurrent with that of the increase of Ca uptake, it may be considered that the contractile mechanism of ABRM would be fully activated by the calcium which entered during potassium contracture.

VON SCHÄDLER\(^3\) has reported that the free calcium concentration necessary for 50% activation of glycerinated ABRM fibers is larger than those of various other muscles. Our findings do not seem to contradict his results.

**SUMMARY**

1. The efflux and uptake of Ca in Mytilus ABRM at rest and during potassium contracture were examined by use of \(^{45}\)Ca.
2. The Ca efflux during potassium contracture was 0.162 p-mol/cm\(^2\). sec. and increased to about 6 times that of the resting state.
3. The resting influx of Ca was 0.028 p-mole/cm\(^2\). sec. The increase of Ca uptake during potassium contracture in 2 min was 0.145\(\mu\) mol/g wet wt. muscle and the Ca influx during the activity was 0.258 p-mol/cm\(^2\). sec. Therefore the Ca influx during potassium contracture increased about 9 times that of the resting state.
4. The activation of the contraction of Mytilus ABRM was discussed on the
basis of these results and other physiological properties. It was suggested that the initiation of contraction of Mytilus ABRM may be associated with Ca entry from the extracellular space.

REFERENCES

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20) Shanes, A.M. Calcium influx in frog rectus abdominis muscle at rest and during


